STUDIES ON DNA METHYLATION AND MECHANISMS OF HYPOMETHYLATION

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Doctor of Philosophy

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The methylation of cytosine residues in DNA is thought to play an important role in the regulation of gene expression, with active genes generally being hypomethylated.

The imidazotetrazinones are a novel group of antitumour agents and hypomethylation has been implicated in their mechanism of action. Studies have been conducted on the mechanism by which they cause hypomethylation, using DNA(cytosine-5)methyltransferase partially purified from murine L1210 leukemia cells. Unmodified calf thymus DNA does not inhibit the transfer of methyl groups from S-adenosyl methionine to M.lysodeikticus DNA by DNA(cytosine-5) methyltransferase. However, when the calf thymus DNA was modified by alkylating agents such as the imidazotetrazinones or nitrosoureas, the treated DNA became an inhibitor of the methylation reaction. This was correlated with the induction of DNA damage, in particular single-strand breaks, since X-irradiated DNA produces a similar effect and neither sonication or treatment with the restriction enzyme MspI caused any inhibition.

Attempts were made to elucidate the strict structure activity relationship amongst the imidazotetrazinones for antitumour activity. The murine colon adenocarcinoma cell line (MAC13) was treated with DNA extracted from GM892 or Raji cells previously treated with either the methyl (temozolomide) or ethyl (ethazolastone) imidazotetrazinone. The DNA extracted from GM892 cells was the most effective growth inhibitor and temozolomide-treated cellular DNA was a more potent growth inhibitor than that from ethazolastone-treated cells. The effect was most potent six hours after drug addition, suggesting a repairable lesion.

The methylation of M.lysodeikticus DNA by DNA(cytosine-5) methyltransferase was inhibited in a potent and specific fashion by polynucleotides containing guanine residues. The inhibitory effect was unaffected by chain length or sugar residue, but was abolished when the O-6 residue of guanine was substituted as in poly[dO^6-methylguanine]$_{20}$. Potent inhibition was also shown by polyinosinic and polyxanthylic acids but not by polyadenylic acid or by the heteropolymers containing adenine and thymine. These results suggested that the 6 position of the purine nucleus is important in binding of the DNA(cytosine-5)methyltransferase to DNA. This was confirmed using synthetic oligonucleotides as substrates for DNA(cytosine-5) methyltransferase. Enzymatic methylation of cytosine was completely suppressed when O^6-methylguanine replaces guanine in 5′-CG-3′ sites.

Key words
DNA alkylation, DNA(cytosine-5)methyltransferase, hypomethylation, imidazotetrazinones, O^6-methylguanine.

'The life so short the craft so long to learn'

Hippocrates

To Mam and Dad,

with love.

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LIST OF ABBREVIATIONS

A	adenine
AAF	N-2-acetylaminofluorene
Ad12	adenovirus 12
AEV	avian erythroblastosis virus
AF	N-2-aminofluorene
aprt	adenine phosphoribosyl transferase
ara-C	1-ß-D-arabinofuranosyl cytosine
ATP	adenosine 5'-triphosphate
BCNU	bis-(2-chloroethyl)nitrosourea
bp	base pairs
BSA	bovine serum albumin
C	cytosine
CAT	catecholaminotranferase
CDSB	carboethoxymethyl dimethylsulphonium bromide
Ci	Curie
CNU	chloroethylnitrosourea
Cys	Cysteine
Da	Daltons
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
dhfr	dihydrofolate reductase
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DPSB	dimethyl propargyl sulphonium bromide
dUMP	deoxyuridine-5'-monophosphate
EBV	Epstein Barr virus
EDTA	ethylenediaminetetraacetic acid
EMS	ethyl methanesulphonate
ENU	N-ethyl-N-nitrosourea
G	guanine
GMP	guanosine 5'-monophosphate
GTP	guanosine 5'-triphosphate
HEBS	2 x HEPES buffered saline
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethane-sulphonic acid]
His	Histidine
HPLC	high performance liquid chromatography
i.p.	intra-peritoneal
mC	5-methylcytosine
5mC	5-methylcytosine
MCTIC	5-[3-(2-chloroethyl)triazen-1-yl]-imidazole-4-carboxamide
MEL	murine erythroleukemia
MLTF	major late transcription factor
MMS	methyl methanesulphonate
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
MNU	N-methyl-N-nitrosourea
Mr	molecular weight
mRNA	messenger RNA
n.d.	not determined
O^6AT	O^6-alkylguanine-DNA alkyltransferase
O^6MG	O^6-methylguanine
P	phosphate
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline

PMSF	phenylmethylsulphonyl fluoride
POPOP	1,4-di-2-(5-phenyloxazolyl)-benzene
PPi	inorganic phosphate
PPO	2,5-diphenyloxazole
Pro	proline
Pu	purine
Py	pyrimidine
RNA	ribonucleic acid
RPMI	Rosewell Park Memorial Institute (medium)
RSV	rous sarcoma virus
SAE	S-adenosylethionine
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SEM	standard error of the mean
s.c.	subcutaneous
SDS	sodium dodecyl sulphate
T	thymine
TBE	Tris-borate electrophoresis
TE	Tris EDTA
TEMED	N,N,N',N',-tetramethyl-ethylenediamine
TK	thymidine kinase
tris	tris(hydroxymethyl)aminomethane base

CHAPTER ONE

INTRODUCTION

1.1 GENERAL INTRODUCTION

The first reported case of a drug being successfully used to treat cancer was by Listauer in 1865 when potassium arsenite was given to a patient with leukemia, with beneficial effects. However, modern cancer chemotherapy started with the use of nitrogen mustard, which was developed from its use as a chemical warfare agent. The review by Gilman and Philips (1946) marks the start of modern cancer chemotherapy, with many of the analogues of nitrogen mustard being used in the clinic today. Since then a large number of clinical trials have been conducted on a range of different classes of antitumour agents, including alkylating agents, antimetabolites, antibiotics and steroids. However, the recurring problem with the various drug candidates are their deleterious side effects resulting from a lack of specificity.

The tumour cell shares many of the biochemical properties of the host cell and so a general problem with cancer chemotherapy is to develop specificity. A strategy suggested by Mihich (1981) to generate drugs with greater specificity towards tumour cells, was to develop a better understanding of the biochemistry of tumour cells in order that new target drugs may be identified.

One such biochemical target may be DNA(cytosine-5)methyltransferase and the level of DNA(cytosine-5)methylation in a cell. Tumours generally have a

decreased 5-methylcytosine content compared to normal cells and tissues. This has been demonstrated by Diala et al. (1983) in which the total genomic DNA methylation was measured in twenty cell lines derived from different types of human tumours and the majority had hypomethylated DNA compared to normal cells and tissues. Further, the 5-methylcytosine content of DNA from 103 human tumours has been determined (Gama-Sosa et al., 1983) and the majority of the metastatic neoplasms had significantly lower genomic 5-methylcytosine contents than did most of the benign neoplasms or normal tissues.

However, despite the DNA hypomethylation generally observed in tumour cells the activity of DNA(cytosine-5)methyltransferase is often greater in tumour cells. This was illustrated by Kautiainen and Jones (1986) in which an increased activity of DNA(cytosine-5)methyltransferase was demonstrated in tumorigenic cells compared to non-tumorigenic cells in culture. An increase in DNA(cytosine-5)methyltransferase has also been demonstrated in malignant mouse hepatic tissues compared to control tissues (Lapeyre et al., 1981).

These biochemical differences form the basis for gaining a better understanding of the biochemistry of DNA(cytosine-5)methylation, in the hope that this may be exploited as a target for the therapeutic intervention of malignant disease.

1.2 Biochemistry of DNA(cytosine-5)methylation

The DNA of prokaryotes contains the methylated bases N^6-methyladenine and 5-methylcytosine (for example *E.coli*; Fujimoto *et al.*, 1965). N^4-Methylcytosine also occurs as a product of DNA modification by the *Bcn* I methylase (Janulaitis *et al.*, 1983). The occurrence of 5-methylcytosine in calf thymus DNA was revealed by Hotchkiss (1948) and remains today as the only modified base found in higher eukaryotes. However, there has been a claim that DNA from human HeLa cells contains 3-methylcytosine, 1-methylguanine, 7-methylguanine, N^2-methylguanine and N^2-dimethylguanine as well as 5-methylcytosine (Culp *et al.*, 1970). This was later discounted by Lawley *et al.* (1972) who detected 5-methylcytosine as the only methylated base in HeLa cell DNA.

5-Methylcytosine arises in DNA by the transfer of methyl groups from *S*-adenosyl-L-methionine catalysed by the enzyme DNA(cytosine-5)methyltransferase (Borek and Srinivasan, 1966). Figure 1 illustrates the enzymatic reaction by which 5-methylcytosine is formed in DNA.

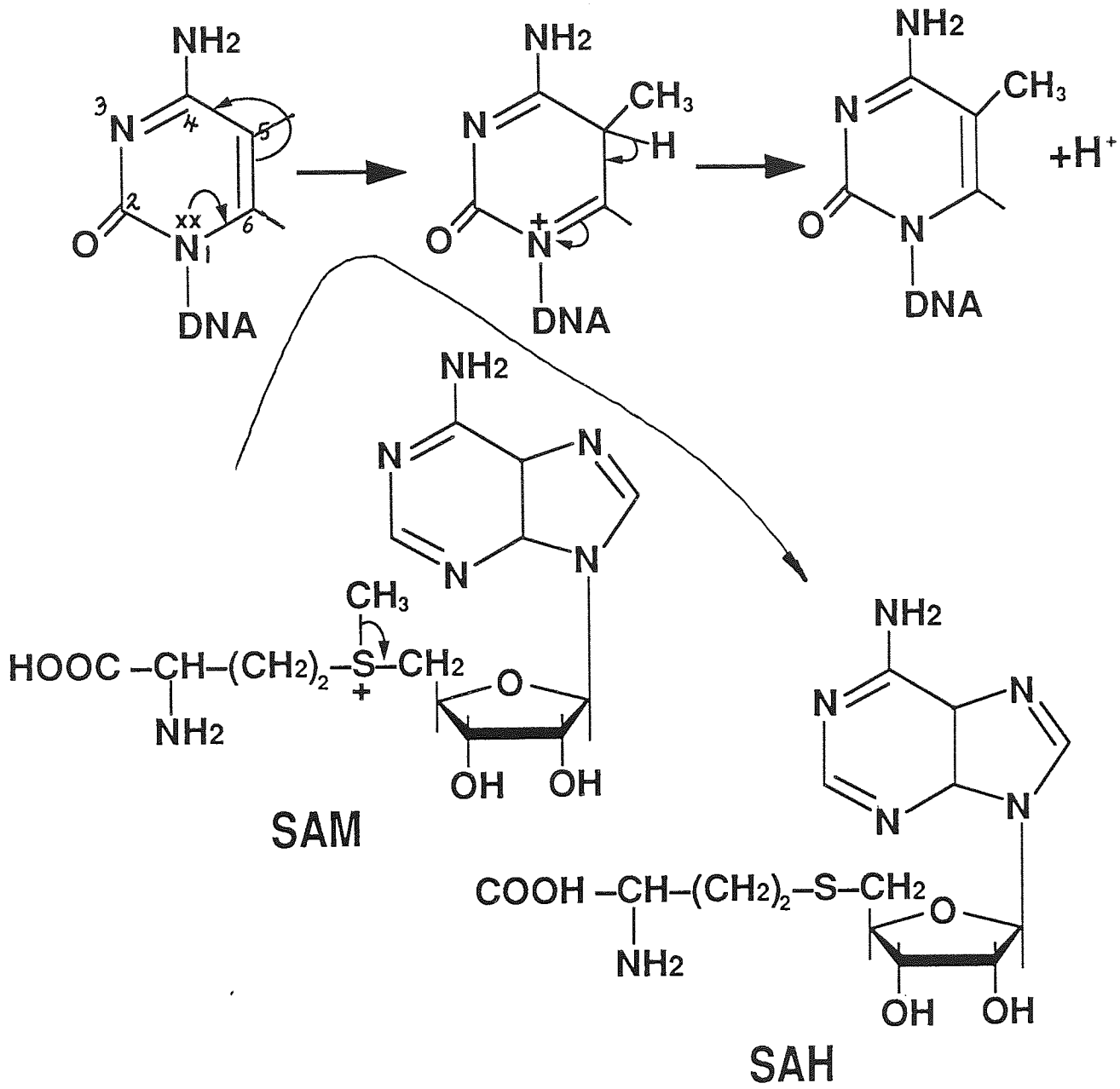


Figure 1: A diagram to illustrate the enzymatic reaction for the formation of 5-methylcytosine in DNA by DNA(cytosine-5) methyltransferase.

Eukaryotic DNA(cytosine-5)methyltransferase is presumed to methylate cytosine by the same mechanism as other enzymes which catalyse electrophilic substitution reactions at the 5-position of the pyrimidine heterocycle (Starzyk et al., 1982).

Figure 2 illustrates the mechanism by which there is nucleophilic attack by the enzyme at position 6 of cytosine to produce a negative charge at the 5-position; this has the effect of activating an inert carbon for reaction with an electrophile. Removal of the proton at carbon-5 and β-elimination produces 5-methylcytosine and the active enzyme.

1.3 Activities of DNA(cytosine-5)methyltransferase

Two activities of DNA(cytosine-5)methyltransferase have been suggested as a mechanism by which patterns of DNA methylation can be changed (Riggs, 1975; Holliday and Pugh, 1975); de novo methylation and maintenance methylation. The theory behind these two activities has been reviewed by Doerfler (1983), Razin and Szyf (1984) and Adams (1990). The principle of these two activities of DNA(cytosine-5)methyltransferase is summarised in figure 3 and will now be discussed.

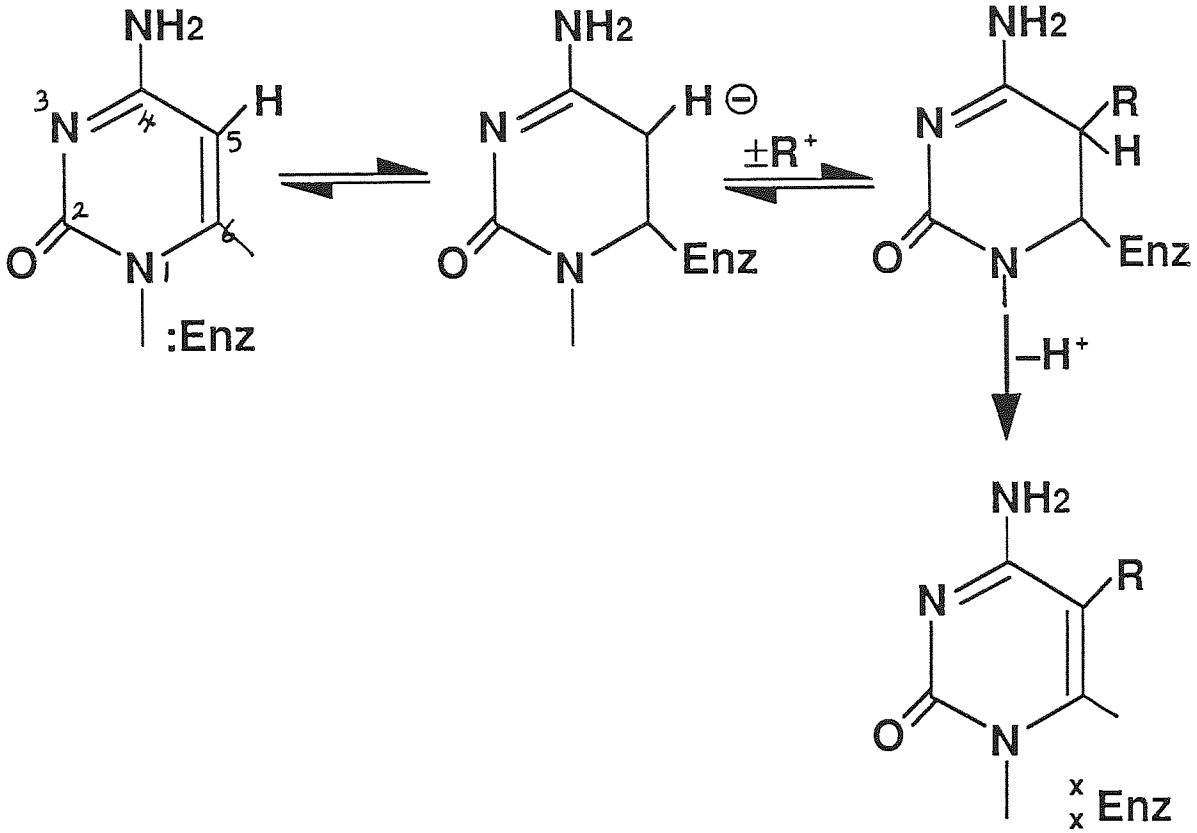


Figure 2: A diagram to illustrate the proposed mechanism for the formation of 5-methylcytosine in DNA by DNA(cytosine-5) methyltransferase.

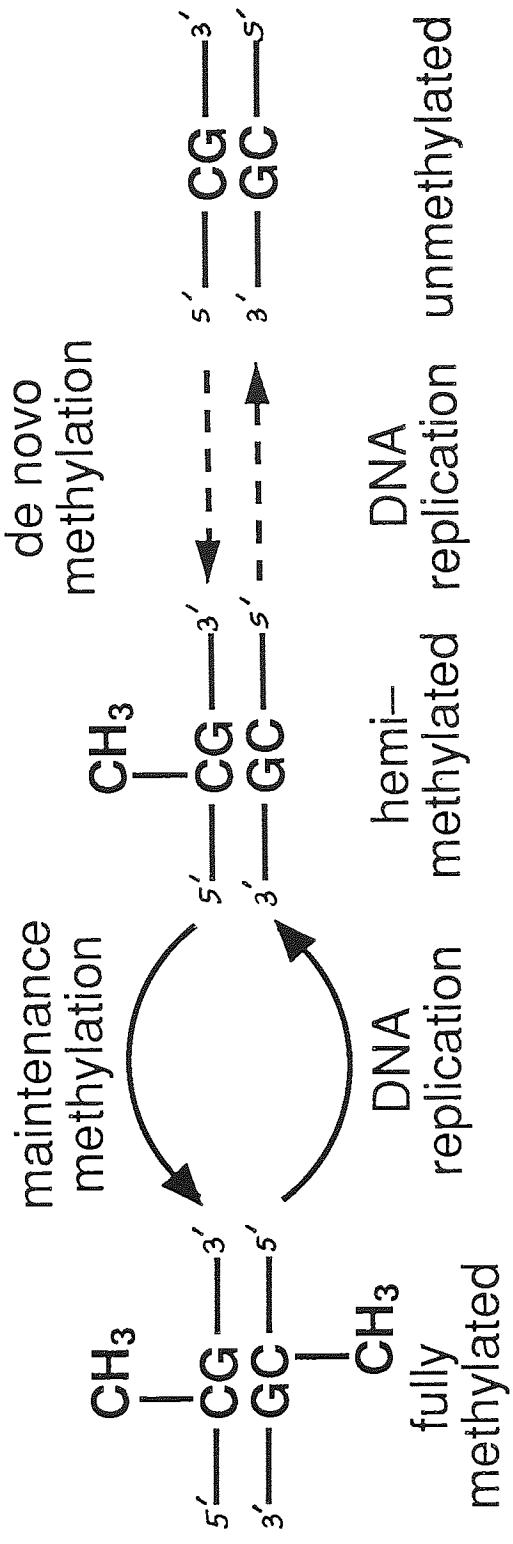


Figure 3: A diagram to illustrate the two proposed activities of DNA(cytosine-5)methyltransferase - maintenance and de novo.

Maintenance methylase

Experiments with asynchronously growing cells, i.e. mouse fibroblasts L929 (Adams, 1971), Chinese hamster ovary cells and a human cell line derived from a retro-orbital haemanginoma (Woodcock *et al.*, 1982) have indicated that enzymatic methylation of DNA occurs shortly after replication of DNA. Further, it has been demonstrated by Wigler *et al.* (1981) that patterns of DNA methylation are inheritable. They showed this by methylating bacteriophage ϕX174 RF DNA and the cloned chicken thymidine kinase (tk) gene *in vitro* using M-*Hpa*II (a modification methylase from *H.parainfluenzae* which methylates the internal cytosine residue of 5'-CCGG-3').

3'-GGCC-5'

After introduction of these DNAs into tk^- cultured mouse cells and cultured for twenty five cell generations, they showed that methylation at *Hpa*II sites was maintained.

The proposal that a maintenance methyltransferase activity exists was further supported by the work of Gruenbaum *et al.* (1982). They showed that hemimethylated DNA was the preferred substrate for DNA(cytosine-5)methyltransferase from mouse ascites

tumour cells. Therefore, after replication of a

5'-mCG-3'

3'-GmC-5' dinucleotide, an unmethylated 5'-CG-3' in the daughter strand of DNA will be paired with the 5'-mCG-3' in the parental strand. Subsequent methylation of the newly synthesised 5'-CG-3' ensures the maintenance of the pattern of DNA methylation. However, if enzymatic DNA methylation is blocked at this stage, then after another round of DNA replication, the result is a daughter strand containing a hemimethylated site 5'-mCG-3'

3'-GC-5'

and an unmethylated daughter strand. Therefore, through an inhibition of maintenance methylation a loss of methyl groups can occur and the pattern of DNA methylation can be changed.

A possible mechanism by which DNA methylation is blocked may be through the presence of DNA binding proteins, for example methylated DNA binding protein (Meehan et al., 1989). If maintenance methylation is in fact blocked by protein factors, one would expect hemimethylated sites to exist in vivo. This has in fact been shown to be true with the existence of hemimethylated sites in the chick vitellogenin gene (Saluz et al., 1986).

This model assumes that in vivo the methyltransferase becomes associated with the DNA at or near the replicating fork. However, maintenance

activity of DNA(cytosine-5)methyltransferase can be demonstrated *in vitro* using a hemimethylated DNA substrate.

De novo methylase

The *de novo* methyltransferase activity of eukaryotic DNA(cytosine-5)methyltransferase can introduce a new pattern of DNA methylation in DNA not previously methylated. A genome-wide *de novo* methylation of DNA is seen in early mouse embryos (Jähner *et al.*, 1982; Lock *et al.*, 1987). *De novo* methylation may be allowed to occur after removal of any constraints imposed on the methyltransferase such as DNA binding proteins. The new pattern of DNA methylation formed by *de novo* methylation would be capable of being passed on to the next generation by maintenance methylation.

In order for a fully unmethylated site in duplex DNA to appear requires two rounds of DNA replication in the absence of DNA methylation (see figure 3). After one division the DNA in the daughter strand will be hemimethylated and after two divisions half of the genes in the next series of daughter strands will be unmethylated. Other mechanisms of demethylation have been suggested, as the above mechanism is a slow process and does not seem sufficient to explain the rapid demethylation seen in some systems, for example the globin gene in stimulated erythroleukemia cells (Razin *et al.*, 1986).

Demethylation of DNA has been suggested to occur through a demethylase enzyme (Gjerset and Martin, 1982). However, doubts have been placed on the credence of these observations since no statistical treatment was applied to these results and the observation has not been substantiated by other workers.

Razin et al. (1986) suggest from their work a mechanism for demethylation whereby 5-methylcytosine is replaced with cytosine. In these experiments mouse Friend erythroleukemia cells were stimulated to differentiate with 5 mM hexamethylene bisacetamide and 12-20 h after treatment the proportion of -CG- dinucleotides methylated fell transiently from over 80% to as low as 40%. At the same time these cells were induced with hexamethylene bisacetamide they were labelled with deoxy[5-^{3}H]cytidine and the density label 5-bromodeoxyuridine. The newly replicated DNA (heavy-light) was separated from the parental DNA by isopycnic centrifugation. In the induced cells there was a drop in the level of DNA(cytosine-5)methylation which corresponded with the incorporation of deoxy[5-^{3}H]cytidine into light-light DNA and this did not occur in the non-induced cells. In parallel experiments, deoxy[G-^{3}H]adenosine was not incorporated into light-light DNA. During the period of de novo DNA methylation that follows the demethylation, the [^{3}H] label was removed from the DNA. Therefore, they suggest that the

demethylation occurs as a result of 5-methylcytosine being replaced by cytosine, possibly through the action of a glycosylase. However, Adams (1990) was unable to confirm this base exchange mechanism of demethylation, i.e. there was no reduction in the proportion of cytosines methylated in MEL cells prelabelled with [^{14}C]deoxycytidine and stimulated with either DMSO or hexamethylene bisacetamide.

1.4 The site of methylation by DNA(cytosine-5)methyltransferase.

In eukaryotes 5-methylcytosine is found predominantly in the dinucleotide 5'-CG-3'. Doskočil and Šorm (1962) showed that 5-methylcytosine occurs predominantly as the dinucleotide 5'-mCPu-3', but they were unable to identify the purine moiety. However, the earlier work of Sinsheimer (1955), who analysed dinucleotides from a DNase I digestion of calf thymus DNA showed that >90% of the 5-methylcytosine residues are found in the sequence 5'-mCG-3' and no 5'-mCA-3' was found, although 5'-CA-3' was abundant. But as pancreatic DNase I acts preferentially on the Pu-Py bond (Young and Sinsheimer, 1965) other methylcytosine containing dinucleotides cannot be excluded. Further evidence to suggest that 5'-CG-3' is the only sequence methylated came from Gruenbaum et al. (1981b) who inserted nicks into DNA using DNase I or by sonication and then used these as primers for E.coli DNA polymerase I using [^{32}P]dGTP,

dATP, dCTP or dTTP. Only the nucleotide 5' of the nick was labelled and their data suggested that 5-methylcytosine is found almost exclusively as 5'-mCG-3'.

However, there have been other reports of mammalian DNA methylation in other 5'-CN-3' dinucleotides (Gruenbaum et al. 1981a; Grafstrom et al., 1985; Sneider, 1980). A DNA(cytosine-5)methyl-transferase extracted from rat liver has been shown to methylate 5'-CA-3' and 5'-CT-3' in SV40 viral DNA in vitro (Simon et al., 1980) and the bovine thymus enzyme (Sano et al., 1983) was capable of methylating the following dinucleotides of M.luteus DNA in vitro, 5'-CG-3', 5'-CC-3', 5'-CT-3' and 5'-CA-3'.

Hubrich-Kühner et al. (1989) carried out studies on the in vitro methylation of viral SV40 DNA by rat liver DNA(cytosine-5)methyltransferase using Maxam-Gilbert sequencing of DNA. They showed that the methyltransferase methylates 5'-CA-3' and 5'-CT-3' at a fifty-fold lower initial rate than 5'-CG-3' but did not observe any methylation of 5'-CC-3'. However, Pfeifer et al. (1985) reported that the methylation of the outer cytosine in 5'-CCCG-3' sequences can occur by a purified methyltransferase. Therefore, it was proposed by Hubrich-Kühner et al. (1989) that the methylation velocity of DNA(cytosine-5)methyltrans-ferase towards the substrate DNA decreases in the order 5'-CG-3'>5'-CA-3'>5'-CT-3'>5'-CC-3' and that the methyltransferase makes its base recognition

through the O^6 carbonyl and the N-7 group of purine residues which both protrude into the major groove of DNA.

This does not mean that eukaryotic DNA(cytosine-5)methyltransferase can methylate all 5'-CG-3' sites within a DNA sequence. Bolden et al. (1985) using single-stranded oligonucleotides has shown that only one of two 5'-CG-3' pairs in a 27mer can be methylated by a partially purified DNA(cytosine-5)methyltransferase from HeLa cells. Further, this methylation was abolished if the other 5'-CG-3' pair was replaced with a 5'-CA-3'. Therefore, there may be more complex structural requirements to determine whether a cytosine can be methylated by the methyltransferase and in this case the methyltransferase may recognise a 5'-CG-3' group as a binding site to methylate the next 5'-CG-3' group at an appropriate distance from it.

Further evidence that the methyltransferase is restricted in terms of which 5'-CG-3' sites are methylated was produced by Ward et al. (1987), using an homologous methyltransferase from murine erythroleukemia cells. They showed that of the ten available 5'-CG-3' pairs in the 368 base pair 5'-region of the mouse β-globin gene, only five to six were methylated, producing one hemimethylated site and two fully methylated sites. The two sites in the

complementary pair that were not methylated were about one hundred base pairs in the 5'-direction from the -CG- cluster that was methylated.

1.5 DNA methylation and gene expression

A large number of studies have now shown that active genes are hypomethylated in cells where the particular gene is expressed and methylated in non-expressing cell types. This correlation between DNA methylation and gene activity has been the subject of a number of reviews (Razin and Riggs, 1980; Razin and Szyf, 1984; Adams and Burdon, 1985; Cedar, 1988). A full discussion of those genes which show a correlation between hypomethylation and gene expression will not be attempted and the reader is referred to these reviews which discuss the subject in detail.

The criterion adopted for saying that a gene is hypomethylated has generally involved the use of methylation sensitive restriction enzymes. This has generally not taken into consideration the number of methylation sites or the region of the gene involved. The promoter region of a gene is a DNA sequence located in front of a gene (5') and controls gene expression. The promoter is the binding site of RNA polymerase in the DNA molecule and serves as the starting point of the synthesis of mRNA. In DNA successive nucleotide units are covalently linked by phosphodiester bridges formed between the 5'-hydroxyl

group of one nucleotide and the 3'-hydroxyl group of the next. There is frequently a stronger correlation between gene activity and hypomethylation when the promoter regions of genes are considered. This is best illustrated in experiments where the expression of a gene is studied after *in vitro* methylation and introduction into cells by transfection. A number of examples which illustrate this correlation are shown in table 1.

However, some studies have shown that there is no correlation between the expression of a gene and hypomethylation (see Adams and Burdon (1985) for further consideration). A number of examples which suggest that there is no correlation between the expression of a gene and the amount of 5-methylcytosine are tabulated in table 2.

Further, 5-methylcytosine has not been detected in the *Drosophila* genome, despite there existing a programme by which genes are switched on and off (Urieli-Shoval *et al.*, 1982). A number of possibilities exist which may explain these discrepancies. Firstly, the expression of these genes may be regulated by mechanisms other than DNA methylation. Secondly, DNA methylation may only be a small part of the 'trigger' to initiate gene expression. Thirdly, the expression of these genes may be controlled by a region a long way from the structural gene and this region may show a good correlation between hypomethylation and gene expression. Fourthly, many of

Table 1. Correlation between hypomethylation in the 5' and promoter regions of genes and gene expression.

Gene	Reference
Globin genes (human)	Busslinger et al. (1983) Yisraeli et al. (1988)
Adenine phosphoribosyl transferase (hamster)	Stein et al. (1983) Keshet et al. (1985)
Dihydrofolate reductase (mouse)	Stein et al. (1983)
Thymidine kinase (viral)	Keshet et al. (1985)
Chloramphenicol acetyltransferase (mouse)	Kruczek and Doerfler (1983)
Eukaryotic adenoviral promoters	Doerfler et al. (1988)
Thyroglobulin (human)	Libert et al. (1986)

Table 2. Examples of genes for which there is no correlation between hypomethylation and gene expression.

Gene	Reference
β-globin (human placenta)	van der Ploeg and Flavell (1980)
Albumin (rat hepatoma)	Ott et al. (1982)
Adenovirus 12 in hamster cells	Kuhlmann and Doerfler (1982)
SV40 or polyoma virus (African green monkey kidney cells, Fischer rat embryo fibroblasts, mouse 3T3 and primary kidney cells).	Graessmann et al. (1983)
Collagen (chick embryo fibroblast cells (CEF), CEF transformed by RSV, erythrocytes, brain and sperm).	McKeon et al. (1982) Chandler et al. (1986)
Alpha foetoprotein/albumin (rat hepatoma, kidney)	Vedel et al. (1983)

these studies rely on the restriction enzymes MspI and HpaII. These enzymes recognise methylation around the sequence CCGG which represents approximately 4% of the total 5'-CG-3' dinucleotides. Therefore, it is possible that a particular gene may have none or only a few of these sites.

Although many studies have shown a correlation between undermethylation and gene expression, they do not establish whether the undermethylation is a cause or a consequence of transcriptional activation. One way that this question has been addressed has been to test the ability of 5-methylcytosine to interfere with the binding of transcription factors. Transcription factors may bind to specific sequences in target promoters and are required for the expression of a particular gene. For example, the transcription factor Sp1 is required for the expression of many viral and cellular genes including housekeeping genes, which encode proteins needed for the basic metabolic processes in the cell. Sp1 activates transcription by binding to specific sites, generally a short distance upstream from the initiation site for RNA synthesis. The recognition sequence for Sp1 is 5'-GGGGCGGGGC-3' and most of the known transcription factor binding sites contain a 5'-CG-3' dinucleotide. The most pertinent evidence to indicate that methylation can block gene expression by interfering with the binding of transcription factors is summarised in table 3.

Table 3. Evidence to indicate that 5-methylcytosine can block gene expression by interfering with the binding of transcription factors.

Factor	Reference
Major late transcription factor to the major late promoter of adenovirus.	Watt and Molloy (1988)
E2 factor to the adenovirus E2 promoter.	Kovesdi *et al*. (1987)
Factor to its binding site in the promoter of the rat tyrosine aminotransferase gene.	Becker *et al*. (1987)
Cyclic AMP-responsive element binding protein to the cyclic AMP-responsive element.	Iguchi-Ariga and Schaffner (1989)
A factor to the late E2A promoter of adenovirus type 2	Hermann *et al*. (1989)
Transcription factor AP-2 to proenkephalin promoter	Comb and Goodman (1990)

However, there is some evidence to indicate that cytosine methylation is not involved in the binding of transcription factors. This evidence has been summarised in table 4.

1.6 An alternative explanation for the effect of cytosine methylation on transcription.

As well as blocking the binding of transcription factors there is some evidence to indicate that cytosine methylation may have an effect on gene expression through the formation of inactive chromatin.

The chromatin of eukaryotic nuclei can be fractionated into regions which have various sensitivities to the action of nucleases *in vitro*. Actively transcribed genes are generally found in the highly sensitive fraction of chromatin and contain a small proportion of 5-methylcytosine. It is generally believed that an inactive gene containing 5-methylcytosine will adopt a nuclease-inaccessible, condensed conformation which may be reversed if the gene becomes transcriptionally active (Adams *et al.*, 1990). When a gene is transfected into an animal cell it associates over 8 h to form chromatin (Graessmann *et al.*, 1985; Buschhausen *et al.*, 1987). A number of reports have been studies on the effect of *in vitro* methylation on gene expression after introduction into cells by transfection. However, genes containing a high proportion of 5-methylcytosine form

Table 4. Evidence to indicate that 5-methylcytosine does not block the binding of transcription factors.

Factor	Reference
Methylation of a single 5′-CG-3′ dinucleotide within a promoter element of the Herpes virus tk gene reduces its transcription in vivo. However, cytosine methylation of these sites does not significantly change the affinity of Sp1 and CTF (CAT binding factor) proteins for their respective recognition sequences.	Ben-Hattar and Jiricny (1988) Ben-Hattar et al. (1989)
Site specific demethylation in the promoter of gamma-globin gene does not alleviate methylation mediated suppression and a minimum methylation free area must be maintained to allow full transcription.	Murray and Grosveld (1987)
Sp1 recognition oligonucleotides were synthesised containing cytosine or 5-methylcytosine at the central position and at another site near the edge of the binding site. There was no difference in the affinity of Sp1 for the methylated and unmethylated DNA as measured using gel retardation binding assays. Synthetic Sp1-binding sites were ligated to a ß-globin TATA box and downstream sequences that allowed synthesis, processing and detection of the transcript. Transcriptional activity was tested in vitro and in vivo and in both cases the presence of 5-methylcytosine in the binding site had no effect.	Holler et al. (1988)
Synthetic oligonucleotides were synthesised to investigate the effect of cytosine methylation on the binding of transcription factor Sp1 to its target sequence. The presence of 5-methylcytosine in the 5′-CG-3′ sequence did not influence Sp1 binding as measured using gel retardation binding assays.	Harrington et al. (1988)

inactive chromatin and so an inhibition of the activity of transfected DNA by methylation is frequently seen. In this case the site of methylation in the gene may not be important, with methylation at the 3' end of the gene being as effective as at the 5' end (Busslinger et al., 1983; Keshet et al., 1985).

In support of the theory that 5-methylcytosine affects the formation of chromatin, Murray and Grosveld (1987) have shown that a minimum length of DNA free of 5-methylcytosine is required for transcription of the gamma-globin gene. Further, transcription of the gene occurs if all 5'-CG-3' dinucleotides are removed from the promoter region. This suggests that a sequence-specific transcription factor is not involved but rather the inhibition of transcription may be due to binding of a methylcytosine-binding protein which seeds the formation of inactive chromatin. Along the same lines, Ben-Hattar and Jiricny (1988) have shown that methylation of any one of four 5'-CG-3' sites in the promoter region of the Herpes simplex tk gene reduced the transcription of the gene in Xenopus oocytes. All four sites lie within a nuclease hypersensitive and therefore active region of chromatin. Therefore, a reduced transcription brought about by methylation may occur as a result of a change in the chromatin assembly rather than through an interference with the binding of transcription factors.

What is the evidence for the existence of a methylcytosine-binding protein, which may be involved in seeding the formation of inactive chromatin? A protein isolated from human placenta has been shown to bind preferentially to 5-methylcytosine-rich DNA (Huang et al. 1984). More recently, Meehan et al. (1990) have purified a protein which binds to DNA containing greater than 15 5'-mCG-3' dinucleotides. These proteins may ensure suppression of transcription by stabilising a higher order folding of chromatin.

Studies on CpG islands also support the theory that DNA methylation is correlated with a change in the structure of chromatin rather than the binding of transcription factors. CpG islands are DNA sequences in which the dinucleotide 5'-CG-3' is abundant and non-methylated. A number of tissue-specific genes do not observe the inverse correlation between methylation and gene expression and these gene promoter regions are within CpG islands, which remain clear of detectable methylation in both expressing and non-expressing cell types (reviewed by Bird, 1986; Bird, 1987). Tazi and Bird (1990) have studied the chromatin structure in the vicinity of transcription sites and they revealed that CpG island chromatin was different from the main form of chromatin: almost devoid of histone H1; histones H3 and H4 were heavily acetylated; and regions of nucleosome-free DNA were present. A lack of histone H1 suggests that CpG

island chromatin is not tightly packaged since histone H1 is known to participate in the folding of chromatin (reviewed by Pederson et al., 1986). It has been proposed that acetylation of histones may be important in transcriptionally active chromatin (Allfrey, 1977) which may explain the high level of acetylation of histones H3 and H4 seen in CpG island chromatin.

Although CpG islands are normally free of methylation even when the gene is transcriptionally silent in the animal, it has recently been observed for cells grown in culture, in particular mouse NIH3T3 and L cells, that greater than half of the islands are heavily methylated (Antequera et al., 1990). The methylated islands were those present in genes that are probably not required in culture and it follows that these cell lines may have lost the ability to express a high proportion of the genes available for expression in the animal. These results suggest that methylation has a major role in suppressing the transcription of CpG island-associated genes. Further, they found that the presence of 5'-mCG-3' dinucleotides at the α-globin CpG island and at eight other randomly selected CpG islands protected the cleavage of the dinucleotides 5'-mCG-3' in the nucleus by MspI. As Msp1 can cleave 5'-mCG-3' in protein-free DNA, they concluded that methylation of CpG islands causes an alteration in chromatin structure. Therefore, these results

support the theory that DNA methylation is correlated with a change in the structure of chromatin rather than with transcription.

It is clear from these observations that the role of DNA methylation in gene expression is complex and that generalisations cannot easily be made.

1.7 DNA methylation and cancer

There is a large amount of experimental evidence which demonstrates altered levels and patterns of DNA methylation in tumour cells (reviewed by Riggs and Jones, 1983; Jones, 1986; Adams and Burdon, 1985). A general trend has been observed for tumour cells to contain hypomethylated DNA when compared to the normal cellular counterparts. Diala et al. (1983) measured the percentage of 5-methylcytosine in twenty cell lines derived from different types of human tumours and found that the majority of the tumour cells had decreased levels of methylated DNA compared to the normal tissues. In a larger study reported by Gama-Sosa et al. (1983), the 5-methylcytosine content of 103 human tumours was determined. Normal tissue had an average of 4.37% cytosines methylated, whilst the cytosines in DNA from primary malignant tumours and metastases were 4.06% and 3.92% methylated respectively.

However, care must be taken when considering data from tumour cells grown in culture as the methylation status of these cells may not necessarily

reflect the situation in the animal. For example, it has been observed by Shmookler-Reis and Goldstein (1982) that the longer human diploid fibroblasts are maintained in culture, the more hypomethylated the DNA becomes. Further, it has been shown by Antequera et al. (1990) that in permanent cell lines (mouse NIH3T3 and L cells), CpG islands have become methylated that are normally non-methylated in cells of the animal. Therefore, by some unknown mechanism cells in culture may have a disturbance of those factors which are normally involved in the maintenance of the pattern of DNA methylation.

An important question to ask at this point is at what stage in the development of a tumour does the DNA cytosine lose its methyl groups? This question has been addressed by Bert Vogelstein and colleagues in particular with respect to the development of colon cancer in humans. It was shown be Feinberg and Vogelstein (1983a) that the DNA from three out of four primary human colonic tumours and one lung carcinoma was substantially hypomethylated at specific sites within growth hormone and globin genes. These observations were particularly significant because they were compared with normal mucosal tissue which was removed at the same time from the region in the vicinity of the tumour. Feinberg and Vogelstein (1983b) extended their studies to the methylation status of the two cellular oncogenes, c-Ha-ras and c-Ki-ras. They compared

primary human carcinomas with adjacent analogous normal tissues and showed that the c-Ha-ras gene was hypomethylated in six from eight carcinomas (five colonic adenocarcinomas and one small cell lung carcinoma) whilst the c-Ki-ras gene was hypomethylated to a lesser extent in two colonic adenocarcinomas. The degree of hypomethylation appeared to be greater in the metastases than in the primary tumours suggesting that a further loss of methyl groups may occur through the metastatic process.

In a further study by Goelz et al. (1985) the DNA from 23 human neoplastic growths was examined, including benign colon polyps and malignant carcinomas and was shown to be hypomethylated at specific sites within growth hormone, gamma-globin, α-chorionic gonadotrophin and gamma-crystallin genes. The DNA from benign polyps was hypomethylated to a degree similar to that in malignant tissue and they concluded that the loss of methyl groups in human colonic tumours occurred at a stage early in the development of the benign adenoma.

Further, it has been shown by Feinberg et al. (1988) that on average there was a reduction in the content of 5-methylcytosine in colon adenomas and adenocarcinomas of between 8 and 10% respectively when compared to normal tissues. In these studies there was no significant difference between benign and malignant tumours.

A similar investigation has been carried out on the methylation status of various stages of neoplasia in the human prostate (Bedford and van Helden, 1987). These investigations were not carried out to the same extent as those with colon cancer described previously, but it was shown that the proportion of 5-methylcytosine in DNA was significantly lower in prostates with benign prostatic hyperplasia and in metastatic tumour than normal prostate tissue. These results add support to the theory that hypomethylation occurs at an early stage in the development of a tumour and they further suggest a correlation between hypomethylation and a worsening pathological condition from normal (i.e. normal → hyperplasia → metastatic cancer).

Despite these observations which indicate that DNA loses methyl groups at an early stage in the development of a tumour, not all tumours are actually hypomethylated. Table 5 summarises some of the papers which indicate that tumours or their associated genes are in fact hypermethylated.

Therefore the evidence to date suggests that the majority of tumours and tumour cell lines are hypomethylated and where there is hypermethylation of a particular gene this generally occurs in the presence of genomic DNA hypomethylation (see Silverman et al. (1989) with respect to the calcitonin gene).

Table 5 Hypermethylation in tumours, tumour cell lines or their associated genes.

Tumour/gene	Reference
Almost twice as much 5MC was detected in the DNA of the hamster kidney fibroblast cell line (BHK21) transformed by polyoma virus compared with the normal BHK21 cells grown in culture.	Rubery and Newton (1973) Cato et al. (1978)
The untransformed hamster fibroblast cell line (BHK21) contains 2.22% 5MC in the DNA whilst the DNA of hamster cells transformed by adenovirus type 12 contains 3.11 and 3.14% 5MC (HA12/7 and T637 cells respectively).	Günthert et al. (1976)
The use of methylation-sensitive restriction enzymes has shown that the DNA of avian erythroblastosis virus transformed chicken erythroblasts was more methylated than the normal erythroblast DNA in the region of the globin genes.	Marcaud et al. (1981)
In human colonic adenomas, colon carcinomas and established colon carcinoma cell lines, the calcitonin gene is hypermethylated in comparison with normal adult tissues. However, this hypermethylation of the calcitonin gene occurs in the presence of genomic DNA hypomethylation.	Silverman et al. (1989)
The DNA in the short arm of chromosome 11 is hypermethylated in a range of human tumour cell lines.	de Bustros et al. (1988)
In human lung cancers (small cell, adeno-, squamous and large cell carcinomas) and in lymphomas, the calcitonin gene is hypermethylated in comparison with normal adult tissues.	Baylin et al. (1986)

A possible explanation for the hypermethylation observed in some regions of DNA in tumour cells is that they represent the location of tumour suppressor genes. The short arm of chromosome 11 in humans is thought to harbour tumour suppressor genes and this is the region where hypermethylation of the calcitonin gene (Baylin et al., 1986) and other genes in human cancers has been demonstrated (de Bustros et al., 1988). The hypermethylation and inactivation of tumour suppressor genes may be a further mechanism for the development of a tumour. For example, in Wilms' tumour the non-expression of a tumour suppressor gene in chromosome 11 may be responsible for the development of the tumour (Weissman et al., 1987).

It would appear therefore that DNA methylation is involved in the development of tumours, whether this is hypomethylation and the expression of aberrant genes or hypermethylation and the inactivation of tumour suppressor genes. However, it is not known whether a tumour occurs as a result of alterations in the level of methylation or whether the change in methylation occurs as a consequence of other factors involved in tumour development.

1.8 Inhibition of DNA methylation by chemical agents.

Cell transformation is a multi-stage process (Farber, 1984) involving at least three stages: initiation, promotion and progression. Initiation is believed to result from the fixation of promutagenic DNA damage which arose via exposure to chemical carcinogens or by radiation or by spontaneous events such as error-prone DNA replication. Clonal expansion of the initiated cells leads to preneoplastic development and possibly the formation of benign tumours. The production of a malignant tumour from a benign tumour may involve a further genetic change followed by clonal expansion.

Chemical carcinogens can initiate the transformation of cells to the neoplastic state. It has been shown previously that DNA(cytosine-5)methylation is involved in gene expression. Therefore an alteration in the pattern of DNA methylation produced by chemical agents may be responsible for the aberrant expression of genes seen in cancer. It is possible that an interference with DNA methylation may affect the carcinogenic process at the initiation, promotion and progression stage.

Chemical carcinogens may disrupt the proportion of 5-methylcytosine in DNA by one of three mechanisms: reaction with the enzyme; reaction with the DNA substrate; or interaction with the cellular

metabolism involved in the control of DNA methylation. The latter has limited relevance to this thesis and will not be discussed further.

Reaction with the enzyme

DNA(cytosine-5)methyltransferase contains thiol groups within cysteine residues at the active site (Bestor et al., 1988). These thiol groups are reactive sites for a number of carcinogens which may cause inactivation of the enzyme. A complete irreversible inhibition of rat liver DNA(cytosine-5)methyltransferase was produced after in vitro treatment with MNNG, whilst a similar treatment of the DNA substrate did not produce any inhibition (Drahovský and Wacker, 1975). However, the drug concentration used was 2 mM and whether these results are significant for such a potent carcinogen is dubious.

Cox (1980) also demonstrated that MNNG caused inactivation of DNA(cytosine-5)methyltransferase by direct interaction with the protein, but once again this was at high concentrations of the drug. In these experiments it was shown that the thiol group is a possible site for inactivation of the methyltransferase as 0.1 mM iodacetamide, which is known to react with thiol groups of proteins, caused about 45% inhibition of the methyltransferase in vitro. Cox (1980) further illustrated that carcinogens which do not react with sulfhydryl groups

such as N-nitrosodiethylamine, N-nitrosodimethylamine and methylazoxymethanol have no direct effect on the activity of the methyltransferase.

Acrolein, the reactive metabolite of cyclophosphamide, which is known to react with thiol groups causes a 30-50% inhibition of methyltransferase activity in vitro at a concentration of 10 μM (Cox et al., 1988). Chan et al. (1983) tested the ability of a number of carcinogens to inhibit DNA(cytosine-5)methyltransferase in vitro and demonstrated a 50% inhibition with 4.3 mM N-acetoxy-N-acetyl-2-aminofluorene, 47 mM MNU and 2.8 mM MNNG. The thiol containing compounds dithiothreitol, β-mercaptoethanol and reduced glutathione when added to the assay mixture in increasing amounts protected the enzyme from carcinogen damage. Consequently, intracellular thiols may play an important role in protecting DNA enzymes from damage.

The above examples demonstrate that certain chemical agents which react with thiol groups can inactivate DNA(cytosine-5)methyltransferase. However, large concentrations of drug were used to produce an effect using in vitro enzyme preparations and it is unlikely that such concentrations could be attained in the cell. Intracellular glutathione protects the cell from this form of carcinogen damage by a scavenger-type reaction (Chasseaud, 1979). Therefore, it is unlikely that an inhibition of DNA(cytosine-5)methyltransferase by this mechanism is

a particularly significant carcinogenic event, which would also depend on the relative rate of methyltransferase turnover.

Reaction with DNA

A number of carcinogens have been shown to interfere with DNA(cytosine-5)methylation through reaction with the DNA substrate. Initial experiments were carried out by Drahovsky and Morris (1972) in which they showed that chemical modification of E.coli DNA by complete depurination with formic acid, by deamination of deoxycytidylate residues with sodium bisulphite or by alkylation of deoxyguanylate residues with dimethyl sulphate abolished the ability of the DNA to accept methyl groups by transfer from rat liver DNA(cytosine-5)methyltransferase. These results did not provide many clues to indicate a particular site of modification responsible for the inhibition of the methyltransferase. However, as one would expect these results do suggest that cytosine and guanine residues are of some importance.

Contrary to these observations, Pfohl-Leszkowicz et al. (1983a) showed that alkylation of chicken erythrocyte DNA or poly(dG-dC).poly(dG-dC) with up to 100 mM dimethyl sulphate had no effect on the ability of beef brain DNA(cytosine-5)methyltransferase to methylate this substrate, despite the level of modification of guanine to 7-methylguanine being as high as 30% and 40% respectively. However, modification

of these substrates with MNU produced methylphosphotriesters and O^6-methylguanine in addition to 7-methylguanine. A 1% alkylation of the bases in DNA or poly(dG-dC).poly(dG-dC) by MNU caused a 40% and 38% inhibition respectively of their ability to behave as substrates for DNA(cytosine-5)methyltransferase.

Acrolein (the reactive metabolite of cyclophosphamide), as well as being able to inhibit DNA(cytosine-5)methyltransferase by reaction with critical sulfhydryl groups, is able to cause a reduction in the ability of M.lysodeikticus DNA to accept methyl groups by transfer from rat bladder methyltransferase (Cox et al., 1988).

Hemimethylated DNA prepared from treating mouse embryo cells in culture with low levels of 5-azacytidine (2 μM) has been used as a substrate for maintenance methylation. In this assay methyl groups are transferred specifically to cytosine residues in the hypomethylated strand (Taylor and Jones, 1982). Treatment of such a DNA substrate with a wide range of carcinogens has been shown to inhibit in vitro methylation by mouse spleen DNA(cytosine-5)methyltransferase (Wilson and Jones, 1983). The most potent inhibitor of DNA methylation was nitrogen mustard followed in order by NAAF, 1,3-bis(2-chloroethyl)-1-nitrosourea and MNNG. However, all the agents investigated which covalently react with DNA caused an inhibition of the methyltransferase to

some extent, including EMS, ENU, ENNG, 9-amino-acridine and benzo(a)pyrene diolepoxide. The induction of alkali-labile sites and single-strand breaks by depurination with weak acid (pH 1.6) or by UV-irradiation of bromouracil-containing DNA also reduced its ability to accept methyl groups in vitro, but the methyltransferase reaction was considerably less sensitive to thymine dimers or to double-strand breaks. The degree of methyltransferase inhibition that they observed with the carcinogens was greater than that which could be accounted for by alkali-labile DNA lesions and therefore it is likely that the carcinogen-DNA adduct may have played a role.

There are a number of possible mechanisms which may account for the inhibition of the methyl-transferase produced with these carcinogens. Firstly, the carcinogen may react with important binding sites for the methyltransferase. Secondly, large bulky carcinogen adducts may prevent the scanning action of the enzyme. Thirdly, the carcinogen adduct may increase the attraction between the methyltransferase and the DNA substrate.

The inhibition of enzymatic DNA(cytosine-5)methylation by reaction of N-substituted aromatic compounds with DNA has been investigated in some detail. One such compound, 2-(acetylamino)fluorene is a potent liver carcinogen, which after administration and metabolic activation in rats (see Singer and Grunberger, 1983 for discussion of

metabolism) produces the following adducts in DNA; 70% non-acetylated C-8 guanine adducts (N-(deoxyguanosin-8-yl)-2-aminofluorene or dG-C8-AF and 30% acetylated adducts (24% N-(deoxyguanosin-8-yl)-2-acetylaminofluorene or dG-C8-AAF and 2% 3-(deoxyguanosin-N^2-yl)-2-acetylaminofluorene or dG-N^2-AAF) (Kriek, 1974; Westra et al., 1976).

2-(N-Acetoxyacetylamino)fluorene or AcO-AAF is a model of the ultimate carcinogen AAF, in that it reacts with DNA to give essentially the acetylated adducts whilst N-hydroxy-2-aminofluorene or N-OH-AF is a model of AAF which reacts with DNA to give the non-acetylated adducts dG-C8-AF (Tang and Lieberman, 1983).

Salas et al. (1979) showed that treatment of chicken erythrocyte DNA with AcO-AAF inhibited the transfer of methyl groups by rat brain DNA(cytosine-5)methyltransferase. Pfohl-Leszkowicz et al. (1981) investigated the mechanism of inhibition in more detail and showed that the enzymatic methylation of chicken erythrocyte DNA-AAF, that contained 5% of modified bases was approximately 15% of the control. Further, they found that the AAF-modified DNA had a higher affinity for the enzyme than the native DNA. It was suggested that the inhibition may be due to bulky AAF-guanine residues on the substrate DNA, which may prevent the scanning action of the enzyme.

Pfohl-Leszkowicz et al. (1982; 1983b) compared the enzymatic methylation (rat brain DNA(cytosine-5)methyltransferase) of chicken erythrocyte DNA modified by AcO-AAF and MNU. They showed that the inhibition of methylation was much greater for MNU-alkylated DNA than for DNA-AAF; 50% inhibition was achieved with 0.15% of modified bases for the MNU-methylated DNA compared with 0.9% for DNA-AAF. However, AAF-modified DNA was capable of inhibiting the enzymatic methylation of unmodified DNA in competition assays, whereas MNU-alkylated DNA did not inhibit the methylation of native DNA (Pfohl-Leszkowicz et al., 1983b). This illustrates two mechanisms by which modified DNA may inhibit DNA(cytosine-5)methyltransferase. Firstly, with MNU-modified DNA the specific sites of alkylation prevents the methyltransferase from recognising binding sites. Secondly, with AAF-modified DNA the bulky AAF groups bound to guanine in DNA may block the mechanism, (as proposed by Drahovský and Morris (1971)), by which the methyltransferase 'walks' along the DNA.

The conclusion that bulky adducts in DNA are capable of inhibiting the action of DNA(cytosine-5)methyltransferase has been complicated by the report of Pfohl-Leszkowicz et al. (1984). They investigated chicken erythrocyte DNA modified in vitro with N-2-aminofluorene which gives only the deacetylated adduct dG-C8-AF. They observed that DNA

modified in this way was hypermethylated compared to native DNA when treated with the methyltransferase, which contrasts with the hypomethylation observed with DNA-AAF. They suggest that the deacetylated adduct changes the secondary structure of the DNA in such a way to facilitate the mechanism by which DNA(cytosine-5)methyltransferase 'walks' along the double helix.

There is a discrepancy between these results and those observed by Ruchirawat et al. (1984), in which the aminofluorene adduct severely impaired the methylation of the polynucleotide poly(dC-dG)-AF. This observation would add support to the theory that bulky adducts inhibit the movement of the methyltransferase and as a consequence reduce the extent of enzymatic DNA methylation. However, the results of Pfohl-Leszkowicz et al. (1981, 1984) in which DNA-AAF was hypomethylated and DNA-AF was hypermethylated by DNA(cytosine-5)methyltransferase are supported by the report of Boehm et al. (1983). They showed that treatment of murine cells in culture with N-AcO-AAF, which produces AAF and AF adducts in DNA, produced two sets of cells, one with hypomethylated DNA and one with hypermethylated DNA.

5-Azacytidine is a potent inhibitor of enzymatic DNA methylation, which is also thought to act through its effect on the DNA substrate. However, the mechanism of inhibition is different from those examples previously discussed, in that a covalent

complex is believed to form between the methyltransferase and the DNA containing 5-azacytosine. The evidence which led to this conclusion will now be reviewed.

5-Azacytidine differs from cytidine only by the replacement of the 5-carbon atom with a nitrogen and can be incorporated into both RNA and DNA in place of cytidine. The treatment of cultured mouse embryo cells with 5-azacytidine in which a 5% substitution of cytosine residues by 5-azacytosine occurred, resulted in greater than 80% inhibition of DNA methylation (Jones and Taylor, 1980). 5-Azacytidine has been used to inhibit the methylation of newly replicated DNA to prepare hemimethylated DNA, which is a good substrate for DNA(cytosine-5)methyltranferase (Jones and Taylor, 1981). The best *in vitro* substrate for the methyltransferase is produced using low concentrations of 5-azacytidine (approximately 2 μM), in which the extent of substitution of 5-azacytosine for cytosine is in the order of 1-2% and approximately 60% of the methylation sites are in the hemimethylated state (Jones and Taylor, 1981; Taylor and Jones, 1982). However, DNA containing higher levels of 5-azacytosine (5% of total cytosine) was a poor substrate for DNA(cytosine-5)methyltransferase *in vitro* and resulted in the formation of a tight

complex between the methyltransferase and the DNA substrate, which could not be dissociated (Taylor and Jones, 1982).

Evidence for a tight binding of the methyltransferase to DNA containing 5-azacytosine was also provided by Tanaka et al. (1980). These authors observed a rapid decrease in extractable methyltransferase activity after treatment of Ehrlich ascites cells in culture with 5-azacytidine. Further, DNA prepared from E.coli K12 cells which had been grown in the presence of 5-azacytidine (10 μg/ml) has been shown to cause irreversible inhibition of bacterial DNA(cytosine-5)methyltransferase in vitro (Friedman, 1981).

The mechanism of inhibition of DNA(cytosine-5)methyltransferase by DNA containing 5-azacytosine is thought to be the same for bacterial and mammalian enzymes (Santi et al., 1983; Santi et al., 1984). It was proposed that the methyltransferase adds to the 6-carbon atom of 5-azacytosine in the manner for the normal enzymatic reaction (figure 2) to form the 5,6-dihydropyrimidine adduct. In cytosine this would produce a negative charge at carbon 5 and activate it for reaction with the electrophilic methyl group from SAM. However, with 5-azacytosine the reaction would be blocked with the formation of the 5,6-dihydropyrimidine adduct. Therefore, it was proposed that

the 5-azacytosine in DNA blocks DNA methylation by forming a covalent bond with the methyltransferase, which consequently depletes the cell of enzyme.

In conclusion it would appear that chemical agents can inhibit DNA(cytosine-5)methyltransferase through an effect on the DNA substrate by three mechanisms: reaction with important binding sites in the DNA for the methyltransferase; bulky chemical adducts which prevent the scanning action of the methyltransferase; covalent bond formation between the methyltransferase and the modified-DNA.

Effect of carcinogens on DNA(cytosine-5)methylation in cells.

It has been shown previously that a number of carcinogens can inhibit DNA(cytosine-5)methylation using *in vitro* enzyme preparations and isolated DNA. However, it is important to determine whether these carcinogens can also inhibit DNA methylation in living cells and some pertinent examples are summarised in table 6.

In contrast to these results Krawisz and Lieberman (1984) showed that MNU and N-AcO-AAF did not cause any detectable change in the 5-methylcytosine content of newly replicated DNA in Raji cells (human lymphoblastoid cell line), S49 cells (a mouse thymic lymphoma cell line) and human diploid fibroblasts even at levels of damage that inhibited replication by 95%. Diala and Hoffman

Table 6. Inhibition of DNA methylation in living cells by chemical carcinogens.

Carcinogen	System	Reference
Ethionine	Novikoff rat hepatoma cell line.	Sneider *et al.* (1975)
	Regenerating rat liver.	Cox and Irving (1977).
	Murine Friend erythroleukemia cell line.	Christman *et al.* (1977)
MNU	Human Raji (Burkitts lymphoma) cell line.	Boehm and Drahovský (1981a)
Benzo(a) pyrene	murine embryo fibroblasts 3T3	Wilson and Jones (1983)
Temozolomide	Human leukemia cell line, K562.	Tisdale (1985)
	K562	Tisdale (1986)
	Human lymphoblastoid cell line GM892.	Tisdale (1989)
Ethazolastone	Human lymphoblastoid cell line GM892.	Tisdale (1985)
MNNG	Human Raji cell line (Burkitts lymphoma).	Boehm and Drahovský (1981b)

(1982) observed no change in the amount of 5-methylcytosine in the DNA of mouse embryo 3T3 cells after treatment with the carcinogens benzo(a)pyrene and methylcholanthrene.

Injection of the carcinogen dimethylnitrosamine into rats after partial hepatectomy, produced no effect on DNA(cytosine-5)methylation in rat liver, which is the target organ for the carcinogen (Craddock and Henderson, 1979). However, in this system MMS which does not induce liver tumours caused an increase in the level of DNA(cytosine-5)methylation.

Despite the fact that in living cells in culture and <u>in vivo</u> the effects on DNA(cytosine-5)methylation may not be obvious, a low level of inhibition of DNA(cytosine-5)methyltransferase may have occurred which remained undetectable in the total genome.

CHAPTER TWO

MATERIAL AND ANIMALS

2.1 Purchased Chemicals, Reagents, Materials and Animals.

Aldrich Chemical Company, Poole, Dorset

25% Tetrapropylammonium hydroxide solution in water (w/v)

Trimethylsulphonium iodide

2,6-diaminopurine

Amersham International, Amersham, Bucks, UK

S-Adenosyl-L-[methyl-^{3}H]methionine (83-85 Ci/mmol)

N-[^{3}H]Methyl-N-Nitrosourea (15 Ci/mmol)

T4 Polynucleotide kinase (*E.coli* B)

[Methyl-^{3}H]Thymidine (79 Ci/mmol)

[6-^{3}H]Uridine (17 Ci/mmol)

American Bionetics Inc. (ABN), Hayward, California, USA

O^6-methylguanosine cyanoethyl phosphoramidite

Bantin and Kingman Ltd., Hull, Humberside

BDF_1 Mice

BDH Chemicals Ltd., Poole, Dorset

Acetic acid, glacial (analar)

Ammonium formate (analar)

Ammonium sulphate (analar)

m-Cresol

Dimethyl sulphoxide (analar) (DMSO)

Diphenyloxazole (scintran) (PPO)

1,4-Di-2-(5-phenyloxazolyl)-benzene (scintran) (POPOP)

2-Ethoxyethanol

Ethylenediaminetetraacetic acid, disodium salt (EDTA)

2-Mercaptoethanol

N,N'-methylenebisacrylamide (electran)

Sodium acetate

Sodium dihydrogen orphophosphate (analar)

Sodium dodecyl sulphate (Biochemical grade)

Sodium phosphate (analar)

Trichloroacetic acid (analar)

Bio-Rad Laboratories Ltd., Watford, Herts.

Bradford dye reagent

Boehringer Corporation Ltd., Lewes, Sussex

S-Adenosyl-L-methionine, HSO_4 salt

DNA Molecular weight markers

Lambda phage DNA

Restriction Enzymes -HpaII, MspI

Fisons Scientific Equipment, Loughborough, Leics.

Acetic acid, glacial

Ammonia solution, specific gravity 0.88 (analytical grade)

Boric acid

Citric acid

Formic acid (analytical grade)

Hydrochloric acid (analytical grade)

2-Mercaptoethanol (biochemical grade)

Methanol (HPLC grade)

Optiphase 'Hisafe III' scintillation fluid

Optiphase 'MP' scintillation fluid

Perchloric acid (analytical grade)

Sodium cacodylate

Sodium chloride

Sodium hydroxide

Sucrose

Toluene (scintillation grade)

Fluka Chemicals Limited, Glossop, Derbyshire

Phenol

Polycytidylic acid, potassium salt (poly[C])

Polyguanylic acid, potassium salt (poly[G])

Gibco, Paisley, Scotland

Donor horse serum

Foetal calf serum

L-glutamine (200 mM)

Penicillin (10000 IU/ml) and streptomycin (10000 UG/ml)

RPMI 1640 (with 25 mM HEPES and L-glutamine)

Trypsin-EDTA solution (10 x)

IBF Biotechnics, Villeneuve-la-Garenne, France

Ultrogel ACA34

Lancaster Synthesis Limited, Morecambe, Lancs

Carboethoxymethyl dimethylsulphonium bromide

Dimethyl propargyl sulphonium bromide

Millipore UK Ltd., Harrow, Middlesex

Millipore 0.45 μm filters

New England Nuclear, Du Pont (UK) Ltd., Southampton, Hants.

Adenosine 5'-[gamma-^{32}P] triphosphate, triethylammonium salt (6000 Ci/mmol)

Nensorb 20 Nucleic Acid Purification Cartridges

Oxoid Laboratories Ltd., Basingstoke, Hants.

Phosphate buffered saline (PBS) tablets

Pharmacia LKB Biotechnology, Milton Keynes, Bucks.

Agarose -M

Polydeoxyguanylic acid, potassium salt (poly[dG])

Sigma Chemical Company Ltd., Poole, Dorset

Adenine

Albumin, bovine serum

p-Aminosalicylic acid

Blue dextran

Bromophenol blue

6-Chloroguanine

Cytosine

2'-Deoxycytidine, free base

Deoxyribonuclease I

Dithiothreitol

DNA, calf thymus

DNA, calf thymus (single-stranded)

DNA, M.lysodeikticus (1% RNA, 2-4% protein)

DNA, E.coli (1% RNA, 1-2% protein)

Dowex-50 H^+ form

Ethidium bromide

Ethyl methanesulphonate (EMS)

Glycerol

Glycine

Guanine

N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid] (HEPES)

8-Hydroxyquinoline

Imidazole

L-Methionine-S-methyl sulphonium iodide

3-Methyladenine

S-Methylcysteine

5-Methylcytosine

7-Methylguanine

Methyl methanesulphonate (MMS)

Ninhydrin

N-Nitroso-N-ethylurea (ENU)

N-Nitroso-N-methylurea (MNU)

Nonidet-NP40

Phenylmethylsulphonyl fluoride (PMSF)

Polyadenylic acid (5'), potassium salt (Poly[A])

Polydeoxyadenylic acid-polythymidylic acid, sodium salt (Poly[dA].Poly[dT])

Polydeoxyguanylic-polydeoxycytidylic acid, sodium salt (Poly[dG].Poly[dC])

Polyinosinic acid (5'), potassium salt (Poly[I])

Polyuridylic acid (5'), potassium salt (Poly[U])

Potassium chloride

Potassium hydroxide

Protein molecular weight markers cytochrome C, horse heart (12400), albumin, bovine serum (66000), β-amylase, sweet potato (200000), apoferritin, horse spleen (443000), carbonic anhydrase (29000)

Spermidine

Stannous chloride

N,N,N',N'-tetramethylethylenediamine (TEMED)

Thymidine

Thymine

Triethylamine

Tris(hydroxymethyl)aminomethane (Tris), base

Xylene cyanole

Whatman Labsales Ltd., Croydon, Surrey

Whatman 3MM chromatography paper

Whatman GF/C glass microfibre filters, 2.5 cm

Whatman DE52 anion exchanger

Whatman DE/81 2.3 cm filters

Whatman Partisil 10SCX 25 cm analytical column

2.2 Treatment of DNA obtained from Sigma

Due to considerable protein contamination in Calf Thymus DNA and significant quantities of protein in M.lysodeikticus DNA, all DNAs obtained from Sigma were purified before use.

The DNA was dissolved in 50 mM KCl at a concentration of 2.0 mg/ml. In polypropylene tubes, 0.1 volumes of 10% SDS was added to the DNA solution and to this was added an equal volume of phenol reagent. The mixture was vortexed for 5 min and then spun at 2000 rpm for 30 min using an Heraeus lab top centrifuge. The upper aqueous layer was then removed, taking care not to remove any of the interfacial material. The DNA was then precipitated by adding 1.5 volumes of 2-ethoxyethanol. The DNA was then pelleted by centrifugation at 4000 rpm for 10 min using an Heraeus lab top centrifuge. Depending on the amount of contaminating protein the above series of steps was repeated an appropriate number of times. The DNA was then washed three times with 70% (v/v) ethanol/ 2% (w/v) sodium acetate, two times with absolute ethanol and once with diethyl ether, by resuspending and sedimenting after each wash. After removing the excess ether by drying in a water-bath at 60^{o}C for 10 min, the DNA was suitable for use.

From experience this procedure significantly increases the ability of the DNA to behave as a substrate for DNA(cytosine-5)methyltransferase and was therefore routinely carried out.

2.3 DNA molecular weight markers

Lambda *Hind* III/*EcoRI* (kDa)

21226, 5148, 4973, 4277, 3530, 2027, 1904, 1584, 1330, 983, 831, 564, 125.

Lambda *Hind* III (kDa)

23130, 9416, 6557, 4361, 2322, 2027, 564, 125

2.4 Synthesis of O^6-methylguanine

O^6-Methylguanine was prepared by the method of Balsiger and Montgomery (1960) and Demple *et al*. (1983).

Sodium methoxide was prepared fresh by slowly adding 5.0 g sodium to 100 ml of redistilled absolute methanol. This freshly prepared solution of sodium methoxide was then slowly added to a flask containing 440 mg 6-chloroguanine. After the 6-chloroguanine had dissolved, the solution was refluxed for 12 hours. This was then cooled to 4°C and neutralised by the addition of 6.0 M HCl. Sodium chloride was then allowed to crystallise out by storing at -20°C over-night. The solution was then filtered and the solid precipitate was discarded and the filtrate was concentrated to dryness using a rotary evaporator with a water-bath temperature of 60°C.

The solid residue contains *O*^{6}-methylguanine and NaCl and this was dissolved in a minimum volume of boiling water, whilst stirring on a magnetic stirrer. This was then slowly cooled down to room temperature, allowing the *O*^{6}-methylguanine to crystallise out and

leaving the NaCl in solution. The $\underline{O}^6$-methylguanine was then separated by filtration and this process of recrystallisation was repeated a further three times, to ensure that all the NaCl had been removed.

It was verified by paper chromatography that all the 6-chloroguanine had been converted to $\underline{O}^6$-methylguanine, in which there was a good separation between 6-chloroguanine and $\underline{O}^6$-methylguanine.

Solvent system for paper chromatography,

Isopropanol: conc.NH_3: H_2O (7:1:2)

The $\underline{O}^6$-methylguanine was quantitated by its ultraviolet absorption, a neutral aqueous solution of A_{280} = 7.9 contains 1 mM $\underline{O}^6$-methylguanine.

2.5 Donated Chemicals

Mitozolomide, temozolomide and ethazolastone were synthesised and donated by May and Baker, Dagenham, England. They were dissolved in DMSO at a concentration of 20 mg/ml and were stored frozen at -20°C.

Chlorambucil was kindly donated by Professor M J Tisdale of Aston University.

L-methionine-$\underline{S}$-[^{3}H-methyl]methylsulphonium iodide (specific activity 70Ci/mmol) and a range of peptides was kindly donated by Mr G M Currie (British Technlogy Group student).

2.6 Stock Solutions and Buffers

Ethidium Bromide Reagent

Tris base	10 mM
EDTA	1 mM
Ethidium bromide	5.0 mg/l

Adjusted to pH 7.6 with conc. HCl

Cell Lysing Medium

Tris base	10 mM
EDTA, disodium salt	10 mM
nonidet NP-40	5.0 ml/l

Adjusted to pH 7.5 with conc. HCl

Buffer for Washing Nuclei

Tris base	20 mM
Sucrose	0.25 M

Adjusted to pH 7.8 with conc. HCl

0.9% Saline

Sodium chloride	9.000 g
Water	to 1.000 l

Low salt restriction buffer

Tris base	10 mM
$MgCl_2$	10 mM
β-mercaptoethanol	5 mM

Adjusted to pH 7.5 with conc. HCl

Buffer M

Tris base	50 mM
EDTA, disodium salt	1 mM
Dithiothreitol	1 mM
Glycerol	100 ml
Phenylmethylsulphonyl fluoride	60 mg/l
Distilled water	to 1.000 l

DNA Methylase Assay Buffer

Tris base	20 mM
EDTA, disodium salt	1 mM
Dithiothreitol	5 mM
Adjusted to pH 7.8 with conc. HCl	

5% TCA

Trichloroacetic acid	50.0 g
Distilled water	to 1.00 l

PPO/POPOP Scintillant

2,5-Diphenyloxazole (PPO)	4.0 g
1,4-Di-2-(5-phenyloxazolyl)-benzene (POPOP)	0.1 g
Toluene	to 1.00 l

Phenol Reagent

Phenol	500 g
Distilled water	62.0 ml
m-Cresol	62.0 ml
8-Hydroxyquinoline	0.62 g

Tris-Borate Electrophoresis (TBE) Buffer

(5x concentration)

Tris base	54.0 g/l
Boric acid	27.5 g/l
EDTA, disodium salt	3.7 g/l

Adjusted to pH 8.0

Agarose Gel Loading Buffer

(6x concentration)

Bromophenol blue	0.25 g
Xylene cyanole	0.25 g
Sucrose	40.0 g
Water	to 100 ml

Reagent A (for the Purification of Nucleic Acids using a Nensorb 20 cartridge)

Tris base	0.1 M
Triethylamine	10 mM
EDTA, disodium salt	1 mM

A stock buffer of 0.1 M Tris-HCl, 1 mM disodium EDTA, pH 7.7 was prepared. To 100 ml of stock buffer was added 140 μl triethylamine and this was adjusted to pH 7.7 with conc. HCl.

10x Kinase Buffer

Tris base	0.5 M
EDTA, disodium salt	1 mM
Magnesium Chloride	0.1 M
Dithiothreitol	50 mM
Spermidine	1 mM

Adjusted to pH 7.6 with conc. HCl.

TE (pH 7.9)

Tris base	10 mM
EDTA, disodium salt	1 mM

Adjusted to pH 7.9 with conc. HCl.

Phenol

The term 'phenol' was used to describe phenol which was equilibrated with TE (pH 7.9).

HEPES (DNA repair) buffer

HEPES	70 mM
dithiothreitol	10 mM
EDTA, disodium salt	1 mm
spermidine hydrochloride	50 μM

adjusted to pH 7.8 with 10 mM KOH

0.1 M Sodium Citrate Buffer (pH 2.7)

Solution A 0.1 M citric acid

Solution B 0.1 M sodium citrate

48.0 ml of solution A was mixed with 2.0 ml of solution B and made up to 100 ml with distilled water. Any adjustment in pH that was necessary to produce pH 2.7 was made using conc. HCl.

0.1 M Sodium Citrate Buffer containing 0.4 M NaCl (pH 3.0)

Solution A 0.1 M citric acid

Solution B 0.1 M sodium citrate

46.0 ml of solution A was mixed with 4.0 ml of solution B and made up to 100 ml with distilled water. Any adjustment in pH that was necessary to produce pH 3.0 was made using conc. HCl.

Ninhydrin Reagent

1) Citric acid buffer pH 5.0

205 ml of 0.2 M citric acid

295 ml of 0.2 M sodium citrate

Adjusted to pH 5.0 with 10.0 M NaOH

2) Dissolve 0.80 g of $SnCl_2.2H_2O$ in 500 ml of 0.2 M citric acid pH 5.0

Add this to 20 g of ninhydrin dissolved in 500 ml of methoxyethanol. Bubble N_2 (g) through for 30 min.

Buffer I

Tris base	50 mM
dithiothreitol	3 mM
EDTA, disodium salt	1 mM
BSA	1 mg/ml

adjusted to pH 8.3 with conc. HCl.

2 x HEPES - buffered saline (HeBS)

NaCl	16.4 g
HEPES acid	11.9 g
Na_2HPO_4	0.21 g

The solution was made up to 1.000 l and titrated to pH 7.05 with 5.0 M NaOH and then filter sterilised using a 0.2 μM Acrodisc filter (Gelman Sciences, U.K.).

Incubation Buffer (Protein/Nucleic Acid Binding Assay)

Imidazole	0.1 mM
EDTA, disodium salt	20 mM
Dithiothreitol	0.5 mM
S-Adenosyl-L-methionine	10 μM

Imidazole was stored as a 10 mM stock and S-adenosyl-L-methionine was stored as a 1 mM stock, 10.0 ml of each gave the appropriate concentration when made up to 1.000 l.

Adjusted to pH 7.5 with conc. HCl.

0.6% Sarkosyl + 0.5 M NaCl

N-Lauroylsarcosine, sodium salt	6.0 g
Sodium chloride	29.2 g

Made up to 1.000 l with distilled water.

0.5% SDS

Sodium dodecyl sulphate	5.0 g
Distilled water	to 1.000 l

EDTA Solution pH 10.0 (Alkaline Elution)

EDTA, disodium salt	20 mM
Sodium hydroxide	40 mM

Adjusted to pH 10.0 by the addition of conc. HCl.

Alkaline Elution Solution

EDTA, disodium salt	5.8 g
Tetrapropylammonium hydroxide	70.0 g
Distilled water	to 1.000 l

Adjusted to pH 12.1 by the addition of a high pH alkaline elution solution.

Acrylamide Stock I Solution

Acrylamide	44.0 g
N,N'-methylenebisacrylamide	0.8 g
Distilled water	to 100 ml

The solution was filtered through a Millipore 0.45 μM filter and then stored in the dark at +4^{o}C.

Acrylamide Stock II Solution

Acrylamide	30.0 g
N,N'-methylenebisacrylamide	0.8 g
Distilled water	to 100 ml

The solution was filtered through a Millipore 0.45 μM filter and then stored in the dark at +4°C.

Reservoir Buffer Stock (10x)

Tris base	0.25 M
Glycine	1.92 M
SDS	1.0%

Adjusted to pH 8.3 with conc. HCl.

When required this solution was diluted 10 x.

SDS-PAGE Loading Buffer (5x)

Tris base	0.315 M
SDS	10%
β-Mercaptoethanol	25%
Glycerol	50%
Bromophenol blue	0.5%

Adjusted to pH 6.8 with conc. HCl.

The appropriate volume of this solution was added to the sample and diluted with water so that the final volume is 5 x greater.

Coomassie Blue Gel Stain

Coomassie brilliant blue R	1.37 g
Methanol	250 ml
Glacial acetic acid	50 ml
Distilled water	250 ml

Gel destain

Acetic acid	70 ml
methanol	500 ml
Distilled water	to 1.000 l

Electroblot Buffer

Tris base	3.03 g
Glycine	14.41 g
Methanol	200 ml

Adjusted to pH 8.8 with conc. HCl.

CHAPTER THREE:

THE PREPARATION AND CHARACTERISATION OF DNA(CYTOSINE-5)METHYLTRANSFERASE

3.1 METHODS

3.1.1 The Preparation of DNA(cytosine-5)methyltransferase

The procedure was based on the method described by Turnbull and Adams (1976), with modifications. The DNA(cytosine-5)-methyltransferase used in these studies was prepared from the L1210 murine leukemia tumour, which was grown *in vivo* or *in vitro*.

Growth of L1210 in vitro

This cell line was maintained as a suspension culture in exponential growth at a density between 0.8×10^5 and 1.0×10^6 cells/ml. Cells were grown in RPMI 1640 medium (with 25 mM HEPES and L-glutamine), supplemented with 10% horse serum, under an atmosphere of 5% CO_2 in air. Under these conditions the cells were found to have a doubling time of 12-14 h.

Growth of L1210 in vivo

The growth of the L1210 tumour *in vivo* was carried out by Mr M P Wynter and Mr W A Fleary from Aston University. 2.0×10^5 Cells in a total volume of 0.1 ml were implanted i.p. and grown for seven days. The mice used were the BDF_1 strain of a weight between 20-25 g and both males and females were used. After seven days the tumour was removed and suspended in 0.9% saline.

Partial purification of DNA(cytosine-5)methyl-transferase

The nuclei were first of all prepared using the following procedure.

The cells were washed in 0.9% saline at 4°C and then pelleted by centrifugation at 1500 rpm for 5 min in an Heraeus bench top centrifuge. The cells were then suspended in 10 ml of cell lysing medium and homogenised using a Dounce teflon homogeniser for 1 min. The nuclei were then pelleted by centrifugation at 4000 rpm for 20 min using an Heraeus bench top centrifuge. The nuclei were then washed by resuspending and resedimentation in 0.25 M sucrose buffer. The nuclei were then suspended in 10 volumes of buffer M containing 0.4 M NaCl. If the nuclei were not to be used immediately they were stored frozen at -70°C, in which state the methyltransferase activity was maintained for at least 1 month. Glycerol present in the buffer at 10% acted as a cryogenic preservative.

During the preparation of DNA(cytosine-5)methyl-transferase, all the procedures were carried out on ice at 4°C. The nuclei were suspended in 10 volumes of buffer M containing 0.4 M NaCl and stirred on ice for 1 hour. Approximately 2 ml of DE52 slurry in buffer M containing 0.4 M NaCl was added to bind the DNA, stirred for a further 5

min and was then centrifuged at 3000 rpm for 5 min to remove the DNA. The supernatant was then made 30% saturated with respect to ammonium sulphate and stirred at 4°C for 15 min. This was then centrifuged at 12000 rpm for 30 min at 4°C, using an AP Pegasus 65 centrifuge. The supernatant was then made to 60% saturation with respect to ammonium sulphate and stirred at 4°C for 15 min. The suspension was then centrifuged at 12000 rpm for a further 30 min at 4°C, using an AP Pegasus 65 centrifuge, after which the supernatant was discarded and the precipitate was redissolved in a minimum volume (less than 1 ml) of buffer M containing 0.4 M NaCl.

This preparation was then applied to a column of Ultrogel AcA34 (dimensions 350 mm x 25 mm) and the enzyme was eluted with buffer M containing 0.4 M NaCl, collecting 1 ml fractions using a Pharmacia FRAC-100 programmable fraction collector. The fractions after the void volume (which had been previously determined using a solution of dextran blue and sucrose), were assayed for DNA(cytosine-5)methyltransferase activity using 10 µl of each fraction in the standard DNA(cytosine-5)methyltransferase assay (section 3.1.3). The peak fractions of highest activity (see fig. 4) were then pooled and dialysed against buffer M containing 50% glycerol for 6 hours. This material was then stored at -20°C and used as the enzyme preparation in this study.

3.1.2 Determination of the approximate molecular weight of DNA(cytosine-5)methyltransferase.

The column of Ultrogel AcA34 was calibrated against the following standards of indicated molecular weight - apoferritin (443 kDa), β-amylase (200 kDa), albumin (66 kDa) and measuring the absorbance at 280 nm. The void volume (Vo) of the column was determined using blue dextran. By plotting the log mr versus Ve/Vo, where Ve = elution volume, a straight line was produced for the standards. Using this formula, the approximate molecular weight of DNA(cytosine-5)methyltransferase was determined from its elution volume (data not shown).

3.1.3 DNA(cytosine-5)methyltransferase assay.

Each assay contained 20 μg of M.lysodeikticus DNA (2.0 mg/ml in water), 1.0 μCi S-adenosyl-L-[methyl-^{3}H] methionine (83-85 Ci/mmol) and the DNA (cytosine-5)methyltransferase preparation made up to 100 μl with assay buffer. After incubation at 37°C for 4 h in a shaking water-bath, the reaction was stopped by adding 40 μg calf thymus DNA (2.0 mg/ml) to act as a carrier and 200 μl of 1.0 M NaOH. The mixtures were then heated to 65°C for 20 min in a water-bath, after which they were placed on ice and the DNA was precipitated by adding 80 μl of 5.0 N $HClO_4$. After at least 1 h the DNA was filtered onto a Whatman GF/C glass microfibre filter and washed

with cold 5% TCA (w/v) and absolute ethanol. After air drying the filters overnight, the radioactivity was determined by adding 8 ml of toluene/PPO/POPOP scintillant and counting on a Packard Tri-Carb 2000CA liquid scintillation counter.

3.1.4 SDS-Polyacrylamide gel electrophoresis

Preparation of SDS-polyacrylamide gels for electrophoresis.

A 7.5% running gel was prepared by mixing the following:

Acrylamide stock 1 solution	7.5 ml
1.5 M Tris.Cl, pH 8.8	15.0 ml
10% SDS (w/v)	1.2 ml
Distilled water	17.5 ml

The mixture was degassed under vacuum and then polymerisation was initiated by adding 112 μl of TEMED and 160 μl of a 10% (w/v) aqueous solution of ammonium persulphate, with gentle stirring. Using a 25 ml syringe, the mixture was immediately poured between the plates of a Biorad Protean 16 cm electrophoresis system and left for 1 h to polymerise. A layer of butanol was layered on the top of the gel to maintain a straight surface.

The stacking gel was prepared by mixing the following:

Acrylamide stock II	3.0 ml
0.5 M Tris.Cl, pH 6.8	4.5 ml

10% SDS	180 µl
Distilled water	9.6 ml

This mixture was degassed under vacuum and 48 µl of TEMED and 60 µl of a 10% (w/v) aqueous solution of ammonium persulphate was added with gentle stirring. The mixture was then poured immediately onto the top of the running gel using a syringe until the substance was 1 cm from the top of the plate. A comb with 5 mm teeth was inserted between the plates to provide the wells. The gel was left for a further 2 h to polymerise after which the comb could be removed and the gel covered with reservoir buffer.

Preparation and running of protein samples on SDS-PAGE

The protein concentrations of the samples were calculated by the Bradford method (Bradford, 1976). The appropriate volume of loading buffer was mixed with 40 µg of the protein sample and then heated for 10 min at 100°C in a water-bath to denature the proteins, followed by centrifugation for 30 s using a microfuge to produce an homogenous sample. The samples were then loaded onto the gel as well as molecular weight markers for comparison. Electrophoresis through the stacking gel was at the constant voltage of 150 V and 250 V through the resolving gel, until the blue dye front was 1 cm from the bottom of the gel.

The gel was then fixed in a 50% TCA (w/v) solution for 10 min, until the blue dye front turned yellow and then stained for 1 h in coomassie blue gel stain. The gel was then placed in destain until the background was almost colourless and the protein bands were clearly visible.

3.1.5 DNA base analysis by high performance liquid chromatography (HPLC).

After incubation of M.lysodeikticus DNA in the standard DNA(cytosine-5)methyltransferase assay, the DNA was precipitated by adding 0.1 volumes of 2.5 M sodium acetate and 2.5 volumes of cold absolute ethanol. The DNA was then pelleted by centrifugation at 3000 rpm for 10 min using an Heraeus bench top centrifuge and washed by resuspending and then resedimentation at 3000 rpm using an Heraeus bench top centrifuge, three times with 70% (v/v) ethanol, 2% (w/v) sodium acetate and two times with absolute ethanol and then air dried.

DNA hydrolysis

The DNA (100 μg) was placed in a pyrex glass tube (150 x 18 mm), to which was added 1-2 ml of 90% formic acid and the tube was then sealed using an oxygen/natural gas flame torch. The tube was then placed within a metal cage and immersed into a stirring oil bath at 180^{o}C. Hydrolysis of the DNA was carried out for 25 min, after which the tubes

were removed and allowed to cool for at least 30 min. The seal was then broken and the formic acid was removed under a stream of $N_2(g)$ whilst warming in a 60°C water-bath.

Isocratic method

HPLC separations were performed on a system consisting of a Waters Maxima 820 Chromatography Workstation, a Waters model 510 pump, a Waters Lambda-Max model 480 Spectrophotometer (at 254 nm) and a Waters automatic sample injector WISP 710B. A Whatman Partisil 10SCX column was used with an isocratic mobile phase of 0.04M ammonium formate pH 2.0. The flow rate was 1.0 ml/min with a run time of 10 min. All buffers were degassed under vacuum immediately prior to use.

The residue was then redissolved in 0.1 M HCl and the equivalent of 20 μg of DNA was injected onto the HPLC column. Fractions were collected every 30 s directly into liquid scintillation glass vials using a Pharmacia FRAC-100 programmable fraction collector and the counts were determined in either Optiphase MP or Optiphase 'HiSafeII' scintillation fluid.

3.1.6 Determination of protein by the Bradford method

This assay was based on the method described by Bradford (1976).

From a stock solution of BSA (0.1 mg/ml) a calibration curve for a range of protein concentrations was produced by dilution with water, 0.2-25 μg/ml. To 0.8 ml of protein solution was added 0.2 ml of Bradford reagent, which was then vortexed and then read on a spectrophotometer at 595 nm against a blank containing water in place of the protein solution.

3.1.7 Statistical analysis

All results are expressed as mean ± standard error of the mean (SEM) for at least three separate determinations.

3.2 RESULTS AND DISCUSSION

The murine leukemia L1210 cell line was used in these studies as the source of DNA(cytosine-5)methyltransferase, because it is a fast growing tumour *in vivo* and large quantities of material could therefore be quickly attained. It is also a tumour that is routinely used within the Laboratories at Aston University and consequently expertise existed amongst the staff for its maintenance.

The enzyme from this tumour has not previously been described but a purification scheme based on the method of Turnbull and Adams (1976) was used with modifications. In particular, the latter stages of purification including phosphocellulose chromatography were not included. Although attempts were made to purify the enzyme further, these were abandoned due to substantial loss of enzyme activity. DNA(cytosine-5)methyltransferase is an unstable enzyme and activity was lost during the preparation due to the long procedures and the time to assay fractions before continuing to the next step in the protocol. A number of steps were taken to maintain enzyme activity.

Phenylmethylsulphonyl fluoride (PMSF) is a protease inhibitor and was included in all stages of the purification. Dithiothreitol was included to reduce oxidation of thiol groups within the active site of the enzyme and the enzyme preparation was stored at -20°C in 50% glycerol. Even taking these

precautions, the enzyme preparation had to be used within three days, due to the rapid reduction in enzyme activity with time.

In order to maximise activity and reduce the amount of contaminating protein, only the peak of enzyme activity from the Ultrogel AcA34 column was used (fig. 4). However, it can be seen from the SDS-PAGE profile of the enzyme used in this study (fig. 5), that a large number of components are present. The intensity profile from the densitometer trace (fig. 6), confirms that the enzyme preparation is impure.

It was attempted to extend the purification of the DNA(cytosine-5)methyltransferase beyond that illustrated in fig. 5, but various procedures such as the use of phosphocellulose and DEAE cellulose resulted in enormous losses of enzyme activity. The use of dye ligand chromatography with Cibacron blue F3GA agarose, as used by Bestor and Ingram (1983) was not attempted and may be a useful 'next step' for any future work.

The approximate molecular weight of the enzyme was determined by the crude method of determining the elution volume of the enzyme on a column of Ultrogel AcA34 calibrated with a range of standard proteins (albumin, bovine serum (66 kDa), β-amylase, sweet potato (200 kDa) and apoferritin, horse spleen (443 kDa). The void volume (Vo) of the column was determined using blue dextran ($2x10^6$ Da). By plotting

log mr versus Ve/Vo, where Ve = elution volume, a straight line was produced for the standards. Using this formula, the approximate molecular weight of DNA(cytosine-5)methyltransferase after purification on the Ultrogel AcA34 column, was shown to be approximately 95 kDa. The SDS-PAGE profile of the enzyme (fig. 5), shows the enzyme preparation to be very impure with a large number of bands present. One of the prominent bands has a molecular weight within the approximate region of 95 kDA.

Ideally, the SDS-PAGE gel should have been run immediately after the final purification step. However, due to limitations on time the polyacrylamide gel was run between 36-48 h after the final enzyme preparation step. Therefore, it is possible that some proteolytic degradation of DNA(cytosine-5)methyltransferase may have occurred. The two most prominent high molecular weight bands have a molecular weight of approximately 120 kDa and 90 kDa.

A wide variation in the molecular weight of DNA(cytosine-5)methyltransferase from various sources has been reported by other workers. The one reported here is slightly lower than other mammalian DNA(cytosine-5)methyltransferases so far characterised, such as bovine thymus cells Mr 130 kDa (Sano et al., 1983), human placenta Mr 120 kDa (Yoo et al., 1987) or Mr 135 kDa (Pfeifer et al., 1983) and HeLa cells Mr 120 kDa (Roy and Weissbach, 1975). None of

these researchers present any information regarding the electrophoretic purity of their particular enzyme preparations.

A number of reports do present photographs of SDS polyacrylamide gels of homogenous enzyme preparations. This includes murine erythroleukemia cells (Bestor and Ingram, 1983) in which two species of DNA(cytosine-5)methyltransferase (molecular weight 150 and 175 kDa) were resolved by the technique of dye-ligand chromatography and mouse ascites tumour cells (Adams et al., 1986) in which two species of DNA(cytosine-5)methyltransferase were resolved of molecular weight 160 and 185 kDa, by a complex series of protein purification steps which included the use of tRNA-sephadex. The DNA(cytosine-5)methyltransferase preparation from a Novikoff rat hepatoma cell line (Sneider et al., 1975) was clearly very impure from the SDS-PAGE gel that they present.

These reports where highly purified DNA(cytosine-5)methyltransferase were prepared, use the enzyme in only a few limited enzyme studies. The expense in terms of time and resources is likely to be very large. A notable example would be the case of DNA(cytosine-5)methyltransferase from MEL cells (Bestor et al., 1988), which was purified to homogeneity for sequencing purposes. In this case MEL cells were grown to approximately 1.0×10^6 cells/ml in 300 l of Dulbecco's minimal essential

medium supplemented with 3% (v/v) foetal calf serum and 5% (v/v) horse serum to yield 450 μg of pure protein.

Therefore, although I have presented evidence indicating that the enzyme preparation used in these studies was impure, it is likely to be no worse than the majority of other workers who have studied the *in vitro* properties of DNA(cytosine-5)methyltransferase.

The specific activity of DNA(cytosine-5)methyltransferase varied from 1500-3000 pmol/mg protein, under the conditions of the assay which were for 20 μg *M.lysodeikticus* DNA with a 4 h incubation (APPENDIX 1). This value is comparable to that used by other workers. The eukaryotic DNA(cytosine-5)methyltransferase (prepared from mouse KrebsII ascites tumour cells) previously commercially available from Amersham International plc, had a reported specific activity of 3000 pmol/mg protein (in the assay 20 μg *E.coli* DNA were incubated for 1 h with the enzyme). The DNA(cytosine-5)methyltransferase from P815 mouse mastocytoma cells or human placentas, as used by Palitti *et al*. (1987) had a specific activity of 2100 units/mg protein and 400-500 units/mg protein respectively (in the 1 h assay *M.luteus* DNA was at a concentration of 12 μg/ml). The enzyme preparation from murine ascites cells (Turnbull and Adams, 1976) had a specific activity of 643 units/mg protein (in the assay 40 μg *E.coli* DNA were incubated for 1 h with the enzyme), whilst the

highly purified enzyme from the same cells (Adams et al., 1986) had a specific activity of 11500 units/mg protein (in the assay 5 μg denatured M.luteus DNA was incubated for 1 h with the enzyme).

Therefore, taking account of the differences in DNA substrates and the length of time that incubations were performed, the specific activity of the enzyme preparation used in these studies was comparable to that used by other workers. This does not include the highly purified forms of enzyme reported by some workers.

Characterisation of DNA(cytosine-5)methyltransferase

In the early stages of the project an assessment was made of various nucleic acid substrates, in order to identify a DNA substrate with a high methyl acceptance ability, that could be used as the standard substrate throughout the work. The intention was to minimise any variation in protein/DNA interactions that may occur between the DNA(cytosine-5)methyltransferase and the DNA substrate. The results are presented in table 7 and M.lysodeikticus DNA appears as the best substrate with the conditions used.

An assessment was also made of the DNA substrates in single-stranded form. Single-stranded regions in the DNA were produced by placing an aqueous solution of the DNA in a water-bath at 100°C for 10 min and then rapidly cooling on ice.

The results in table 7 show that the heat-denatured DNA is a better substrate than the native form, but to differing extents depending on the substrate. Human (Raji cell line) DNA was shown not to be a substrate, presumably due to there being few available methylatable cytosines. E.coli DNA shows little variation after heat denaturing, whilst salmon testes DNA, calf thymus DNA and M.lysodeikticus DNA are clearly better substrates after heat denaturing.

The effect of using a heat-denatured DNA substrate has been shown by other workers to have various effects on its ability to behave as a substrate for DNA(cytosine-5)methyltransferase. Other factors are likely to influence this interaction, such as the source of the enzyme, its purity and the 'quality' of the DNA substrate. Adams et al. (1979) using DNA(cytosine-5)methyltransferase from mouse Krebs II ascites cells (molecular weight 184 kDa) studied the effect of heat denaturing on the ability of a range of DNA substrates to behave as substrates for the enzyme. They showed that native calf thymus DNA and Xenopus laevis DNA are better substrates than their heat-denatured counterparts. There was very little change with bacteriophage DNA after heat treatment. Heat-denatured mosquito (Aedes albopictus) and E.coli DNA were shown to be better substrates than their respective native DNAs.

Roy and Weissbach (1975) using DNA(cytosine-5)methyltransferase isolated from HeLa cells showed that the enzyme methylates single-stranded DNA (M.luteus, E.coli and HeLa cell) to an extent up to three times greater than that of the corresponding double-stranded DNA. Pfeifer et al. (1983) using DNA(cytosine-5)methyltransferase isolated from human placenta and Sneider et al. (1975) using enzyme from a Novikoff rat hepatoma cell line also showed that the enzyme methylates heat-denatured DNA better than native DNA.

Turnbull and Adams (1976) treated E.coli, salmon testes and calf thymus DNA with deoxyribonuclease from N.crassa, which degrades only single-stranded DNA. DNA treated with this enzyme was compared with native DNA and it was shown that DNA(cytosine-5)methyltransferase from mouse Krebs II ascites cells (molecular weight 80 kDa) preferentially methylates single-stranded regions in native E.coli DNA but for eukaryotic DNA, methylation occurred in double-stranded regions. This enzyme shares characteristics with the enzyme used in these studies.

Therefore, the source of DNA appears to be important in determining whether the single- or double-stranded form is the preferred substrate for DNA(cytosine-5)methyltransferase. The concentration of DNA has been shown by Palitti et al. (1987) to be important in determining whether single-stranded or double-stranded DNA was the best substrate for

DNA(cytosine-5)methyltransferase prepared from human placenta or P815 mouse mastocytoma cells. At concentrations of 10 μg/ml and 50 μg/ml double-stranded M.luteus DNA was a better substrate than single-stranded DNA, whilst at a concentration of 300 μg/ml single-stranded M.luteus DNA was a better substrate than double-stranded DNA. In my experiments the concentration of DNA used was 200 μg/ml and similar to the results of Palitti et al. (1987) at a concentration of 300 μg/ml, single-stranded M.luteus DNA was a better substrate than double-stranded DNA. The important observation made by Palitti et al. (1987) that the ability of single-stranded or double-stranded DNA to behave as a substrate for DNA(cytosine-5)methyltransferase varies with the concentration of DNA used in the assay, was not investigated in this thesis. These authors make the conclusion that the interpretation of experiments from laboratories in which the extent of DNA methylation was expressed at a particular concentration of either double-stranded or single-stranded DNA should now be questioned in light of the effect that DNA concentration can have.

However, the purpose in this thesis of assaying various DNA substrates was to identify a DNA substrate with the greatest methyl acceptance ability that could be used throughout as a standard substrate. Therefore, in order to reduce any

variations that heat denaturing may have and in order to maximise methyl acceptance ability, native M.lysodeikticus DNA was used.

The reason why there are wide variations in the ability of various DNAs to behave as substrates for DNA(cytosine-5)methyltransferase, is presumably due to the differences in the molar proportions of the bases in DNAs from different species. M.lysodeikticus DNA has a high content of C and G (72%), whilst calf thymus DNA, salmon sperm DNA and E.coli DNA have a C and G content of 40%, 44% and 51% respectively (Davidson, 1965). The high G and C content of M.lysodeikticus is therefore likely to be the reason why it is a good acceptor of methyl groups from SAM and DNA(cytosine-5)methyltransferase and why it is widely used as a substrate in such studies.

Some basic experiments were then conducted to determine whether the methyltransferase preparation had any DNA(cytosine-5)methyltransferase activity and to confirm that the in vitro activity of the enzyme was dependent on the level of the substrates, DNA and S-adenosyl-L-methionine. It was not proposed to carry out a complex characterisation study of the enzyme, as this has been done by other workers (for example, Adams et al., 1979; Palitti et al., 1987).

The results in figures 7, 8, 9, 10, characterise the enzyme with respect to the concentration of S-adenosyl-L-methionine, enzyme, DNA and time respectively. As DNA(cytosine-5)methyltransferase

has two substrates, DNA and S-adenosyl-L-methionine and the activity can vary depending on the nature of the DNA, it was important to determine optimum conditions for future experiments and reduce any variations.

Figure 7 is a graph of the relationship between the concentration of SAM versus the methyl groups transferred to 20 μg DNA per mg of protein in the methyltransferase preparation. A straight line was produced with no plateau in the graph with concentrations of SAM up to 10 μM. Attempts at deriving a Km value from a Lineweaver-Burk plot had to be abandoned as there were not sufficient points to generate an accurate estimate. The y axis has the units of pmol-methyls transferred/mg protein in the methyltransferase preparation, as an investigation was being made of the ability of the enzyme measured in terms of protein to transfer methyl groups from SAM to the DNA. Therefore, it was decided that future experiments should be conducted with the maximum specific activity of SAM within reasonable economic limits and consequently 1 μCi (12.5 pmol) were used in each assay.

Figure 8 shows the relationship between the mass of enzyme protein versus the amount of methyl groups transferred from 1 μCi (12.5 pmol) SAM to 20 μg DNA which was expressed as pmol/mg DNA. This experiment was limited by the amount of enzyme preparation that could be put into a 100 μl incubation mixture. Under

the conditions of the assay using 20 μg DNA and 1 μCi SAM (12.5 pmol) a curve was produced, which did not reach a plateau for methyl incorporation. Therefore, in the following experiments in this thesis the amount of enzyme that was used in each assay was kept constant for a particular experiment or series of experiments. This amount of enzyme was based on its ability to transfer methyl groups during standard conditions, i.e. from 1 μCi SAM to 20 μg DNA during a 4 h incubation period and was not based on a standard amount of protein. This was necessary as the activity of the enzyme per unit protein dropped over a period of days.

Figure 9 is a graph to show the relationship between the mass of DNA and the methyls transferred from 1 μCi SAM (12.5 pmol) by a fixed amount of enzyme (mass of protein not recorded). Under the conditions used, incorporation of ^{3}H-methyl groups plateaued at approximately 10 μg DNA. Therefore, in order that DNA was not a limiting factor 20 μg of DNA were used in most enzyme assays.

Figure 10 is a graph to show the relationship between the methyls transferred to 20 μg DNA by 1 μCi (12.5 pmol) SAM by a fixed amount of enzyme over various time periods. In this graph, a lag period of approximately 0.5 h was observed followed by linearity in the reaction for about 2 h. After 3 h the curve had plateaued and no more ^{3}H-methyl groups were incorporated. In order that time was not a

limiting factor in the reaction, most incubations in the following experiments were carried out for 4 h. Turnbull and Adams (1976) observed methylation of calf thymus DNA occurring for 50 h without addition of further enzyme. However, they used 40 μg calf thymus DNA and 3.3 μCi SAM (1 μCi/nmole).

Before the enzyme preparation could be described as having DNA(cytosine-5)methyltransferase activity, it was important to be certain that the only product of reaction in DNA was 5-methylcytosine. When M.lysodeikticus DNA was methylated by the methyltransferase and S-adenosyl-[^{3}H-methyl]-L-methionine, isolated and hydrolysed to the bases, it can be seen from fig. 11 that the incorporated radioactivity co-chromotographs with the 5-methylcytosine marker (see APPENDIX 2 for the separation of the bases by HPLC). A small amount of thymine occurs which is the product of deamination of the 5-methylcytosine at the 4-position. The thymine represents approximately 6% of the total activity.

Incubation of M.lysodeikticus DNA with the methyltransferase in the presence of sodium chloride produced a 50% inhibition of the methylation reaction at a concentration of 150 mM (fig. 12). However, low concentrations of sodium chloride caused a marked stimulatory effect up to 90 mM. This result is similar to that obtained by Drahovský and Morris (1971) using E.coli DNA and Turnbull and Adams (1976) for heat-denatured calf thymus DNA (90 mM NaCl did

not produce an increase in the methylation of native calf thymus DNA). This suggests that M.lysodeikticus DNA has a proportion of single-stranded regions like E.coli DNA and heat-denatured calf thymus DNA, and the enzyme methylates the DNA mainly in these regions.

The enzyme is stimulated maximally towards M.lysodeikticus DNA by 90 mM NaCl, probably caused by disaggregation of the enzyme to active monomer, as suggested by Adams et al. (1979). The enzyme requires regions of single-stranded DNA with which to interact (Drahovský and Morris, 1971) and these are available when the duplex 'breathes', stimulated by low salt and high temperature (Adams et al., 1979). Salt stabilises the DNA double helix by preventing unwinding, which as suggested by Drahovský and Morris (1971) is a pre-requisite for methylation. The stimulatory effect observed using NaCl up to 90 mM caused by disaggregation of the enzyme is opposed by concentrations of NaCl above 0.1 M when the NaCl prevents breathing of the DNA. The enzyme was extracted in the presence of 0.4 M NaCl and in the absence of salt, aggregation of the enzyme may occur to produce higher molecular weight enzyme.

Therefore, the standard assay conditions for further experiments on this enzyme preparation were 20 μg M.lysodeikticus DNA, 1 μCi SAM (12.5 pmol) and a 4 h incubation period. These parameters are

similar to the suggested assay conditions for eukaryotic DNA methylase from Amersham International and similar to those used by Tisdale (1988, 1989).

It must be emphasised that there are a number of problems associated with the use of this enzyme preparation. Firstly, the enzyme used in these studies is isolated from murine tumour tissue and may have characteristics different from DNA(cytosine-5)methyltransferase isolated from normal tissue. Tumour tissue is often used as the source of the enzyme (for example Sneider et al., 1975; Bestor and Ingram, 1983; Adams et al., 1979; Adams et al., 1986; Bolden et al., 1986a) because higher levels are frequently present (Lapeyre et al., 1981; Kautiainen and Jones, 1986).

Secondly, the enzyme preparation was impure. Proteins affecting the methylation of DNA may be present in the enzyme preparation such as methylated DNA binding protein (Huang et al., 1984; Meehan et al., 1989) which bind to areas rich in 5-methyl-cytosine. The first attempts have been made to prepare homogenous mammalian DNA(cytosine-5)methyl-transferase using the techniques of recombinant DNA technology, by having completed the cloning and sequencing of the cDNA encoding DNA(cytosine-5)methyltransferase of mouse cells (Bestor et al., 1988). However, the incorporation of this gene into

cells for over production has proved to be extremely toxic to the cells (Bestor, T. personal communication).

Thirdly, as well as being impure, the enzyme used may be a product of partial proteolysis of the enzyme present *in vivo*. The mouse ascites DNA(cytosine-5)methyltransferase purified by Adams *et al*. (1986) may provide the best comparison with this enzyme, which has a molecular weight of 185 kDa. Proteolytic degradation of this enzyme gave polypeptides of 170, 100 and 50 kDa. It has been suggested (Adams *et al*., 1983; Adams *et al*., 1986) that proteolysis of the enzyme may remove a domain of the enzyme, such as to increase the capacity of the enzyme to carry out *de novo* methylation of the double-stranded DNA. Therefore, the *in vitro* observations may not reflect the *in vivo* situation, but specific proteolysis may occur *in vivo* when a switch from maintenance to *de novo* methylation occurs. The experiments reported in this thesis are a study of the *de novo* activity of DNA(cytosine-5)methyltransferase.

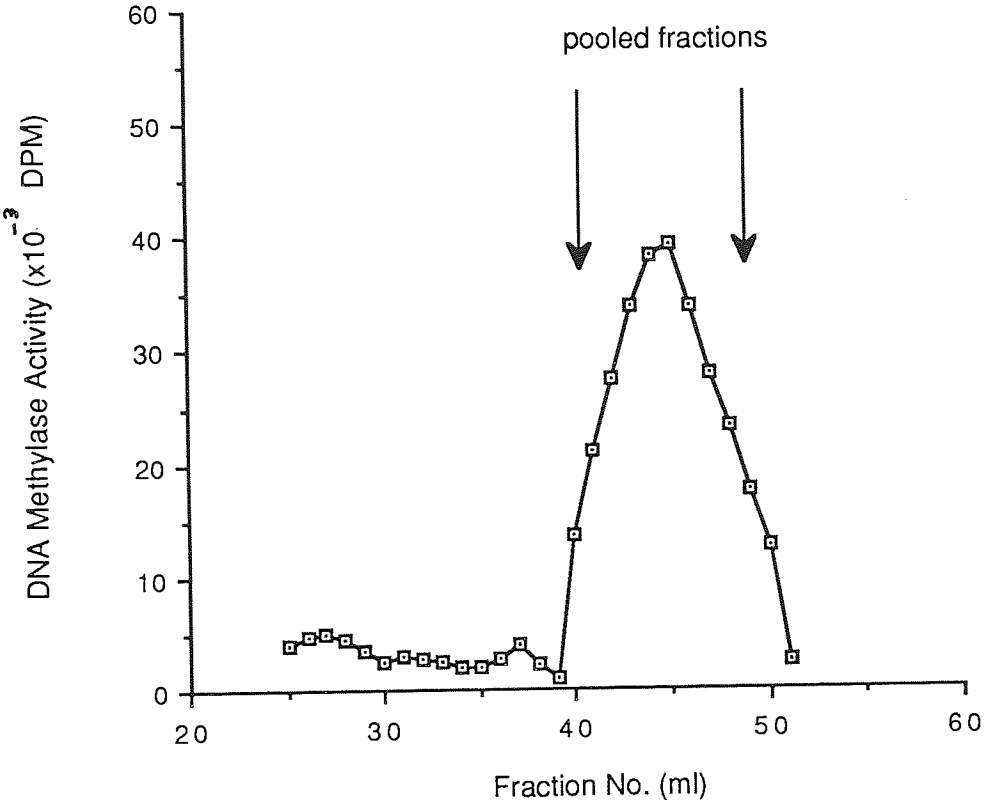


Figure 4: Ultrogel AcA34 column chromatography of DNA(cytosine-5) methyltransferase.
The chromatography was carried out using buffer M containing 0.4 M NaCl and 1.0 ml fractions were collected, of which 10 µl of each fraction was assayed for DNA(cytosine-5)methyltransferase activity. This graph is representative of a typical methyltransferase preparation.

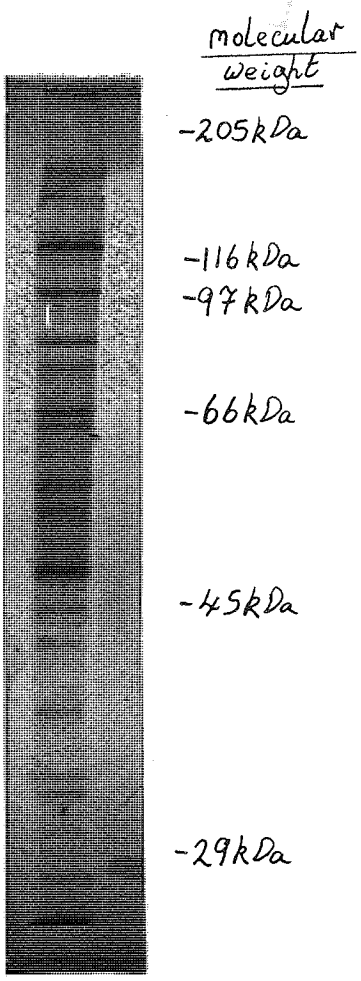


Figure 5: SDS-Polyacrylamide gel electrophoresis of partially purified DNA(cytosine-5)methyltransferase.
The concentration of acrylamide was 7.5%. This gel was run approximately two days after the methyltransferase was prepared.

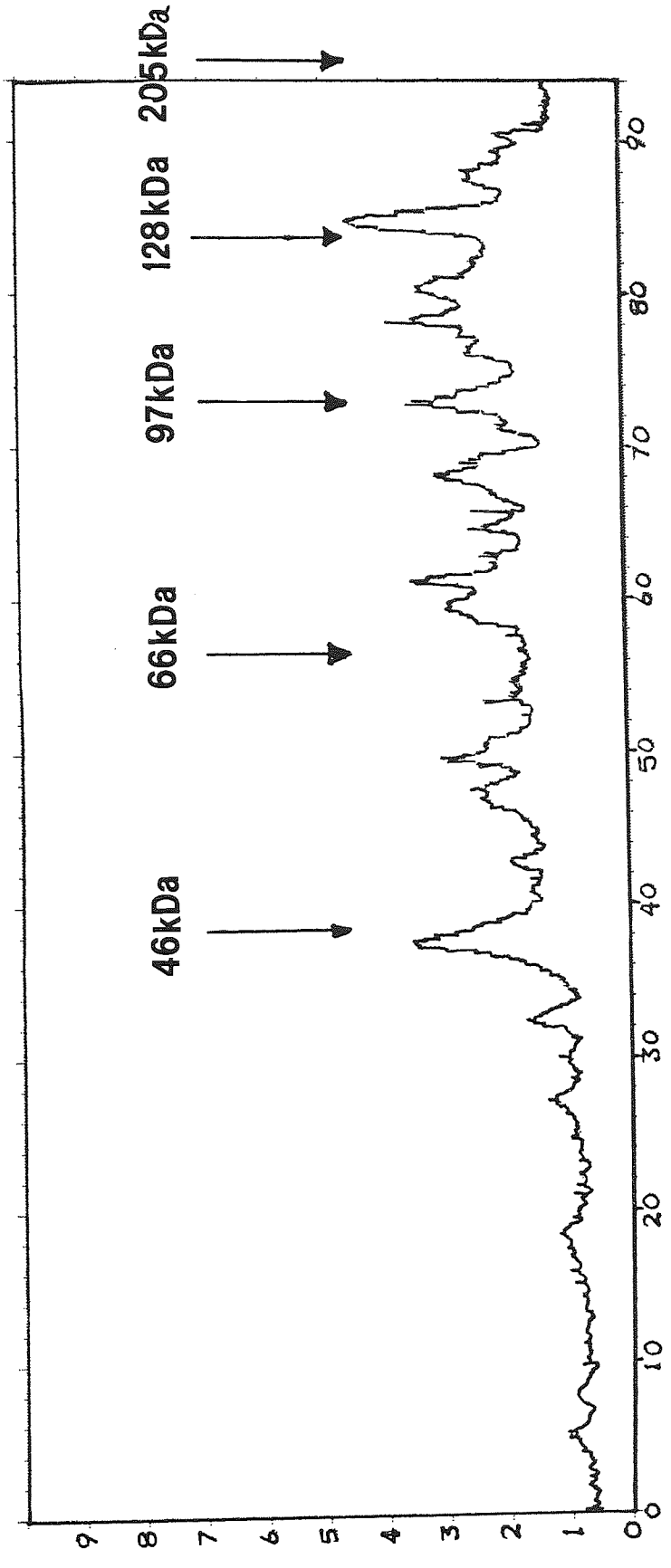


Figure 6: Densitometer trace of the DNA(cytosine-5) methyltransferase preparation run on an SDS-PAGE gel (fig 5). The intensity profile was generated using an LKB ultrascan.

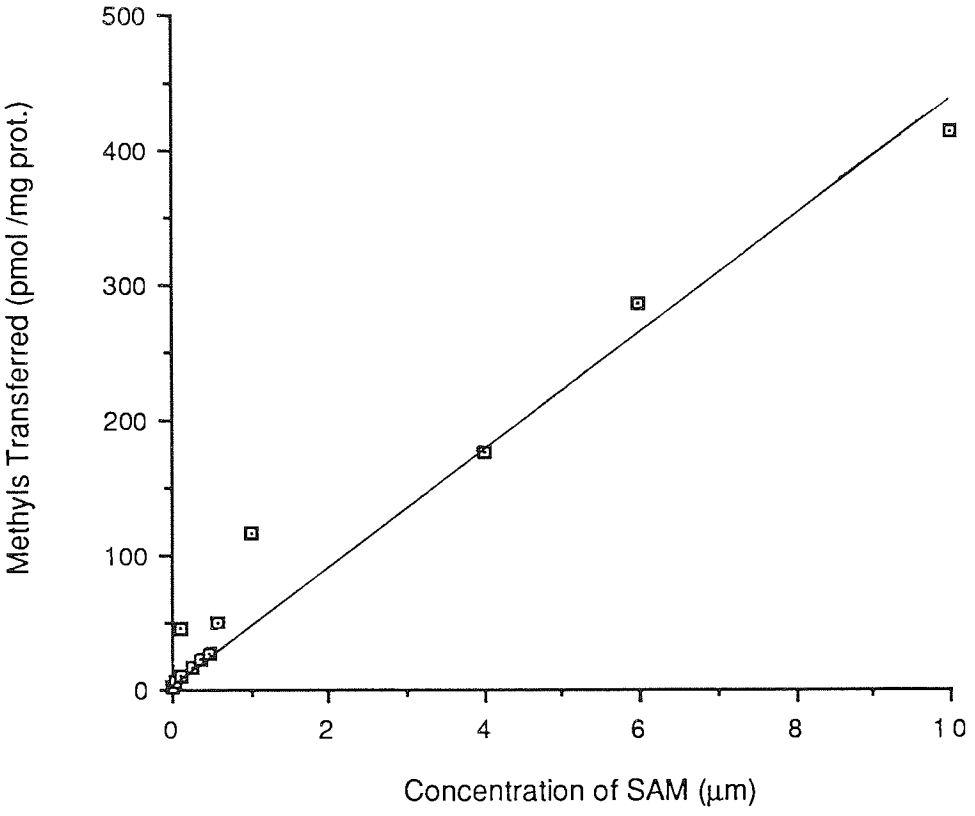


Figure 7: Characterisation of DNA(cytosine-5)methyltransferase with respect to the concentration of S-adenosyl-L-methionine.
Incubations were carried out at 37°C for 4 h using 20 µg M.lysodeikticus DNA, DNA(cytosine-5)methyltransferase and various concentrations of SAM. Assays were carried out in a total volume of 100 µl.
This data represents a typical experiment and each point is the mean of duplicate values.

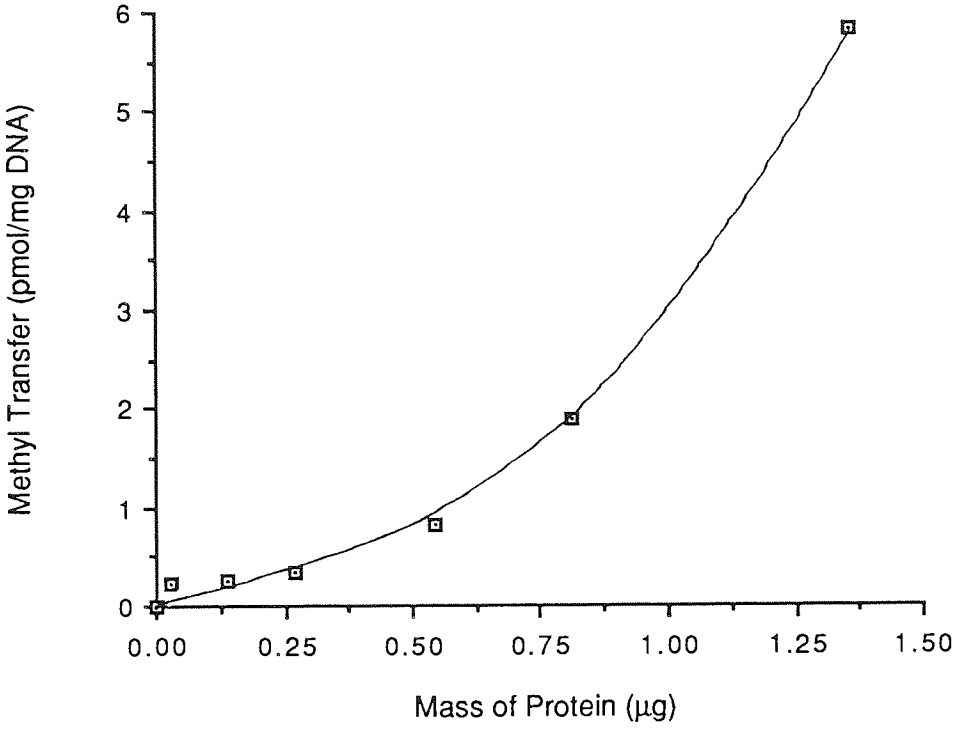


Figure 8: Characterisation of DNA(cytosine-5)methyltransferase with respect to the amount of enzyme.
Incubations were carried out at 37°C for 4 h with 20 µg M.lysodeikticus DNA, 1 µCi SAM and increasing amounts of DNA(cytosine-5)methyltransferase. Assays were carried out in a total volume of 100 µl. A control incubation was carried out in which the enzyme was incubated with [^{3}H]SAM in the absence of DNA. This data represents a typical experiment and each point is the mean of duplicate values.

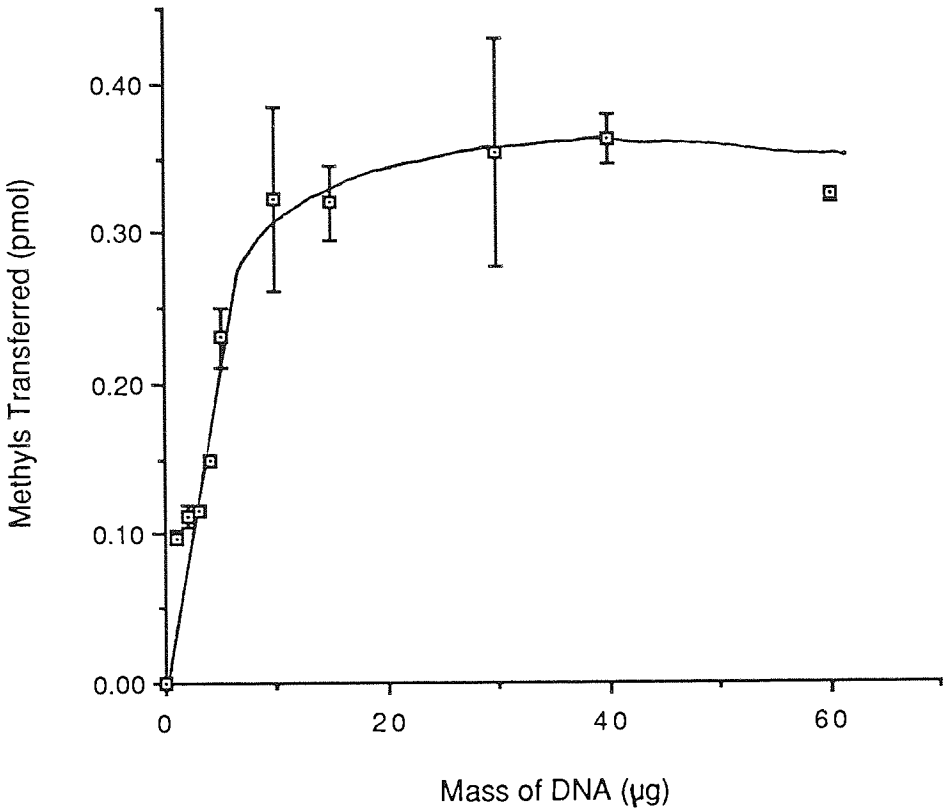


Figure 9: Characterisation of DNA(cytosine-5)methyltransferase with respect to the amount of DNA.
Incubations were carried out at 37°C for 4 h with 1 µCi SAM, a fixed amount of enzyme and increasing amounts of M.lysodeikticus DNA.
Assays were carried out in a total volume of 100 µl.
This represents the mean of three experiments ± SEM.

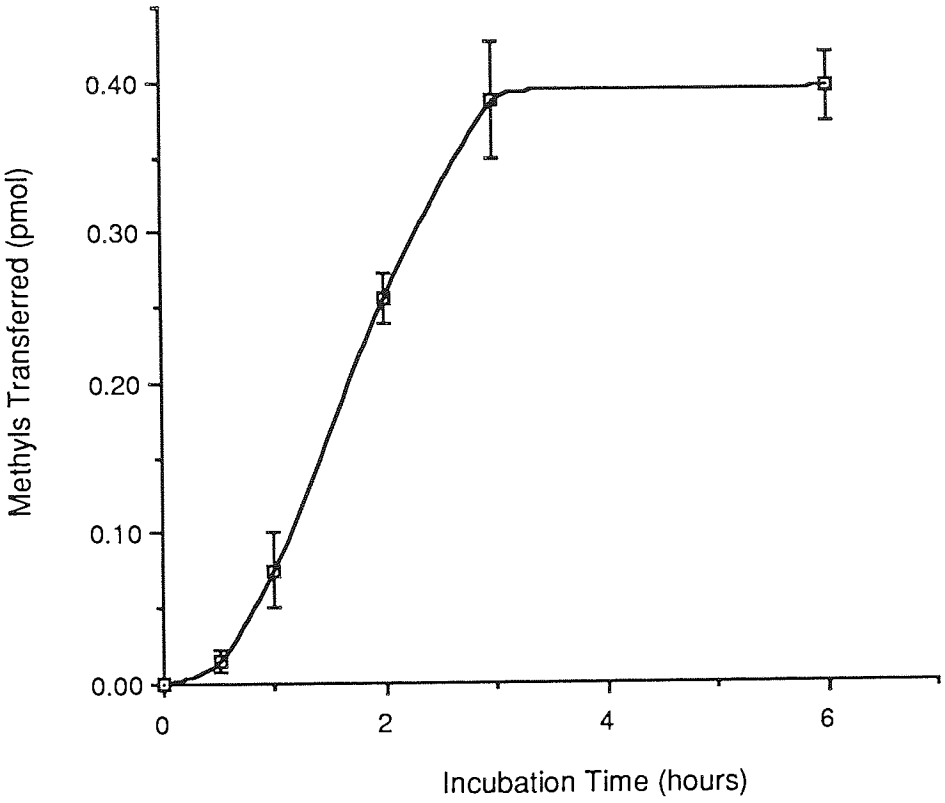


Figure 10: Characterisation of DNA(cytosine-5)methyltransferase with respect to time.
Incubations were carried out at 37°C with 20 µg M.lysodeikticus DNA, 1µCi SAM and a fixed amount of enzyme for various periods of time. Assays were carried out in a total volume of 100 µl.
This represents the mean of three experiments ± SEM.

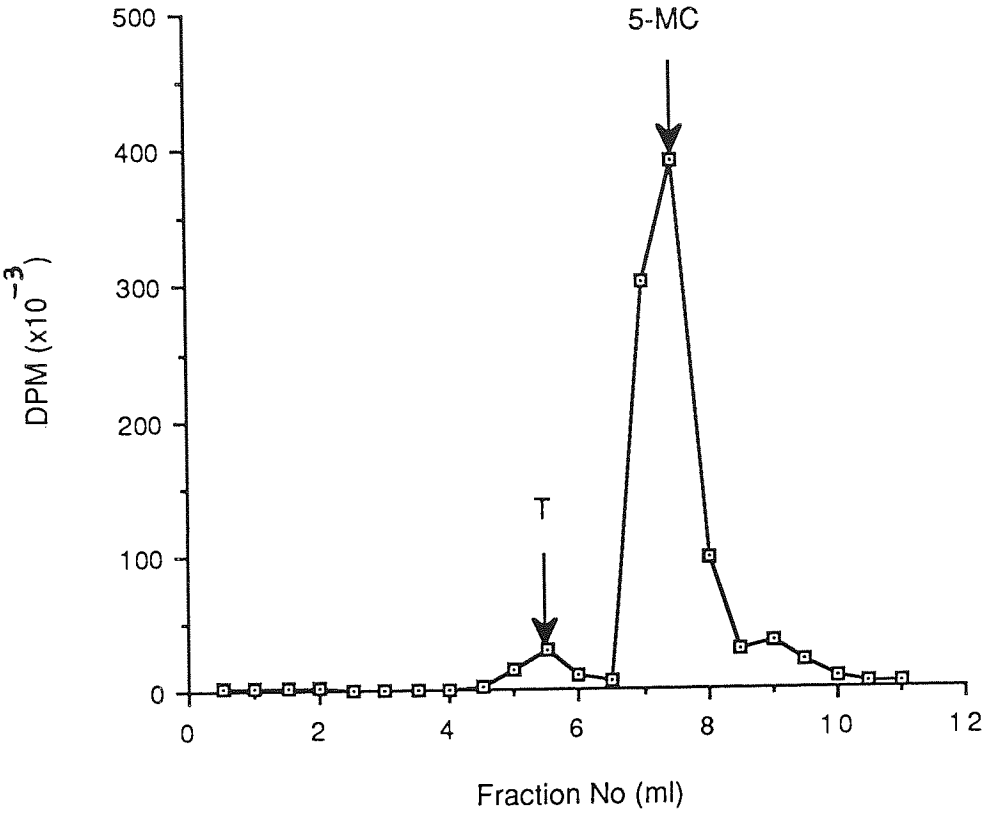


Figure 11: Identification of the product of methylation. M.lysodeikticus DNA was treated with DNA(cytosine-5)methyl-transferase and [^{3}H]SAM. The DNA was hydrolysed to its constitutive bases with 90% formic acid which were then separated by HPLC.

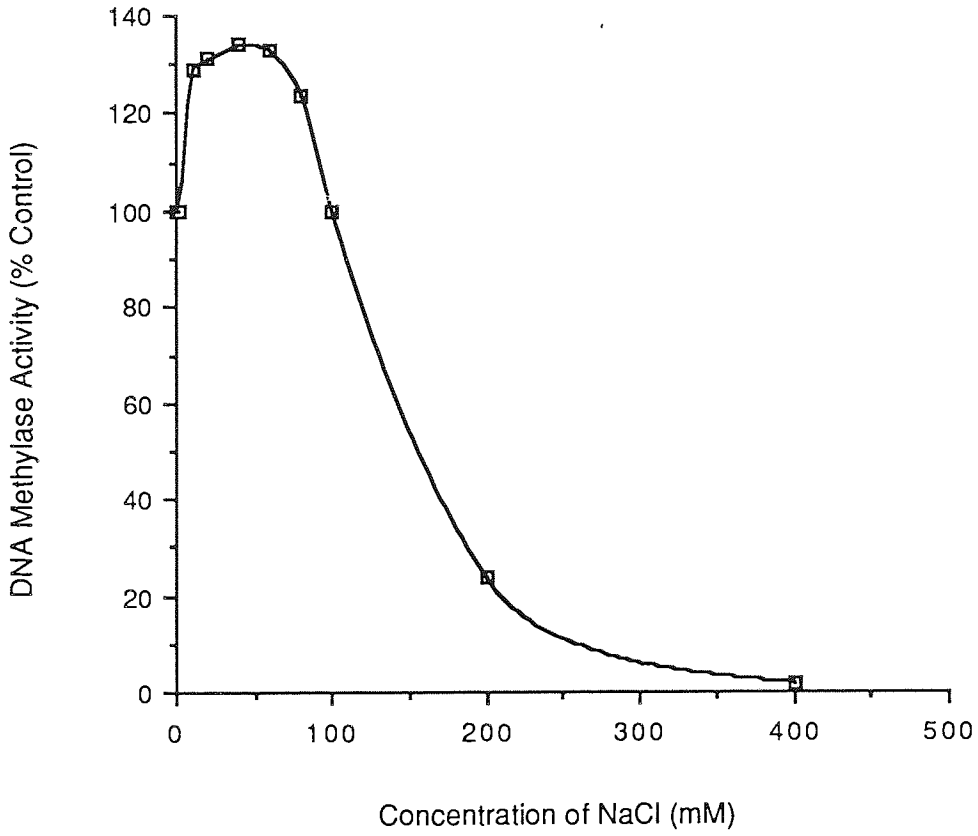


Figure 12: Effect of sodium chloride on the methylation of native M.lysodeikticus DNA.
Incubations were carried out at 37°C for 4 h with 20 µg M.lysodeikticus DNA, 1µCi SAM and a fixed amount of methyltransferase. There was a pre-incubation of 5 min before adding the salt. Assays were carried out in a total volume of 100 µl.
This is a representative experiment and each point is the mean of duplicate values.

DNA Substrate	Heated	(pmol /4h)	(pmol/ mg prot).	As a % of *M.lysodeikticus*
M.lysodeikticus	N	0.035	161.3	100
	Y	0.053	244.2	100
E.coli	N	0.020	92.2	57
	Y	0.023	106.0	44
Calf thymus	N	0.001	4.6	2
	Y	0.017	78.3	31
Salmon testes	N	0.004	18.4	13
	Y	0.008	36.9	15
Raji cell line	N	0	0	0
	Y	0	0	0

Table 7: Methyl acceptance ability of various nucleic acid substrates by DNA(cytosine-5)methyltransferase.
Incubations were carried out at 37°C for 4 h with 20 µg *M.lysodeikticus* DNA, 1 µCi SAM and DNA(cytosine-5)methyltransferase. Heat treatments were carried out at 100°C in a water-bath for 10 min followed by rapid cooling on ice.
Each value is the mean of duplicate samples carried out with the same batch of enzyme at the same time.

CHAPTER FOUR

STUDIES ON THE MECHANISM OF INDUCTION OF DNA(CYTOSINE-5) HYPOMETHYLATION BY 3-SUBSTITUTED IMIDAZOTETRAZINONES

4.1 INTRODUCTION

The imidazotetrazinones are a group of broad spectrum antitumour agents with activity against a range of murine tumours and human xenografts (Hickman et al., 1985; Fϕdstad et al., 1985; Stevens et al., 1984; Stevens et al., 1987). Structure-activity studies show that 3-(2-chloroethyl) (mitozolomide) and 3-methyl (temozolomide) are active antitumour agents whilst 3-ethyl (ethazolastone) and higher homologues are inactive (Stevens et al., 1987). Ring opening occurs under alkaline conditions to form the corresponding alkyl triazene (Baig and Stevens, 1987) which are the active biological moieties (Horgan and Tisdale, 1984).

The antitumour activity of the 3-substituted imidazotetrazinones is thought to be due to their reaction with DNA. Mitozolomide ring opens to produce 5-[3-(2-chloroethyl)triazen-1-yl]imidazole-4-carboxamide (MCTIC) and at equimolar concentrations both mitozolomide and MCTIC have similar in vitro cytotoxicities and produce similar levels of DNA interstrand cross-linking (Gibson et al., 1984a). The cytotoxic effects of mitozolomide were studied in the normal (IMR-90) and SV40-transformed (VA-13) human embryo cells, which differ in their ability to repair O^6-alkylguanine lesions (Mer+ and Mer- respectively). The VA-13 cell line which is deficient in the ability to repair O^6-alkylguanine lesions (Mer-) were more sensitive to the cytotoxic effects of mitozolomide

than the repair proficient IMR-90 (Mer+) cells (Gibson et al., 1984b). Mitozolomide and MCTIC produce cross-linking in the Mer- cells which was dependent on the drug concentration, while in the Mer+ cells little or no cross-link formation was detected (Gibson et al., 1984b). Therefore, DNA interstrand cross-link formation may be the mechanism for the in vitro cytotoxicity of mitozolomide and this probably occurs as a consequence of an initial alkylation at the O^6-position of guanine followed by a delayed reaction with the opposite strand.

The 3-methyl substituted (temozolomide) and the 3-ethyl substituted (ethazolastone) imidazotetrazinones are chemically incapable of cross-linking DNA. The cytotoxic effects of the imidazotetrazinones were studied in a range of cell lines with varying capacities to repair O^6-alkylguanine modifications (Tisdale, 1987). Cell lines proficient in the repair of O^6-alkylguanine lesions (Mer+) were least sensitive to the cytotoxic effects of mitozolomide and temozolomide than cells lacking the repair enzyme (Mer-). Ethazolastone did not show any differential cytotoxicity between Mer+ and Mer- cell lines. Depletion of O^6-methylguanine-DNA methyltransferase in the Mer+ cell lines by treatment with the free base O^6-methylguanine, produced an increased sensitivity to both mitozolomide and temozolomide but not to ethazolastone (this does not include the Burkitt's lymphoma Raji cell line). Therefore,

although temozolomide is incapable of cross-linking DNA, alkylation of guanine at the O^6-position is probably involved in the cytotoxicity.

Studies on the K562 human erythroleukemia cell line have shown that growth inhibition produced by temozolomide is accompanied by a proportional increase in the number of haemoglobin-producing cells (Tisdale, 1985). No such effect was observed with ethazolastone even at equitoxic drug concentrations. Further, it was shown that the amount of 5-methylcytosine three days after treatment with temozolomide decreased from 3.5% to 2.2% (Tisdale, 1986) using a concentration of temozolomide (73.5 μM) which was capable of inducing haemoglobin synthesis without substantial cytotoxicity. It was concluded by Tisdale (1986) that the temozolomide-induced differentiation of K562 cells to cells with erythroid characteristics may be due to hypomethylation of DNA.

In order to investigate the mechanism of DNA(cytosine-5) hypomethylation, Tisdale (1988) alkylated calf thymus DNA *in vitro* with temozolomide or ethazolastone and the alkylated DNA was shown to inhibit the transfer of methyl groups from SAM to *M.lysodeikticus* DNA by DNA(cytosine-5)methyltransferase. In this system, 10 μg of calf thymus DNA, which is a poor substrate for DNA(cytosine-5)-methyltransferase, was added to 20 μg *M.lysodeikticus* DNA, which is an excellent substrate for the enzyme, and the calf thymus DNA was shown not to affect the

methylase reaction. However, when the calf thymus DNA was modified *in vitro* with temozolomide or ethazolastone, the alkylated-DNA was shown to inhibit the transfer of methyl groups from SAM to *M.lysodeikticus* DNA by DNA(cytosine-5)methyltransferase. In this *in vitro* system, ethazolastone-alkylated calf thymus DNA was a more effective inhibitor of DNA(cytosine-5) methylation than temozolomide-alkylated calf thymus DNA. This was a reverse of the order of potencies of the free drugs against tumour cells in culture (Bull, 1988). Further, Tisdale (1989) showed that DNA isolated from the human lymphoblastoid cell line GM892 (Mer-) previously treated with temozolomide, also inhibited the methylation of *M.lysodeikticus* DNA, whilst DNA from untreated GM892 cells had no effect. The inhibitory effect of DNA from GM892 cells was maximal 6 h after drug addition and was proportional to the concentration of the drug.

The aim of this section was to continue the work of Tisdale and explore the mechanism by which the alkylated DNA causes the observed *in vitro* inhibition of DNA(cytosine-5)methyltransferase. The approach taken was to determine whether the inhibition was unique for imidazotetrazinone-alkylated DNA and then attempt to identify the particular base modification or DNA damage responsible for the inhibition.

4.2 METHODS

4.2.1 Determination of DNA using the ethidium bromide-fluorometric assay.

Ethidium bromide intercalates into DNA and a measure of the amount of DNA may be determined by the amount of fluorescence.

A series of standard tubes were prepared from the standard calf thymus DNA solution (0.5 mg/ml), ranging from 0-25 μg/ml. Ethidium bromide reagent was added to give a total volume of 3.0 ml. The tubes were vortexed and the solution was transferred to a Hellma quartz Suprasil cell with a light path of 10 mm, and then read on a Perkin Elmer LS-5 Luminescence Spectrometer. The samples were read against a blank containing 3.0 ml of ethidium bromide reagent and the DNA concentration was calculated from the standard curve. The following settings were used - excitation wavelength of 360 nm, emission wavelength of 590 nm, excitation slit at 10 and emission slit at 5.

4.2.2 Alkylation of DNA in vitro.

Temozolomide, ethazolastone, MNU and ENU were dissolved in DMSO, chlorambucil was dissolved in ethanol and MMS and EMS were diluted with DMSO such that the concentration of solvent did not exceed 3% when added to the DNA solution. Calf thymus DNA (600 μg) or poly[dA].poly[dT] (600 μg) dissolved in 50 mM

phosphate buffer (pH 8.4) was treated with the indicated concentration of drugs for 2 h at 37°C in a total volume of 1 ml.

To the solution was added 100 μl of 2.5 M sodium acetate and the DNA was precipitated by adding 2.5 volumes of cold absolute ethanol. The DNA was then pelleted by centrifugation at 3000 rpm for 10 min using an Heraeus bench top centrifuge. The precipitation process was repeated twice by resuspension and resedimentation in water and then three times with absolute ethanol. After drying the DNA overnight, the DNA was redissolved in distilled water at a concentration of 2 mg/ml.

4.2.3 X-Irradiation of DNA.

Calf thymus DNA at a concentration of 2 mg/ml in 50 mM KCl(aq) in glass vials was exposed to ^{137}Cs gamma radiation to produce a range of doses. This was performed at the Paterson Institute, Manchester.

4.2.4 Deoxyribonuclease treatment of DNA.

DNaseI from bovine pancreas was reconstituted with cold 0.15 M NaCl to give a concentration of 10,000 K/ml. Calf thymus DNA was dissolved in 5 mM $MgSO_4$, 0.1 M CH_3COONa (pH 5.0) at a concentration of 0.4 mg/ml. Various amounts of DNaseI in the range 10-1000 K were added to 2.5 ml of the DNA solution and incubated at 25°C for 30 min. The reaction was stopped by adding phenol reagent and the DNA was

extracted twice and precipitated by adding 0.1 volumes of 2.5 M sodium acetate and 2.5 volumes of cold absolute ethanol. The precipitation process was repeated twice and the DNA was washed with absolute ethanol and dried with diethyl ether. The DNA was redissolved in water at a concentration of 2.0 mg/ml according to the DNA/ethidium bromide fluorometric assay.

4.2.5 Sonication of DNA

Calf thymus DNA at a concentration of 2.0 mg/ml in water was sonicated using a sonic probe at an amplitude of 20 microns for various periods of time.

4.2.6 Restriction enzyme (MspI) treatment

1 ml of a solution of calf thymus DNA (0.5 mg/ml) in low salt restriction buffer was treated with the restriction enzyme MspI for 2 h at 37°C. The amount of enzyme used in the incubations was 10, 25, 50, 75, 100 and 200 Units. The reaction was stopped by adding phenol reagent and the DNA was extracted twice and precipitated by adding 0.1 volumes of 2.5 M sodium acetate and 2.5 volumes of cold absolute ethanol. The precipitation process was repeated twice and the DNA was washed with absolute ethanol and dried with diethyl ether. The DNA was redissolved in water at a concentration of 2.0 mg/ml according to the DNA/ethidium bromide fluorometric assay.

4.2.7 Experiment to determine the effect of modified DNA on DNA(cytosine-5)methyltransferase activity.

DNA(cytosine-5)methyltransferase was prepared as described in section 3.1.1.

DNA was modified as described in section 4.2.2 to 4.2.6 and was redissolved in distilled water at a concentration of 2 mg/ml. To the standard DNA(cytosine-5)methyltransferase assay described in section 3.1.3 was added 10 μg of the modified DNA.

4.2.8 Experiment to determine the effect of free drug or base on DNA(cytosine-5)methyltransferase activity.

DNA(cytosine-5)methyltransferase was prepared as described in section 3.1.1.

To the standard DNA(cytosine-5)methyltransferase assay described in section 3.1.3 was added the solution of drug or base which did not constitute more than 10% of the assay volume. Temozolomide, ethazolastone, mitozolomide, MNU and ENU were dissolved in DMSO and chlorambucil was dissolved in ethanol. The alkyl sulphonates were diluted with DMSO. The purine and pyrimidine bases were dissolved in 0.01 M HCl. Appropriate controls were performed using the solvent alone in the incubation.

4.2.9 Preparation of [^{3}H]labelled DNA.

DNA was prepared from the L1210 murine leukemia cell line. L1210 cells were maintained as a suspension culture in exponential growth at a density between 0.8 x 10^5 and 1.0 X 10^6 cells/ml. Cells were grown in RPMI 1640 medium (with 25 mM HEPES and L-glutamine), supplemented with 10% horse serum, under an atmosphere of 5% CO_2 in air. Under these conditions the cells were found to have a doubling time of between 12-14 h.

200 ml of cells were set up at a density of 1.0 x 10^5 cells/ml, gassed with 5% CO_2 in air and incubated for 1 h. To the cells was added 0.5 μCi/ml [^{3}H]thymidine (79 Ci/mmol) and incubated for 48 h.

The DNA was then prepared from the cells using a method based on that described by Warren (1984). The cells were pelleted by low speed centrifugation (1500 rpm) using an Heraeus bench top centrifuge, and washed by resuspending in 0.9% saline. To the cell pellet in a Universal container was added between 1-2 ml of 6% p-aminosalicylic acid and vortexed for 5 min. To this was added 0.1 volumes of 10% SDS and incubated at 37^{o}C for 5 min. The solution clarified and became more viscous as lysis proceeded. After transferring to polypropylene tubes, an equal volume of phenol reagent was added and the mixture was vortexed for 5 min. The mixture was then centrifuged at 2000 rpm for 30 min in an Heraeus bench top centrifuge. The upper aqueous layer was removed

using a Pasteur pipette taking care not to disrupt the interfacial material. The DNA was then precipitated from the aqueous phase by adding 1.5 volumes of ethoxyethanol and gently agitating. The DNA was then either removed using a glass spatula or separated by centrifugation at 3000 rpm in an Heraeus bench top centrifuge. The DNA was then washed by resuspending and then resedimentation at 3000 rpm using an Heraeus bench top centrifuge, three times with 70% (v/v) ethanol 2% (w/v) sodium acetate and two times with absolute ethanol and then air dried.

Traces of phenol were removed from the DNA by mixing them with an equal volume of water saturated diethyl ether and separated by standing for 5 min. The upper layer was removed and discarded and repeated a further two times. Traces of ether were then removed by heating in a water-bath at 70°C for 10 min.

The amount of DNA was calculated using the ethidium bromide-fluorometric assay (section 4.2.1).

4.2.10 In vitro alkylation of labelled DNA.

The drugs were dissolved in DMSO so that the final concentration of DMSO did not exceed 1% of the reaction volume. The DNA was alkylated to give the same degree of chemical reaction for all the drugs. These values were calculated from the published

figures shown in table 8. The alkylations were carried out in 50 mM phosphate buffer, pH 8.4 for 2 h, at 37°C.

Table 8: Extent of reaction of alkylating agents with DNA

Drug	Extent of Reaction (mmol alkyl gp/mol DNA-P)	Final Concn. of Drug
MMS	5.3	5.0 mM
EMS	0.6	46.0 mM
MNU	55.0	0.5 mM
ENU	4.6	6.0 mM
Swenson and Lawley (1978)		
Temozolomide	16.49	1.5 mM
Ethazolastone	2.43	11.0 mM
Bull (1988)		

4.2.11 Protein/nucleic acid binding assay.

The method for determination of protein binding to nucleic acid was based on that described by Tisdale (1988).

Each assay consisted of 5.0 μg [^{3}H]labelled DNA with 10 μl of the DNA(cytosine-5)methyltransferase preparation, made up to a total volume of 200 μl with

incubation buffer. The binding reaction was performed at 37°C for 20 min. The complexes were then incubated at 4°C prior to being washed onto a Millipore HA 0.45 μm filter with the following agents of increasing dissociating strength:incubation buffer; 1.0 M NaCl; 0.6% sarkosyl + 0.5 M NaCl; 0.4% SDS. The filters were then air dried and 8.0 ml of PPO/POPOP/toluene scintillant was added and the radioactivity determined by liquid scintillation counting using a Packard Tri-Carb 2000CA liquid scintillation counter.

4.2.12 Horizontal agarose gel electrophoresis for the separation of DNA.

Agarose was added to 100 ml of TBE buffer to give the appropriate concentration of agarose as required. The mixture was then heated to boiling whilst stirring and then cooled to 50°C, and then 10 μl ethidium bromide solution (5.0 mg/ml) was added to give a final concentration of 0.5 μg/ml. The agarose solution was then poured into the gel container with the comb in position to produce the wells, and left for 0.5 h to set. The gel was then placed into the electrophoresis tank ensuring that the gel was covered with TBE buffer containing ethidium bromide (0.5 μg/ml).

1-5 μg of DNA was made up to 20 μl with 1 x TBE buffer and to this was added 4 μl of 6x concentration loading buffer. After heating at 65°C for 10 min,

the DNA samples were inserted into the wells in the gel using a Gilson pipette and then run at a constant voltage of 5.0 V/cm. When the bromophenol blue tracking dye was 2-3 cm from the end of the gel, electrophoresis was stopped and the DNA was revealed using a UVP transilluminator at a wavelength of 302 nm. A photograph may taken be taken at this stage using a Polaroid MP4 close up camera with Polaroid 665 film.

4.2.13 Estimation of DNA damage by alkaline elution.

This method was based on that described by Kohn et al. (1981) and was carried out at the Paterson Institute, Manchester.

[^{3}H]Thymidine-labelled DNA from L1210 cells was prepared as described in section 4.2.9 and this was alkylated with various concentrations (0.001 mM-1.0 mM) of temozolomide or ethazolastone. The drugs were dissolved in DMSO and added to the DNA solution so that the DMSO did not exceed 3% of the reaction volume. The labelled DNA (600 μg) dissolved in 50 mM phosphate buffer (pH 8.4) was treated with the drug for 2 h at 37^{o}C in a total volume of 1 ml. Care was taken to protect the solutions from light using tin foil. The DNA was then precipitated by adding 0.1 volumes of 2.5 M sodium acetate and 2 volumes of cold absolute ethanol, and pelleted by centrifugation at 4000 rpm for 10 min. The DNA was washed twice in

absolute ethanol by resuspending the DNA and then centrifugation. The DNA was stored suspended in ethanol at -20°C until used.

The DNA suspension (10 μg) was layered onto a 25 mm, 2 μm polyethylene chloride filter, within a polyethylene filter holder, which was attached to a 60 ml polyethylene leur lock syringe. The filter and DNA sample were then washed with 5 ml of 0.02 M EDTA solution pH 10.0.

The alkaline elution solution was then pumped through the filter using a peristaltic pump. The solution was then passed from the filter holder to the fraction collector via polyethylene tubing. The pump system was set up so that 16 samples could be run at the same time with the flow rate of 0.035 ml/min constant in all 16 channels.

After 18 h the filters were removed and placed in a scintillation vial and 1.0 ml of 1.0 N HCl was added. The vials were sealed and heated at 60°C for 1 h to depurinate the DNA and then counted. The filter holder and the pump tubing were washed with 0.4 N NaOH, an aliquot of which was counted. All samples were mixed with 10 ml of Ecoscint scintillation fluid, to which 0.7% glacial acetic acid had been added to prevent chemiluminescence.

Using the above procedure it was proposed to measure the level of single-strand breaks, by comparing the elution profiles of control DNA with <u>in</u>

vitro alkylated DNA. As a positive control, DNA treated with 500 rads ^{137}Cs X - irradiation was included.

4.2.14 Statistical analysis

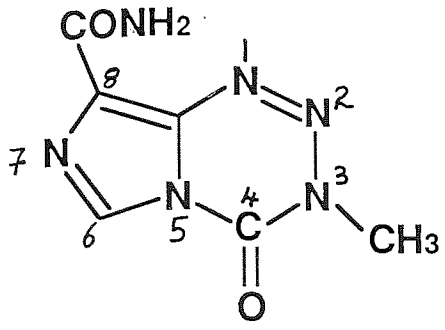
All results are expressed as mean ± standard error of the mean (SEM) for at least three separate determinations.

4.3 RESULTS

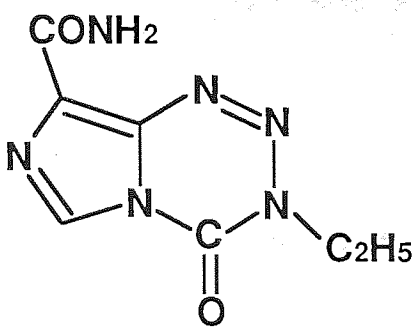
The effect of alkylated calf thymus DNA on DNA(cytosine-5)methyltransferase activity.

The system used to investigate the effect of DNA modifications on the activity of DNA(cytosine-5)methyltransferase was the same as that used by Tisdale (1988). Calf thymus DNA is a poor substrate for DNA(cytosine-5)methyltransferase and when 10 μg are placed in an assay containing 20 μg *M.Lysodeikticus* DNA (a good substrate), the number of methyl groups transferred by the enzyme from SAM to the DNA substrate was unaffected. However, modification of the calf thymus DNA by temozolomide or ethazolastone has been shown by Tisdale (1988) to inhibit transfer of methyl groups. Therefore, this assay provided a system by which the effect of various DNA modifications of a non-substrate DNA can have on the ability of DNA(cytosine-5)methyltransferase to methylate *de novo* the unmodified substrate DNA. The structures of the alkylating agents used in this study are shown in fig. 13.

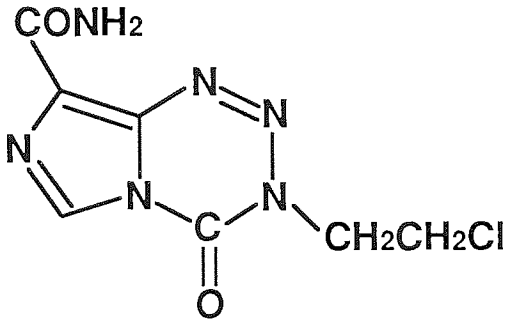
It was first of all important to be able to repeat the results of Tisdale (1988). Calf thymus DNA was alkylated *in vitro* with temozolomide or ethazolastone, and after extensive washing was allowed to compete with *M.lysodeikticus* DNA in the standard DNA(cytosine-5)methyltransferase assay. The results are presented in fig. 14 and this shows that the extent of inhibition of DNA(cytosine-



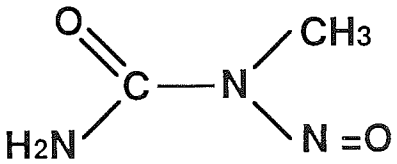
temozolomide



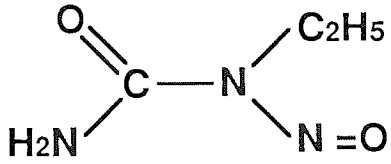
ethazolastone



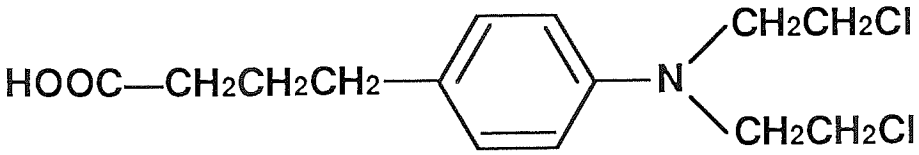
mitozolomide



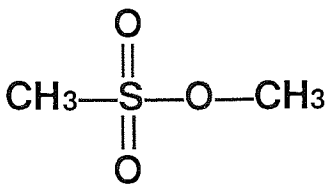
N–methyl–N–nitrosourea



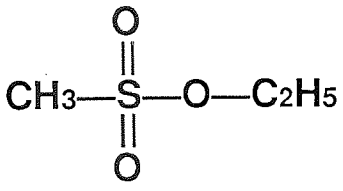
N–ethyl–N–nitrosourea



chlorambucil



methyl methane – sulphonate



ethyl methane– sulphonate

Figure 13: The structure of the alkylating agents used in this study.

5)methylation is proportional to the concentration of temozolomide or ethazolastone to which the calf thymus DNA was exposed. In these experiments the amount of DNA(cytosine-5)methyltransferase used was such that 0.1 pmol of methyl groups were transferred to 20 μg M.lysodeikticus DNA under the conditions of the assay. This was the same as that used by Tisdale (1988) (personal communication).

The concentration of drug used in the reaction with calf thymus DNA was plotted on the x-axis as a logarithmic scale, as this allows the construction of a straight line through the experimentally derived points. Wilson and Jones (1983) also use a logarithmic scale for demonstrating the dose-dependent inhibition of methyltransferase activity after alkylation of hemimethylated DNA with various agents. The inhibition of DNA(cytosine-5)methyl-transferase depends on the degree of DNA damage and the use of a logarithmic scale shows that comparatively low concentrations of drug are required initially to produce a sharp fall in the level of enzymatic DNA methylation.

The results presented in fig. 14 are different to those observed by Tisdale (1988). In particular Tisdale observed complete inhibition of DNA(cytosine-5)methyltransferase activity with calf thymus DNA treated with 0.05 mM ethazolastone, whilst in my system only a 40% inhibition of activity was produced. Tisdale (1988) showed that calf thymus DNA

modified with ethazolastone was more effective as an inhibitor of DNA(cytosine-5)methyltransferase than DNA modified with temozolomide, whilst the results in fig. 14 show a similar degree of inhibition with both agents.

In order to determine whether a particular base modification was responsible for the inhibition, it was necessary to calculate the extent of chemical alkylation. The extent of chemical modification of the in vitro treated DNA was deduced from the data of Bull and Tisdale (1987). The reaction of calf thymus DNA in vitro with 0.25 mM temozolomide or ethazolastone resulted in the binding of approximately 0.53 and 0.10 pmol/μg DNA respectively. Therefore, assuming that these agents alkylate DNA to the same extent under the conditions used here as those used by Bull and Tisdale (1987), and the extent of substitution at various sites in DNA by these agents is the same as determined by Bull (1988), then the extent of reaction at various sites in DNA can be calculated. In this calculation it was assumed that the average molecular weight of a DNA nucleotide is 330. The approximate extents of reaction with DNA were 175 and 33 μmol methyl/mol DNA-P for temozolomide and ethazolastone respectively.

Calf thymus DNAs modified with temozolomide or ethazolastone were similar in the degree of inhibition of DNA(cytosine-5)methyltransferase activity that they produced (fig. 14). It can be seen

from table 9, that for the major bases alkylated there is no correlation between the extent of alkylation and the inhibition of the methyltransferase. The extent of alkylation of the phosphodiester groups was not determined by Bull (1988). However, assuming that temozolomide and ethazolastone alkylate through $CH_3N_2^+$ and $C_2H_5N^+$ ions respectively, approximate values for alkylation of the phosphodiester groups can be taken for MNU and ENU (Beranek et al., 1980). These values are given in table 9, with values of 21 μmol/mol DNA-P methyltriesters and 18 μmol/mol DNA-P ethyltriesters for temozolomide and ethazolastone respectively. Although these values are not in exact agreement, there is a correlation between the extent of alkylation at the phosphotriester groups and the inhibition of DNA(cytosine-5)methyltransferase produced by the alkylated DNA.

Tisdale (1988) showed that calf thymus DNA modified with ethazolastone was a better inhibitor of the methylation of M.lysodeikticus DNA by DNA(cytosine-5)methyltransferase than temozolomide-modified DNA. The conclusion was that since ethazolastone forms predominantly ethylphosphotriesters, this lesion was responsible for the inhibition of the methyltransferase. However, Tisdale's conclusion was not correct as the overall alkylation of the phosphotriesters (table 9) are similar. Nevertheless, the results presented in this

thesis do indicate a correlation between alkylphosphotriester formation and an inhibition of DNA(cytosine-5)methyltransferase and support Tisdale's conclusion despite the different results.

In order to determine whether this effect was specific for the two imidazotetrazinones, the experiment was repeated for other alkylating agents. For the nitrosoureas, MNU and ENU (fig. 15), MNU-alkylated calf thymus DNA was a slightly better inhibitor of DNA(cytosine-5)methyltransferase than ENU-alkylated calf thymus DNA. The extent of reaction at various sites in DNA is shown in table 10. The extent of reaction was determined by Swenson and Lawley (1978) based on 20 mM alkylating agent and a DNA concentration of 15 mM DNA-P/ml. The values for the extent of substitution at various sites in DNA were determined by Beranek et al. (1980).

The inhibition of DNA(cytosine-5)methyltransferase by MNU and ENU-alkylated calf thymus DNA can be correlated with the extent of alkylation at all the major alkylation sites (N-7-guanine, O^6-guanine, N-3-adenine and phosphotriesters).

Calf thymus DNA alkylated with the alkyl sulphonates, MMS or EMS, were poor inhibitors of the methylation of M.lysodeikticus DNA by DNA(cytosine-5)methyltransferase. The results are presented in fig. 16 and it can be seen that there is no significant difference between MMS and EMS-alkylated calf thymus DNA.

Table 9. The extent of alkylation of calf thymus DNA treated with temozolomide or ethazolastone.

Base	Temozolomide		Ethazolastone	
	Percent of total DNA alkylated	Overall alkylation (µmol/mol DNA-P)	Percent of total DNA alkylated	Overall alkylation (µmol/mol DNA-P)
7-alkylguanine	70[1]	122	24[1]	7.9
O^6-alkylguanine	5.3[1]	9.3	5.4[1]	1.8
3-alkyladenine	9.2[1]	16.1	4.9[1]	1.6
Total alkyl-phosphotriester	12[2]	21	55[2]	18

[1] The extent of substitution at various sites in DNA was determined by Bull (1988).

[2] The extent of substitution was not determined by Bull (1988). It was assumed that temozolomide and ethazolastone alkylate through $CH_3N_2^+$ and $C_2H_5N_2^+$ ions as for MNU and ENU and therefore the total phosphotriester was assumed to be similar to that determined by Beranek et al. (1980).

Table 10. The extent of alkylation of DNA treated with dimethyl sulphate, ethyl methane sulphonate, N-methyl-N-nitrosourea and N-ethyl-N-nitrosourea.

	MNU		ENU		MMS		EMS	
	A	B	A	B	A	B	A	B
7-alkylguanine	66.4[1]	36.5	11.0[1]	0.5	81.4[1]	4.3	58.4[1]	0.4
O^6-alkylguanine	5.9[1]	3.2	7.9[1]	0.4	0.3[1]	0.02	2.0[1]	0.01
3-alkyladenine	8.4[1]	4.6	2.8[1]	0.1	11.3[1]	0.6	4.2[1]	0.03
Total alkyl phosphotriester	12.1[1]	6.7	55.4[1]	2.5	0.8[1]	0.04	12.0[1]	0.07

A. Percentage of total DNA alkylated.
B. Overall alkylation (μmol/mol DNA-P). The extent of reaction was determined by Swenson and Lawley (1978) based on the use of 20mM alkylating agent and a DNA concentration of 15mM DNA P/ml

Reagent	Extent of reaction (mmol alkyl group/mol DNA-P)
Dimethyl sulphate	5.3
Ethyl methanesulphate	0.6
N-methyl-N-nitrosourea	55.0
N-ethyl-N-nitrosourea	4.6

ENU and MMS (similar to dimethyl sulphate) have a similar reactivity towards DNA (Swenson and Lawley, 1978) with extents of reaction of 5.3 and 4.6 mmol of alkyl group/mol of DNA-P respectively. However, calf thymus DNA alkylated with these agents does not cause the same degree of methyltransferase inhibition. ENU-alkylated calf thymus DNA is a better inhibitor of the methyltransferase than MMS-alkylated calf thymus DNA (fig. 15 and 16). The extent of reaction at various sites in DNA is presented in table 10. The inhibition of DNA(cytosine-5)methyltransferase by ENU and MMS-alkylated calf thymus DNA can be correlated with the extent of alkylation at the phosphotriesters and the O^6 atom of guanine. These observations further support the conclusion that alkylphosphotriesters are responsible for the inhibition of DNA(cytosine-5)methyltransferase by alkylated DNA. This is the first time that a biological effect has been correlated with DNA-alkylphosphotriester formation.

Poly[dA].poly[dT] is not a substrate for DNA(cytosine-5)methyltransferase and when alkylated with MNU it has been reported to form 90% methylphosphotriesters (McCarthy and Lindahl, 1985). Therefore, this provided a system to determine whether alkylphosphotriesters are responsible for the inhibition of the methyltransferase. MNU-alkylated poly[dA].poly[dT] was placed in competition with M.lysodeikticus DNA under the same conditions

inhibition was observed with MNU-alkylated calf thymus DNA. The results are shown in fig. 17, in which there is no correlation between the extent of methyltransferase inhibition and the concentration of MNU used to alkylate the poly[dA].poly[dT]. Therefore, this casts doubts on the conclusion made earlier that alkylphosphotriester formation may be responsible for the inhibition of DNA(cytosine-5)methyltransferase. However, since MNU-alkylated calf thymus DNA was capable of causing a dose-dependent inhibition of DNA(cytosine-5)methyltransferase and MNU-alkylated poly[dA].poly[dT] was not, a property of the synthetic poly[dA].poly[dT] may be responsible for this discrepancy.

This work was then extended to the cross-linking agents mitozolomide and chlorambucil. The mechanism by which mitozolomide cross-links DNA is thought to be similar to other chloroethylating agents. Initial alkylation occurs at the $\underline{O}^6$ position of guanine followed by internal rearrangement to the $\underline{N}$-1 position of guanine, followed by completion of the interstrand link to the $\underline{N}$-3 position of the complementary cytosine residue (Kohn, 1977). The major reaction of the bifunctional alkylating agent, chlorambucil is thought to be crosslinking between the $\underline{N}$-7 position of guanine on one strand with the $\underline{N}$-7 position of guanine in the opposite strand (Pratt and Ruddon, 1979).

The results for mitozolomide and chlorambucil alkylated calf thymus DNA are shown in figs. 18 and 19 respectively. It can be seen that calf thymus DNA modified by these agents inhibits DNA(cytosine-5)methyltransferase to a similar extent. In these experiments, enzyme activity capable of transferring 0.4 pmol of methyl groups under the conditions of the assay was used, as opposed to 0.1 pmol which was used in the previous experiments. The use of different levels of enzyme activity means that direct comparisons between the various experiments cannot be made. However, the fact that inhibition of the methyltransferase occurred with the use of higher levels of enzyme with chlorambucil and mitozolomide-alkylated DNA, suggests that DNA treated with these agents were better inhibitors than DNA treated with temozolomide or ethazolastone.

Experiments to determine the extent of binding between the alkylated-DNA and protein in the DNA(cytosine-5)methyltransferase preparation.

The inhibition of DNA(cytosine-5)methyl-transferase by alkylated calf thymus DNA, may occur by a stronger binding of the methyltransferase to the alkylated DNA. The system used to assess the stability of the complex between the alkylated DNA and the protein in the enzyme preparation was that used by Tisdale (1988). Labelled DNA was extracted from L1210 cells grown in the presence of

[^{3}H]thymidine. The labelled DNA was then treated with either temozolomide, ethazolastone, MMS, EMS, MNU or ENU. The concentration of alkylating agent used was chosen to give the same extent of reaction with DNA based on published values (Bull, 1988; Swenson and Lawley, 1978) using 20 mM of the agent. The extent of reaction of dimethyl sulphate (similar to MMS), EMS, MNU and ENU with salmon sperm DNA at a concentration of 15 mM DNA-P/ml (equivalent to 5.0 mg/ml based on an average molecular weight of a DNA nucleotide of 330) and an incubation time of 2.5 h was determined by Swenson and Lawley (1978). The extent of reaction of temozolomide and ethazolastone with calf thymus DNA at a concentration of 2.0 mg/ml and an incubation time of 2 h, was determined by Bull (1988). These differences in protocol may affect the extent of reaction with the DNA and no attempt was made to verify the extent of alkylation. However, based on these values the labelled DNA was treated with the alkylating agents so that the overall level of alkylation was the same.

The alkylated DNA (5 μg) was then incubated for 20 min with 10 μl of methyltransferase preparation (approximately 1 μg protein), during which time complexes form between the DNA and the protein. An assessment of the stability of the DNA/protein complex was then made by washing the incubation mixture onto a Millipore HA 0.45 μM filter with solutions of increasing dissociating strength

(incubation buffer, 1.0 M NaCl, 0.6% sarkosyl + 0.5 M NaCl or 0.5% SDS). The amount of DNA in terms of radioactivity trapped on the filter was expressed as a percentage of the total amount of DNA. A control was carried out in which 5 μg of the labelled DNA was washed onto the filter with incubation buffer, and in this case all the DNA was washed through the filter. Therefore, retention on nitrocellulose filters was dependent on the formation of a complex between the DNA and protein.

A study of this type was carried out by Tisdale (1988), in which nuclear proteins were shown to form more stable non-covalent complexes with ethazolastone-alkylated DNA rather than temozolomide-alkylated DNA. However, in these experiments calf thymus DNA was labelled with [^{14}C] temozolomide or ethazolastone, which has a number of advantages. Firstly, calf thymus DNA was used rather than DNA extracted from L1210 cells which makes any comparison between these results and the effect of alkylated calf thymus DNA on methyltransferase activity easier. Secondly, the DNA was labelled with the actual alkylating agent, which is a more precise way of producing DNA with the same extent of alkylation. The high cost of labelled drugs prevented their use in this work.

A crude nuclear extract from GM892 cells was used by Tisdale (1988) as the source of nuclear protein. The proportion of DNA(cytosine-5)methyl-

transferase in this nuclear extract would have been a very small proportion of the total nuclear proteins and so any conclusion made about the ability of the alkylated calf thymus DNA to bind nuclear protein could only be made in a general fashion. Therefore, the attempt to relate this to the binding of DNA(cytosine-5)methyltransferase is rather naive. The results presented in this thesis use a partially purified form of DNA(cytosine-5)methyltransferase, but due to the low purity of the enzyme any variation in binding to the alkylated DNA can only be accounted for in terms of general protein binding and not specific for DNA(cytosine-5)methyltransferase. These results are presented in fig. 20.

Although not as striking as Tisdale's observations, nuclear proteins form more stable non-covalent complexes with ethazolastone-modified DNA than with temozolomide-modified DNA. The percentage of DNA bound after washing with incubation buffer was similar for the two drugs (74% and 67% respectively for temozolomide and ethazolastone). However, on addition of 1 M NaCl the binding of temozolomide-modified DNA was reduced to 44% compared to 58% for ethazolastone-modified DNA, and after addition of 0.6% sarkosyl + 0.5 M NaCl the binding of temozolomide-modified DNA was reduced to 32% compared with 42% for ethazolastone-modified DNA. The

addition of 0.5% SDS reduced the binding of ethazolastone-modified DNA to 3% whilst that for temozolomide-modified DNA was reduced to 12%.

In the case of the nitrosourea modified DNAs, there are only slight differences between the extent of complex formation between the DNA and the nuclear proteins. The percentages of DNA bound to the filter after washing with the incubation buffer were 80% and 79% respectively for MNU and ENU. On the addition of 1.0 M NaCl the binding of MNU-modified DNA was reduced to 48% compared with 52% for ENU-modified DNA and after the addition of 0.6% Sarkosyl + 0.5 M NaCl the binding of MNU-modified DNA was reduced to 32% compared with 40% for ENU-modified DNA. The addition of 0.5% SDS reduced the binding of both modified DNAs to approximately 3-4%. Any differences observed for the binding of nuclear proteins to MNU and ENU-alkylated DNA was considered insignificant as the values fell within the error range of the experiment.

In the case of the alkyl sulphonate-modified DNAs there was an increased binding of the nuclear proteins to the MMS-modified DNA. The percentage of DNA bound to the filter after washing with the incubation buffer were similar, with values of 76% and 77% for MMS and EMS respectively. On the addition of 1.0 M NaCl the binding of EMS-modified DNA was reduced to 48% compared with 60% for MMS-modified DNA and after the addition of 0.6% Sarkosyl + 0.5 M NaCl the binding of EMS-modified DNA was reduced to 33%

compared with 42% for MMS-modified DNA. The addition of 0.5% SDS reduced the binding of both modified DNAs to 3%.

The differences observed in the stability of the complexes were not very great. The most striking difference occurred between temozolomide and ethazolastone and this might initially suggest that a particular modification was responsible for the stronger complex formation. It was suggested by Tisdale (1988) that the stronger affinity of ethazolastone-modified DNA for nuclear proteins over temozolomide-modified DNA, may be due to an increased electrostatic attraction by neutralisation of some of the negative charges on the DNA-phosphate backbone. Although a similar observation was made here, the idea did not hold up when other alkylating agents were tested and no correlation exists between the stability of the DNA/protein complex and a particular DNA modification. The assay is rather crude and a greater degree of enzyme purity is required before any conclusions can be made from such an experiment.

Experiments to investigate whether DNA-strand breakage is responsible for the inhibition of DNA(cytosine-5)methyltransferase.

Calf thymus DNA alkylated with agents such as the imidazotetrazinones has been shown to inhibit the transfer of methyl groups from SAM to M.lysodeikticus DNA by DNA(cytosine-5)methyltransferase. The

observed inhibition may be due to effects such as depurination and strand breakage induced by the alkylating agent, which alter the topological nature of the DNA. Alkylation of N-7 guanine labilises the glycosidic bond between the purine and the sugar to such an extent that considerable spontaneous depurination occurs even at normal physiological pH. In order to investigate whether this form of damage was responsible for the inhibition, damage was made to the calf thymus DNA by treatments such as sonication, the use of X-rays and the use of deoxyribonuclease I. This provided a means to investigate the possibility that drug-induced DNA damage other than alkylation, may be responsible for the inhibition of DNA(cytosine-5)methyltransferase. All the following studies were carried out using methyltransferase capable of transferring 0.4-0.5 pmol of methyl groups from SAM.

Deoxyribonuclease I treatment:

Deoxyribonuclease I (DNase I) causes the appearance of single-strand as well as double-strand breaks. This enzyme was used to determine whether DNA strand-breakage could inhibit the methyltransferase. The digestion of calf thymus DNA by DNase I was monitored using various amounts of the enzyme and the results are illustrated in fig. 21. The change in absorbance for the DNase I-treated DNA plateaued at a unit of enzyme activity of 200 K, at

which point the DNA would exist largely as small oligonucleotides. The DNA concentration was calculated according to the ethidium bromide-DNA fluorometric assay and 10 μg was routinely added to the standard DNA(cytosine-5)methyltransferase assay. The results are illustrated in fig. 22 which shows that inhibition of the methylation of M.lysodeikticus DNA by the methyltransferase occurs, which is dependent on the degree of DNase I treatment to the calf thymus DNA. These results show that the effectiveness of calf thymus DNA as an inhibitor of DNA(cytosine-5)methyltransferase is proportional to the number of DNA strand-breaks which have been produced. Therefore, inhibition of DNA(cytosine-5)methyltransferase may be due to single-strand breaks, double-strand breaks, an increase in the number of DNA molecules, a decrease in the length of the DNA molecule or a combination of these.

Restriction enzyme treatment:

In order to investigate whether specifically double-strand breaks in the calf thymus DNA, were capable of causing the inhibition of the enzymatic methylation of M.lysodeikticus DNA, a restriction enzyme was used to cleave the DNA. The calf thymus DNA was treated with MspI which cleaves the sequence

5'- C↓C G G - 3'

3'- G G C↑C - 5'

The DNA concentration was calculated according to the ethidium bromide-DNA fluorometric assay and 10 μg was routinely added to the standard DNA methylase assay. The results are illustrated in fig. 23 which shows that no inhibition of the methylation of M.lysodeikticus DNA by DNA(cytosine-5)methyltransferase occurs. This result suggests that double-strand breaks are unlikely to be responsible for the inhibition of DNA-(cytosine-5)methyltransferase.

Sonication:

Sonication is another means of producing DNA strand-breaks. A solution of calf thymus DNA was sonicated for various periods of time and 10 μg was routinely added to the standard DNA(cytosine-5)methyltransferase assay. The results are presented in fig. 24 and rather than inhibiting the methyltransferase reaction, there was an increase in the number of methyl groups transferred to the DNA. These results were rather surprising in light of the fact that DNase I-treated DNA was an inhibitor of the methylation reaction. An explanation for this arose when the sonicated calf thymus DNA was assessed for its ability to behave as a substrate for DNA(cytosine-5)methyltransferase. The results in fig. 25 illustrate that the sonicated calf thymus DNA becomes a better substrate for the methyltransferase by a factor of up to five times. An assessment of the amount of DNA by the ethidium bromide-DNA

fluorometric assay was made after the various periods of sonication and these results were expressed as a percentage of untreated DNA. These results are illustrated in fig. 26 and although there is no 'real' loss of DNA, less DNA is detected by this technique. The ethidium bromide-DNA fluorometric assay is based on the intercalation of ethidium bromide into DNA and therefore the apparent 'loss' of DNA shows that the DNA becomes more single-stranded after sonication.

The sonicated-DNA was then treated with an excess of the restriction enzyme MspI which cleaves double-stranded DNA at the sequence

5' - C↓C G G - 3'

3' - G G C↑C - 5'

The restriction enzyme-treated sonicated-DNA was then added to the standard DNA(cytosine-5)methyltransferase assay, and as can be seen from fig. 27, the increase in activity which was observed with sonicated-DNA was reduced (although not completely).

DNA irradiation

Calf thymus DNA in aqueous solution was exposed to various levels of X-radiation. To the standard DNA(cytosine-5)methyltransferase assay was added 10 μg of X-irradiated calf thymus DNA, in order to determine whether such treatment has any effect on the methylation of M.lysodeikticus DNA by DNA(cytosine-5)methyltransferase. The results in

fig. 28 show that X-irradiation does not cause calf thymus DNA to become a better substrate, as was demonstrated for sonicated calf thymus DNA. However, as can be seen from fig. 29, a potent inhibition of the methylation of M.lysodeikticus DNA by the methyltransferase was observed when the X-irradiated calf thymus DNA was added to the standard DNA(cytosine-5)methyltransferase assay. The inhibition of the methyltransferase was dependent on the dose of X-rays and was maximum at an X-ray dose of 200 rads. The data fits a Dixon plot (fig. 29(b)) and therefore the inhibition obeys first-order kinetics. Calf thymus DNA exposed to 200 rads caused an 80% reduction in the methyltransferase reaction when 10 μg were added to the standard assay and an X-ray dose greater than 200 rads did not increase the degree of inhibition beyond 80%.

An assessment of the amount of DNA by the ethidium bromide-DNA fluorometric assay was made after various levels of X-irradiation and these results are illustrated in fig. 30. The ethidium bromide-DNA fluorometric assay is based on the intercalation of ethidium bromide into DNA and although there is no real 'loss' of DNA, the apparent 'loss' of DNA (fig. 30) shows that the DNA is becoming more single-stranded after irradiation. It can be seen from fig. 30 that the maximum 'loss' of double-stranded DNA occurs at a dose of 200 rads.

This is also the dose at which calf thymus DNA causes the maximum inhibition of DNA(cytosine-5)methyltransferase.

This suggests that the inhibition of the methyltransferase may be due to single-strand breaks or a reduction in the length of the DNA. However, there are a very large number of radiation products produced in DNA (Breimer, 1988) which may contribute or be solely responsible for the potent inhibitory effect on methyltransferase activity.

An investigation was carried out to determine whether proteins in the methyltransferase preparation bind more strongly to the X-irradiated DNA, using the same assay as described for alkylated DNA. The results in fig. 31 show that there was a dramatic drop in the amount of DNA bound to the filter after X-irradiation. This is probably due to the fragmentation of the DNA by the X-rays, allowing more to pass through the filter. Therefore, this was an unsuitable method to determine whether the inhibition of DNA(cytosine-5)methyltransferase was due to the enzyme proteins binding more strongly to the DNA.

Agarose gel electrophoresis of the modified-DNAs.

There was no significant change in the molecular weight distribution of calf thymus DNA after treatment with up to 1 mM temozolomide or ethazolastone (fig. 32), MNU or ENU (fig. 33) and MMS or EMS (fig. 34). However, after treatment of calf

thymus DNA with chlorambucil or mitozolomide (fig. 35), there was an increase in the amount of low molecular weight species present. Chlorambucil caused this reduction in the molecular weight at concentrations as low as 0.001 mM and at a chlorambucil concentration of 1 mM the DNA was barely visible by this technique. This is because fluorescence of ethidium bromide intercalated into the DNA was used to reveal the DNA and therefore, this suggests that chlorambucil has degraded the DNA to very low molecular weight DNA and/or the DNA is more single-stranded. The effect of mitozolomide on DNA was less striking, but a reduction in the molecular weight of the DNA was evident at drug concentrations greater than 0.01 mM.

Calf thymus DNA treated with temozolomide, ethazolastone, MNU or ENU was shown earlier in this section to inhibit the transfer of methyl groups from SAM to M.lysodeikticus DNA by DNA(cytosine-5)methyl-transferase. In these assays a low activity of enzyme was used, such that under the conditions of the assay the control value was typically 0.1 pmol. However, calf thymus DNA treated with chlorambucil or mitozolomide was shown earlier in this section to inhibit the methyltransferase using enzyme activities typically capable of transferring 0.4 pmol in the control. A possible explanation for the mechanism of inhibition is that the alkylated DNA competes with the M.lysodeikticus DNA for enzyme binding sites and

therefore the activity of the enzyme used in the assay becomes important. Chlorambucil and mitozolomide-alkylated DNA caused inhibition at the higher enzyme activity whilst temozolomide, ethazolastone, MNU and ENU—alkylated DNA caused inhibition at the lower activity of enzyme. It would appear therefore, that there was a slight correlation between the extent of damage of calf thymus DNA as observed by agarose gel electrophoresis and the ability of that DNA to inhibit the methyltransferase. The correlation is only weak at this stage and so it was necessary to extend the investigation to DNase I-treated, Msp I-treated, sonicated and X-irradiated calf thymus DNA. The most potent inhibitors of the methyltransferase from this group were X-irradiated and DNase I-treated calf thymus DNAs. After treatment of calf thymus DNA with DNase I (Fig. 36), the molecular weight of the DNA dropped to below the smallest molecular weight marker (564 bases). This occurred for all the treatments (25-200 K).

Treatment of calf thymus DNA with 50 rads of X-irradiation (fig. 39) resulted in a drop in the molecular weight of the DNA. However, calf thymus DNA exposed to greater than 100 rads of X-irradiation was not visible by agarose gel electrophoresis. This is because in this system the DNA is made visible by fluorescence of ethidium bromide intercalated into the DNA and if the DNA is single-stranded it would not be made visible. It is noted that the control

DNA in this gel also appears to be degraded. These results add support to the correlation between the inhibition of the methyltransferase and the extent to which the calf thymus DNA has been degraded.

Calf thymus DNA digested with Msp I was analysed by agarose gel electrophoresis and the results are shown in fig. 37. There was a reduction in the molecular weight of the DNA after Msp I treatment which was proportional to the amount of restriction enzyme used. It was shown earlier that calf thymus DNA treated with Msp I does not inhibit the methyltransferase reaction. Therefore, this apparently contradicts the correlation between DNA degradation and the ability to inhibit the methyltransferase. However, restriction enzymes produce only double-strand breaks (Yuan, 1981). Therefore, the actual drop in molecular weight of the calf thymus DNA observed after Msp I treatment is 'real' and is not due to the formation of single-strand regions. This suggests that the inhibition of DNA(cytosine-5)methyltransferase by calf thymus DNA after various modifications was due to single-strand breaks and not double-strand breaks.

It can be seen from fig. 38 that sonication of calf thymus DNA caused a reduction in the molecular weight of the DNA which was proportional to the length of sonication. Sonication would presumably create double-strand and single-strand breaks but, as shown earlier, DNA treated in this way becomes a

better substrate for DNA(cytosine-5)methyltransferase and does not inhibit the methyltransferase reaction. Therefore, in order to explain this result further work would be required.

These observations on modified DNA suggest a correlation between the formation of single-stranded regions in DNA and the ability of that DNA to inhibit DNA(cytosine-5)methyltransferase. However, the concern with this conclusion is that the damage to the calf thymus DNA by temozolomide, ethazolastone, MNU and ENU could not be detected by agarose gel electrophoresis, despite this modified-DNA being able to inhibit the methyltransferase reaction. An attempt was made to assess the level of single-strand breaks in the in vitro drug-treated calf thymus DNAs using the technique of alkaline elution. However, the technique was too sensitive in that the alkaline solution eluted most of the DNA even in the control samples, and no comparisons could be made.

The technique of alkaline elution (Kohn et al., 1981) has previously been used to study the level of DNA damage after drug treatment of cells grown in culture. The preparation and handling of DNA before and after in vitro drug treatment is likely to cause damage to the DNA which is detectable by alkaline elution. This seems very likely as even the control DNA in the agarose gels (fig. 32 to 39) appears degraded (one would not expect the high molecular

weight calf thymus DNA to have a molecular weight less than the highest molecular weight marker, 21226 kb).

The direct effect of free drug on DNA(cytosine-5)methyltransferase).

An investigation was carried out to determine the effect of the alkylating agents, temozolomide, ethazolastone, mitozolomide, MNU, ENU, EMS and MMS on DNA(cytosine-5)methyltransferase activity. The results are shown in table 11. In most cases significant inhibition did not occur until concentrations greater than 1 mM were used and even using 10 mM MNU the methyltransferase activity was unaffected. The inhibition that they do produce is thought to occur by reaction with critical sulfhydryl groups.

The direct inhibition of DNA(cytosine-5)methyltransferase has been observed by Cox (1980) for MNNG and Chan et al. (1983) for MNNG and MNU in which they observed that dithiothreitol provided some protection for the enzyme. In all these cases however, the concentration of agent required to cause methyltransferase inhibition was considerably higher than could be attained in vivo. Tisdale (1989) observed no inhibition of DNA(cytosine-5)methyltransferase when treated in vitro with temozolomide or ethazolastone up to a concentration of 10 mM. Therefore, although it is probable that some of the inhibition may be due to direct

reaction with the enzyme, it is not the major mechanism by which temozolomide and ethazolastone inhibit DNA(cytosine-5)methyltransferase.

The effect of various methylated bases on DNA(cytosine-5)methyltransferase has been investigated (table 12). None of the bases investigated at a concentration of 1 mM had any effect on methyltransferase activity.

4.4 DISCUSSION

The aim of this section was to explore the mechanism by which temozolomide causes DNA hypomethylation. It was a continuation of the work of Tisdale (1986), in which he suggested that temozolomide-induced differentiation of the human erythroleukemia cell line K562 was due to gene hypomethylation and Tisdale (1988), in which calf thymus DNA alkylated *in vitro* with temozolomide or ethazolastone was shown to inhibit the transfer of methyl groups from SAM to *M.lysodeikticus* by DNA(cytosine-5)methyltransferase. However, during the course of this work a paper was published by Zucchetti *et al*. (1989), which claimed that the temozolomide-induced differentiation of the human erythroleukemia K562 cell line, was not mediated by gene hypomethylation. This paper questions the validity of Tisdale's hypothesis, and since the work presented in this thesis was a continuation of Tisdale's, a thorough examination of the paper by Zucchetti *et al*. (1989) was considered necessary.

Examination of the paper by Zucchetti et al. (1989).

Although the results confirm that temozolomide induces the differentiation of K562 cells, they report no change in the (cytosine-5)methylation pattern of the ϵ-globin and gamma-globin genes and the c-*myc* and c-Ha-*ras* oncogenes. The DNAs from

untreated and treated K562 cells were digested with MspI or HpaII and hybridised with either the 0.7 kb BamHI fragment of the ϵ-globin gene, the 3.3 kb HindIII fragment of the gamma-globin gene, the 6.5 kb BamHI fragment of the c-Ha-ras or the 1.4 kb ClaI-EcoRI fragment of the c-myc oncogenes. They report that there was no detectable change in the methylation status of these genes after temozolomide treatment. However, after consideration of their data I suggest that their conclusion was mistaken and in fact the evidence that they present indicates that there were changes in the methylation status of these genes. The appropriate figures of the paper have been reproduced and are presented in APPENDIX 3.

Figure 3 represents the pattern of DNA methylation of ϵ and gamma-globin genes from the DNA of untreated or temozolomide-treated K562 cells. The authors state that the methylation status of these genes was not altered after temozolomide-treatment, but this is clearly not true on examination. The pattern of DNA methylation of ϵ-globin genes after temozolomide treatment reveals bands after HpaII digestion at approximately 7 kb and 12 kb (lane 4) which are only very faint in the corresponding lane (lane 2) for the untreated K562 cells. A very large band at approximately 1 kb also appears after temozolomide treatment (lane 4) which is not present in the corresponding lane for untreated K562 cells

(lane 2). The pattern after MspI treatment is also different for the K562 DNA between untreated and temozolomide-treated cells. The band at approximately 5 kb is very faint for the untreated cellular DNA compared with that for temozolomide-treated DNA.

The pattern of DNA methylation of the gamma-globin genes after temozolomide treatment of K562 cells are not identical. After HpaII digestion the band at approximately 3 kb is very faint for the DNA of cells after temozolomide treatment (lane 5) compared with the DNA from untreated cells (lane 2).

These differences in the pattern of DNA methylation are small, but were overlooked by the authors. The results in particular for the ϵ-probe suggest that the DNA was hypomethylated for the K562 cells after temozolomide treatment.

They then extended their studies to the c-Ha-ras and c-myc proto-oncogenes, which contain many methylated sites. Once again the authors state that there was no difference in the pattern of DNA methylation around the two oncogenes between temozolomide-treated and untreated K562 cells. Figure 4 represents the pattern of DNA methylation of c-Ha-ras and c-myc proto-oncogenes. The pattern of DNA methylation of c-Ha-ras genes clearly reveals a greater proportion of DNA over the lower molecular weight region for DNA from temozolomide-treated cells after HpaII digestion (lane 4) compared with DNA from

untreated cells (lane 2). This is clearly an indication that the c-Ha-ras proto-oncogene was more hypomethylated after temozolomide treatment.

The pattern of DNA methylation of the c-myc oncogene (fig 4B), shows that there is an increase in the amount of DNA in the region 1.5-4.3 kb for DNA from temozolomide-treated cells after HpaII digestion (lane 4) compared with DNA from untreated cells (lane 3). It would appear that after HpaII digestion there has been a shift to a lower molecular weight for temozolomide-treated cells. The use of a densitometer would have been useful in the analysis of these results, which as presented suggest that the c-Ha-ras gene was hypomethylated after temozolomide treatment.

These authors also state that there was no difference found in the methylation status of GCGC sequences as assessed with the use of the restriction enzyme HhaI. This data was not presented and I suspect that it was even less convincing than the data for CCGG sequences, in supporting their argument that temozolomide does not induce DNA hypomethylation.

A comparison was then made of the DNA methylation pattern of c-Ha-ras and c-myc proto-oncogenes of DNA from K562 cells after temozolomide treatment and after 5-aza-2'-deoxycytidine treatment. Clearly a greater degree of gene hypomethylation was demonstrated after 5-aza-2'-deoxycytidine treatment.

However, there is a large difference in the methylation pattern of the c-Ha-ras and c-myc oncogene in the DNA from untreated cells in fig. 4 and fig. 5. One would expect the restriction pattern for the c-Ha-ras or the c-myc probe to remain constant for the DNA from untreated cells and not produce the large differences seen between fig. 4 and fig. 5.

Therefore, I conclude that this paper does not provide any evidence to contradict the hypothesis that temozolomide-induced differentiation of K562 erythroleukemia cells is mediated by gene hypomethylation.

The mechanism by which temozolomide causes hypomethylation can now be discussed. It was reported by Tisdale (1986) that temozolomide, but not ethazolastone, induced the differentiation of K562 erythroleukemia cells grown in culture. He found that after temozolomide treatment (73.5 μM) the content of 5-methylcytosine in DNA after 3 days fell from 3.5 to 2.2%, whilst there was no significant change after ethazolastone treatment (240 μM). In order to investigate this mechanism of hypomethylation, Tisdale (1988) alkylated calf thymus DNA in vitro with temozolomide or ethazolastone and the alkylated DNA was shown to inhibit the transfer of methyl groups from SAM to M.lysodeikticus DNA by DNA(cytosine-5)methyltransferase. Calf thymus DNA modified with ethazolastone was more effective as an

inhibitor of DNA(cytosine-5)methyltransferase than temozolomide-modified DNA, which was a reverse of the order of potency against tumour cells in culture. Most tumour cell lines including Raji, GM892 and K562 (Tisdale, 1987; Bull and Tisdale, 1987) are more sensitive to temozolomide than ethazolastone, one exception being the human lung carcinoma cell line A549.

Further investigations by Tisdale (1989) using the same in vitro assay, showed that DNA extracted from GM892 cells after treatment with temozolomide, also inhibited the transfer of methyl groups from SAM to M.lysodeikticus DNA by DNA(cytosine-5)methyl-transferase. The inhibition was shown to be proportional to the concentration of temozolomide used. The inhibition was maximal 6 h after drug treatment and after 24 h returned to control levels, which suggests that the inhibition was due to a repairable lesion. The effect of temozolomide and ethazolastone on the enzymatic methylation of DNA(cytosine-5) was investigated in the human lympho-blastoid cell line, GM892. It was observed that four days after treatment with temozolomide (206 μM) the content of 5-methylcytosine fell from 3.41 to 2.50% and after five days treatment with ethazolastone (480 μM), the content of 5-methylcytosine fell from 3.53 to 2.29% in the total genome. Hypomethylation of DNA in the K562 erythroleukemia cell line was not

observed after ethazolastone (240 μM) treatment (Tisdale, 1986), although in this case the drug concentration was half of that used to cause hypomethylation in GM892 cells.

The activity of DNA(cytosine-5)methyltransferase incubated *in vitro* with up to 10 mM temozolomide or ethazolastone (Tisdale, 1989) was not affected. However, the results presented in this thesis show that up to 25% of the enzyme activity was lost when the DNA(cytosine-5)methyltransferase assay was carried out in the presence of a drug concentration of 1 mM. DNA(cytosine-5)methyltransferase contains sulfhydryl groups at the active site (Bestor *et al.*, 1988) which is a reactive site for a number of agents.

A complete inhibition of the methyltransferase has been observed after treatment of the enzyme with MNNG (Drahovský and Wacker, 1975), whilst a similar treatment of the DNA substrate did not produce any inhibition. However, the drug concentration used was 2 mM and whether these results are significant for such a potent carcinogen is dubious. Cox (1980) confirmed this observation, but once again this was at high concentrations of the drug. Acrolein, the reactive metabolite of cyclophosphamide, known to react with thiol groups has been shown to cause a 30-50% inhibition of methyltransferase activity at a concentration of 10 μM. The ability of a number of

carcinogens to inhibit DNA(cytosine-5)methyltransferase *in vitro* has been investigated by Chan *et al*. (1983) and a 50% inhibition of activity was observed with 4.3 mM *N*-acetoxy-*N*-acetyl-2-aminofluorene, 47 mM MNU and 2.8 mM MNNG. The significance of these observations is limited, as these very high drug concentrations would not be attained in the cell.

It is unlikely that the hypomethylation observed in GM892 cells (Tisdale, 1989) and K562 cells (Tisdale, 1986) was due entirely to alkylation of the enzyme as the high drug concentrations required were not attained in the cell. However, calf thymus DNA treated *in vitro* with relatively low concentrations of temozolomide or ethazolastone (0.01 mM) caused a significant inhibition of DNA(cytosine-5)methyltransferase. This inhibition has been investigated further in an attempt to identify the DNA modification responsible.

Tisdale (1988) concluded that alkylphosphotriesters were responsible for the inhibition of the methyltransferase, since ethazolastone-modified DNA was a more effective inhibitor than temozolomide-modified DNA and phosphotriesters are the main site of alkylation for ethazolastone (Bull, 1988).

The extent of *in vitro* alkylation at the phosphotriester group in DNA using 0.25 mM temozolomide or ethazolastone was calculated to be 21

μmol/mol DNA-P and 18 μmol/mol DNA-P respectively. Therefore, the fact that ethazolastone-ethylated DNA was more effective than temozolomide-methylated DNA as an *in vitro* inhibitor of the methyltransferase in Tisdale's system, cannot be accounted for in terms of the level of alkylphosphotriester formation. The results presented in this thesis show a better correlation between the degree of phosphotriester alkylation and the ability of the DNA to inhibit the methyltransferase, which were similar for both calf thymus DNA alkylated with temozolomide or ethazolastone. Poly[dA].poly[dT] alkylated with MNU forms 90% methylphosphotriesters (McCarthy and Lindahl, 1985) but was shown only to be a poor inhibitor of the methylation of *M.lysodeikticus* DNA by DNA(cytosine-5)methyltransferase.

It remains a possibility that alkylphosphotriesters in the calf thymus DNA, produced as a result of reaction with temozolomide or ethazolastone, are responsible for the observed inhibition of DNA(cytosine-5)methyltransferase. However, two observations cast doubts on this conclusion. Firstly, the results presented by Tisdale (1988) on the inhibition of DNA(cytosine-5)methyltransferase by calf thymus DNA alkylated with temozolomide or ethazolastone are different from the

results presented in this thesis. Secondly, poly[dA].poly[dT] alkylated with MNU is a poor inhibitor of the methyltransferase.

The *in vitro* inhibition of DNA(cytosine-5)methyltransferase by chemically modified DNA has been observed with other agents. DNA prepared by growing *E.coli* in the presence of 5-azacytidine has been shown to inhibit the methylation of 'native' *E.coli* DNA in an *in vitro* assay (Friedman, 1981). Chicken erythrocyte DNA modified with AAF has been shown to cause the inhibition of the methylation of 'native' DNA by DNA(cytosine-5)methyltransferase (Pfohl-Leszkowicz *et al.*, 1981; Pfohl-Leszkowicz *et al.*, 1983b). In these examples the mechanism of inhibition is thought to involve an increased binding of the methyltransferase to the modified DNA,with the result that less enzyme is available for the methyltransferase reaction. The ability of azacytosine-containing DNA to inhibit the methyltransferase reaction (Friedman, 1981) is thought to be due to covalent bond formation between the enzyme and the *N*-5 position of the cytosine analogue in the DNA (Santi *et al.*, 1983). It is unlikely that stable covalent bond formation occurs between DNA(cytosine-5)methyltransferase and calf thymus DNA alkylated with temozolomide or ethazolastone, since complexes between nuclear proteins and the substituted DNA could be dissociated in the presence of SDS. The

poor purity of the methyltransferase preparation prevents this possibility from being completely excluded.

Pfohl-Leszkowicz et al. (1981, 1983b) proposed another mechanism by which modified DNA was able to inhibit DNA(cytosine-5)methyltransferase. MNU-methylated DNA was shown by Pfohl-Leszkowicz et al. (1983b) to inhibit the enzymatic methylation of native DNA much less than AAF-modified DNA. They suggested that AAF bound to the guanine residues prevented the enzyme from 'walking' along the DNA helix, as the adduct formed was bulkier than that formed with MNU. However, the evidence presented by Pfohl-Leszkowicz et al. (1983b) was not very convincing. Firstly, the radioactivity incorporated into the DNA in these experiments was very low (<1000 CPM) and there was no indication of any statistical considerations. Secondly, they compared AAF-modified DNA with MNU-modified DNA, but they did not present the data for MNU-modified DNA. Therefore, we are not given the opportunity to judge the significance of their claim.

Nuclear proteins in the enzyme preparation were shown to have a slightly stronger affinity for ethazolastone-modified DNA than temozolomide-modified DNA. However, the difference was not as large as reported by Tisdale (1988) and there was no significant difference for the other drug-modified

DNAs. It is possible that, as suggested by Tisdale (1988), the stronger affinity for ethazolastone-modified DNA may occur as a result of an increased electrostatic attraction between the DNA and the DNA(cytosine-5)methyltransferase, due to neutralisation of some of the negative charges on the phosphate backbone of the DNA. Further support for this theory comes from Farrance and Ivarie (1985) who report that poly[dC-dG].poly[dC-dG] when ethylated by EMS at molar ratios of bases to co-polymer of 5 to 7 x 10^3, produces a better substrate for the methyltransferase than unmodified DNA. It may become a better substrate as a result of stronger binding by increased electrostatic attraction between the methyltransferase and the co-polymer. However, EMS-modified calf thymus DNA in my system was a poor inhibitor of the methyltransferase which suggests that although there may be an increased binding of the methyltransferase to the modified DNA, this is only weak. Additionally, MNU-methylated poly[dA].poly[dT] was not a better inhibitor than poly[dA].poly[dT], which further weakens this theory as a possible explanation.

A large study on the inhibition of DNA methylation by chemical carcinogens <u>in vitro</u> has been conducted by Wilson and Jones (1983). However, there were major differences in the assay that they used compared with my system. These workers used hemi-

methylated DNA as the substrate for DNA(cytosine-5)methyltransferase and were therefore measuring the maintenance activity of the enzyme. However, in my system M.lysodeikticus DNA was used as the substrate and consequently, the de novo activity of the enzyme was measured. In the system of Wilson and Jones (1983), the actual substrate for the methyltransferase was treated with the carcinogen, whilst in my system the substrate DNA remained intact and the effect of a DNA (which under normal conditions has no effect on the methyltransferase activity), was alkylated with various drugs and was shown to inhibit the methyltransferase reaction.

Wilson and Jones (1983) showed that modification of hemimethylated DNA by a wide range of carcinogens (MNNG, EMS, ENU, ENNG, BCNU, 9-aminoacridine, nitrogen mustard, N-acetoxy-N-acetylaminofluorene and benzo(a)pyrene diolepoxide) inhibited DNA(cytosine-5)methyltransferase. These authors made no attempt to assess the relative importance of specific base or phosphotriester adducts.

The next approach taken was to investigate whether the observed inhibition of DNA(cytosine-5)methyltransferase with alkylated calf thymus DNA, was due to the formation of some other form of DNA damage, such as apurinic sites or strand breaks. Calf thymus DNA exposed to X-irradiation and calf thymus DNA treated with the enzyme deoxyribonuclease I were

both effective inhibitors of the methyltransferase. X-Irradiation causes a wide variety of DNA damage including double-strand and single-strand breaks (Breimer, 1988). Deoxyribonuclease I causes the formation of single-strand and double-strand breaks. However, calf thymus DNA treated with the restriction enzyme MspI was not an inhibitor of DNA(cytosine-5)-methyltransferase and it recognises only double-stranded DNA to produce only double-strand breaks (Yuan, 1981). Although this evidence is not conclusive, it suggests that the inhibition of methyltransferase activity observed with DNaseI-treated calf thymus DNA and at least some of the inhibition observed with X-irradiated calf thymus DNA is due to the formation of single-strand breaks. Extrapolating this conclusion to the drug-treated DNAs, the inhibition of DNA(cytosine-5)methyl-transferase produced with temozolomide or ethazolastone-treated calf thymus DNA, may in part be due to the formation of single-strand breaks in the DNA by these drugs.

This hypothesis is supported by the evidence of Drahovský and Morris (1971), in which they demonstrated that rat liver DNA(cytosine-5)methyl-transferase binds more strongly to single-stranded DNA than double-stranded DNA, and in mixtures of single-stranded and double-stranded DNA the single-stranded DNA was preferentially methylated.

Therefore, if calf thymus DNA which is a very poor substrate for the methyltransferase, is made through the formation of strand breaks to bind the methyltransferase more firmly, then less enzyme would be available to act on the M.lysodeikticus DNA. The result of this increased binding would be the observed reduction in the level of enzymatic methylation.

As part of the study by Wilson and Jones (1983) on the inhibition of DNA methylation by chemical carcinogens in vitro, an investigation was carried out to determine the importance of strand breaks and apurinic sites. Single-strand breaks were produced by ultraviolet-irradiation of the bromouracil-containing substrate, which was shown by sucrose gradient analysis to contain no double-strand breaks. Inhibition of the methyltransferase reaction was much greater for DNA treated in this way, compared to DNA containing double-strand breaks induced by sonication. Ultraviolet light induces other forms of DNA damage such as thymine dimers (Hanawalt et al., 1979) which may have been responsible for the observed inhibition. They controlled for this by treating substrate DNA and substrate DNA containing bromouracil with ultraviolet light and the methyltransferase reaction was only decreased significantly for the bromouracil- containing DNA. Therefore, the effects of ultraviolet light on

bromouridine-containing DNA were due to the formation of single-strand breaks or other alkali-labile damage and not the formation of thymine dimers.

Therefore, using a different approach Wilson and Jones (1983) have also provided evidence that DNA single-strand breaks are capable of causing inhibition of DNA(cytosine-5)methyltransferase. One can extrapolate from these observations and conclude that temozolomide or ethazolastone treatment of the calf thymus DNA generated enough single-strand breaks to cause the observed inhibition of DNA(cytosine-5)-methyltransferase.

However, no data was generated to give any indication of the level of single-strand breaks. Alkaline elution was used to assess whether the inhibition of DNA(cytosine-5)methyltransferase by damaged DNA could be correlated with the production of single-strand breaks. The technique developed by Kohn et al. (1981) is normally used to measure strand-breaks and crosslinks in cellular DNA after treatment of cells with drug. However, in this system, DNA treated in vitro with drugs was assessed and the level of DNA damage was so great that even in the control the majority of the DNA was washed through the filter.

In mouse L1210 cells treated with 50 μM mitozolomide, Gibson et al. (1984a) found that negligible single-strand breaks occurred up to 9 h

after drug removal. The fact that DNA single-strand breaks are not produced to a great extent in cells treated with 50 μM mitozolomide does not necessarily imply that the same occurs when isolated DNA is treated with 500-1000 times this concentration of drug.

The extent of DNA damage of K562 cells treated with temozolomide or ethazolastone has been investigated by Zucchetti et al. (1989) using alkaline elution. They showed that at drug concentrations of 50 μM, 100 μM and 200 μM, temozolomide causes a greater degree of single-strand breaks in K562 cells than ethazolastone. Therefore, if this was to occur for isolated DNA treated in vitro with these drugs, then one would expect temozolomide-modified DNA to be a better inhibitor of the methyltransferase than ethazolastone-modified DNA. Once again, it does not necessarily follow that isolated DNA would be damaged in the same fashion as cellular DNA.

Bull and Tisdale (1987) have demonstrated that five to six times more temozolomide than ethazolastone binds to isolated calf thymus DNA when using a drug concentration of 0.1 mM. However, when the same drug concentration is used in Raji and GM892 cells the binding of the drugs to the DNA is similar. This indicates that isolated calf thymus DNA and DNA in cells is alkylated to different extents by these

drugs. However, this observation suggests that isolated DNA treated *in vitro* with temozolomide would receive a greater extent of damage in terms of single-strand breaks than ethazolastone-modified DNA. An accurate assessment of these drugs to form single-strand breaks in calf thymus DNA *in vitro* is required before this form of damage can be directly correlated with an inhibition of DNA(cytosine-5)methyltransferase.

At this point it is worth commenting on the observation that calf thymus DNA treated with X-irradiation is an effective *in vitro* inhibitor of DNA(cytosine-5)methyltransferase. This provides an alternative explanation for the radiation-induced hypomethylation of DNA in cell lines as observed by Kalinich *et al*. (1989). These workers demonstrated a dose-dependent decrease in 5-methylcytosine in four normal and tumour cell lines (Chinese hamster ovary, Chinese hamster lung fibroblast, HeLa and a mouse neuroblastoma clone) after exposure to ^{60}Co gamma-radiation. They observed a migration of DNA(cytosine-5)methyltransferase activity from the nuclear to the cytoplasmic component, whilst there was no total loss of enzyme levels. Although this migration of methyltransferase activity may occur, the enzyme may have a stronger affinity for the DNA in the radiation-damaged cells and this may account for the observed hypomethylation.

Wilson and Jones (1983) have shown that the ability of hemimethylated DNA to behave as a substrate for maintenance methyltransferase activity is also inhibited after X-irradiation. However, after a dose of 2250 rads the activity was inhibited by only 25%. This is surprising as such a large dose would have been expected to cause degradation of the DNA.

Thus it has been shown that DNA damaged by drugs, radiation and enzymes can inhibit DNA(cytosine-5)methyltransferase *in vitro*, but can they cause hypomethylation of DNA *in vivo*?

It has been shown that temozolomide (73.5 μM) causes genomic hypomethylation of DNA in the human leukemia cell line K562 (Tisdale, 1988), with a drop in the level of 5-methylcytosine from 3.5% to 2.2% three days after treatment. Hypomethylation of DNA from the human lymphoblastoid cell line GM892 has been shown to occur after temozolomide (206 μM) and ethazolastone (480 μM) treatment, with a drop in the level of 5-methylcytosine from 3.4% to 2.5% four days after treatment and 3.53% to 2.95% five days after treatment respectively.

MNU, which like temozolomide alkylates DNA through formation of the alkyldiazonium ion, has been shown to cause hypomethylation of DNA in the human Raji Burkitt's lymphoma cell line (Boehm and Drahovský, 1981a). MNU at a concentration of 20 μg/ml

was shown by two methods to cause hypomethylation. Firstly, the overall extent of enzymatic DNA methylation was measured by determining the [^{14}C]deoxycytidine derived radioactivity in DNA 5-methylcytosine and cytosine. Secondly, the use of restriction enzymes MspI and HpaII, which can be used to detect enzymatic methylation of the internal cytosine of the sequence 5'-CCGG-3'. However, the report by Krawisz and Lieberman (1984) contradicts these observations, as they show that the carcinogens MNU and N-acetoxy-2-acetylaminofluorene do not cause any detectable change in the 5-methylcytosine levels of newly replicated DNA in Raji cells (a human lymphoblastoid cell line), S49 cells (a mouse thymic lymphoma cell line) and human diploid fibroblasts, even at concentrations that inhibit cell replication by 95%.

In conclusion, DNA treated with a number of alkylating agents, in particular temozolomide and ethazolastone, has been shown to inhibit DNA(cytosine-5)methyltransferase. This may be responsible for the observed hypomethylation of DNA seen after treatment of some cell lines with these drugs (Tisdale, 1988; Tisdale, 1986; Boehm and Drahovský, 1981a), but there are notable exceptions when no effect is observed (Krawisz and Lieberman, 1984). The formation of single-strand breaks in the DNA after treatment with drug is likely to be

important as experiments with modified DNA suggest that this lesion is inhibitory. However, in the absence of any data on the relative number of single-strand breaks formed after *in vitro* drug treatment of the DNA, a direct correlation cannot be made between the *in vitro* inhibition of DNA(cytosine-5)methyl-transferase and the number of single-strand breaks.

The relevance of these *in vitro* observations when applied to the cell are questionable, as extensive damage was produced in the calf thymus DNA before it was capable of inhibiting the methyltransferase. Other factors may be involved in the *in vitro* inhibition of the methyltransferase, such as the formation of the alkylphosphotriester lesion, but the evidence for this being involved is weak. Although treatment of the methyltransferase *in vitro* with temozolomide or ethazolastone produces only a weak inhibition, some enzyme inhibition is likely to occur through this mechanism in cells. Finally, it should once again be acknowledged that the enzyme preparation used in these studies was not homogenous and the presence of other proteins may have affected the methyltransferase inhibition.

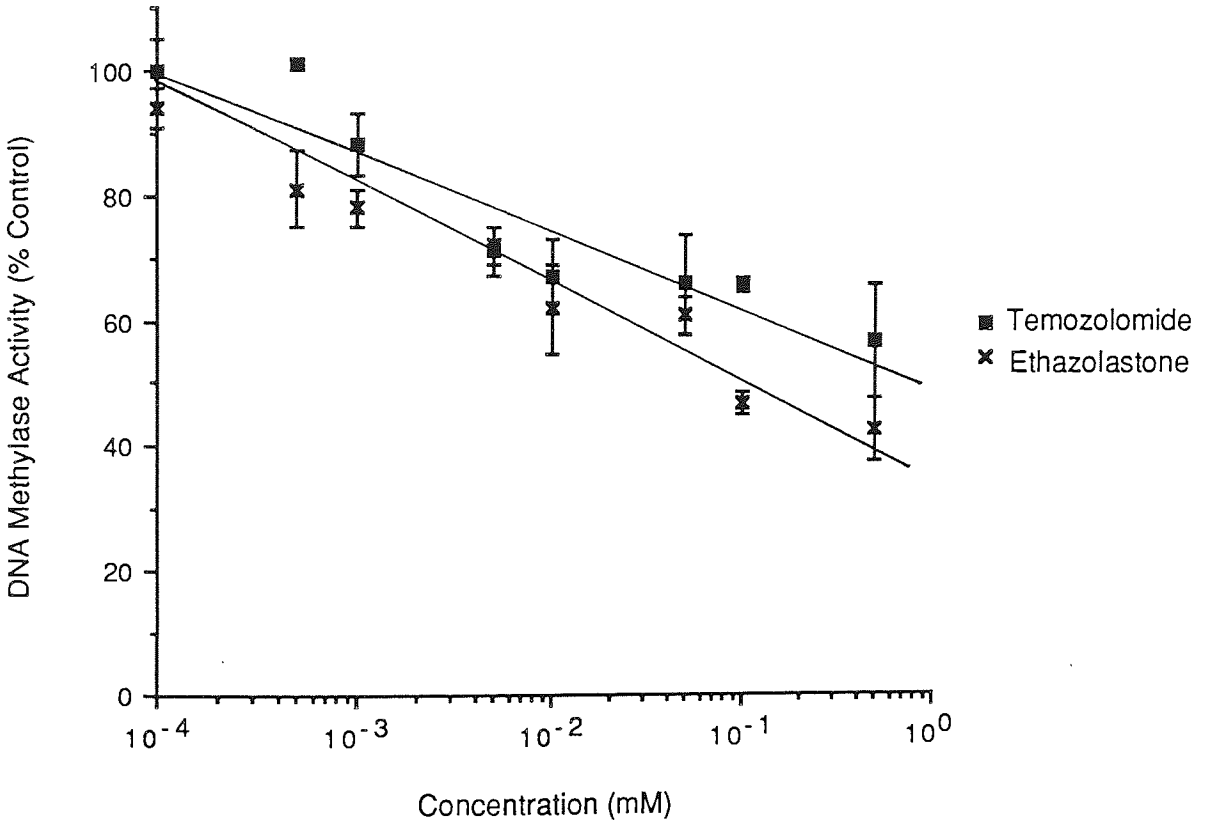


Figure 14: The effect of calf thymus DNA modified with temozolomide or ethazolastone on the methylation of M.lysodeikticus DNA by DNA(cytosine-5)methyltransferase.
Calf thymus DNA was treated with drug as described in section 4.2.2 and 10 µg of the treated-DNA was added to the standard DNA methylase assay which contained 20 µg M.lysodeikticus DNA, 1 µCi SAM and DNA(cytosine-5)methyltransferase capable of transferring 0.1 pmol of methyl groups under the conditions of the assay and incubated at 37°C for 4 h. In this graph the concentration on the x-axis represents the concentration of drug in the reaction with the calf thymus DNA and in this reaction the drugs were dissolved in DMSO which constituted less than 10% of the reaction volume.
The results are expressed as a percentage of the methylation of M.lysodeikticus observed with the untreated calf thymus DNA and represent the results of three experiments expressed as mean ± SEM.

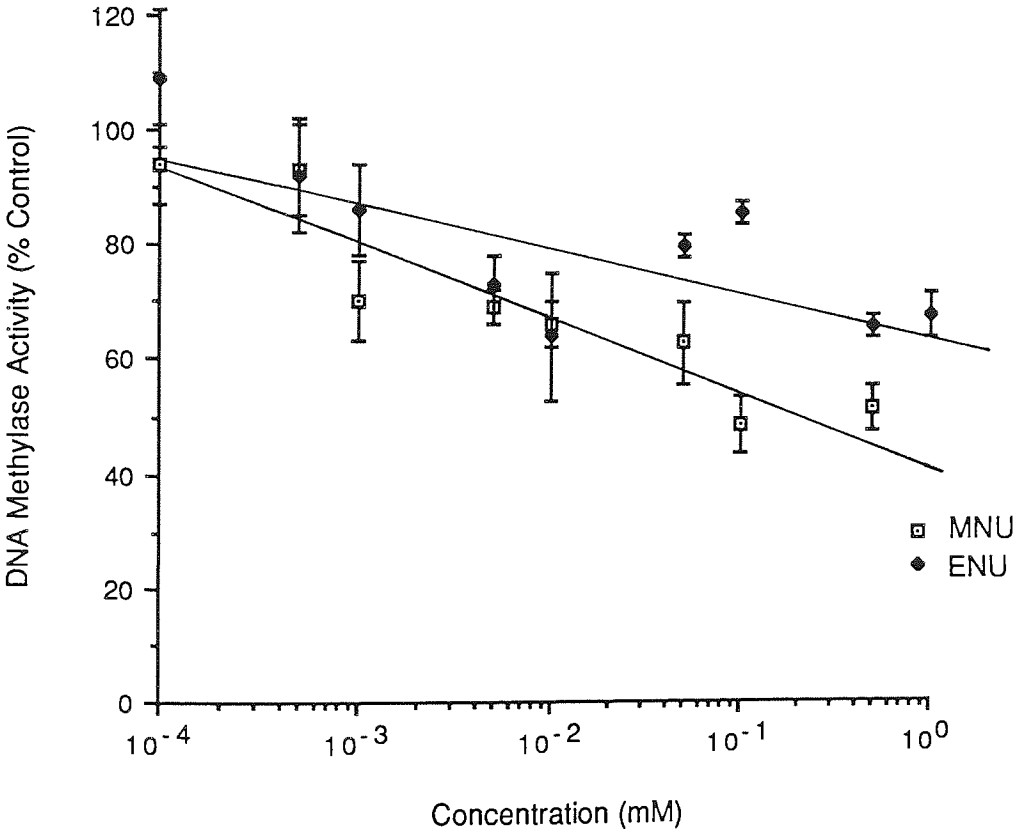


Figure 15: The effect of calf thymus DNA modified with MNU or ENU on the methylation of M.lysodeikticus DNA by DNA(cytosine-5) methyltransferase.
Calf thymus DNA was treated with drug as described in section 4.2.2 and 10 µg of the treated-DNA was added to the standard DNA methylase assay which contained 20 µg M.lysodeikticus DNA, 1 µCi SAM and DNA(cytosine-5)methyltransferase capable of transferring 0.1 pmol of methyl groups under the conditions of the assay and incubated at 37°C for 4 h. In this graph the concentration on the x-axis represents the concentration of drug in the reaction with the calf thymus DNA and in this reaction the drugs were dissolved in DMSO which constituted less than 10% of the reaction volume.
The results are expressed as a percentage of the methylation of M.lysodeikticus observed with the untreated calf thymus DNA and represent the results of three experiments expressed as mean ± SEM.

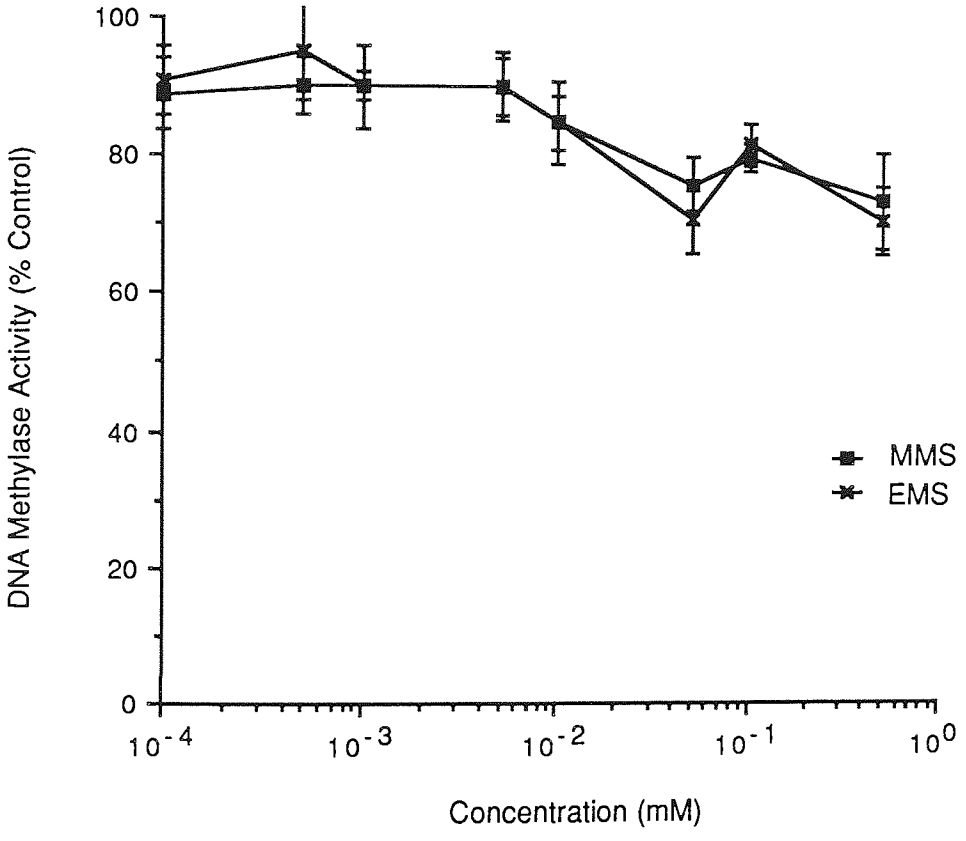


Figure 16: The effect of calf thymus DNA modified with MMS or EMS on the methylation of M.lysodeikticus DNA by DNA(cytosine-5) **methyltransferase.**
Calf thymus DNA was treated with drug as described in section 4.2.2 and 10 µg of the treated-DNA was added to the standard DNA methylase assay which contained 20 µg M.lysodeikticus DNA, 1 µCi SAM and DNA(cytosine-5)methyltransferase capable of transferring 0.1 pmol of methyl groups under the conditions of the assay and incubated at 37°C for 4 h. In this graph the concentration on the x-axis represents the concentration of drug in the reaction with the calf thymus DNA and in this reaction the drugs were diluted in DMSO which constituted less than 10% of the reaction volume.
The results are expressed as a percentage of the methylation of M.lysodeikticus observed with the untreated calf thymus DNA and represent the results of three experiments expressed as mean ± SEM.

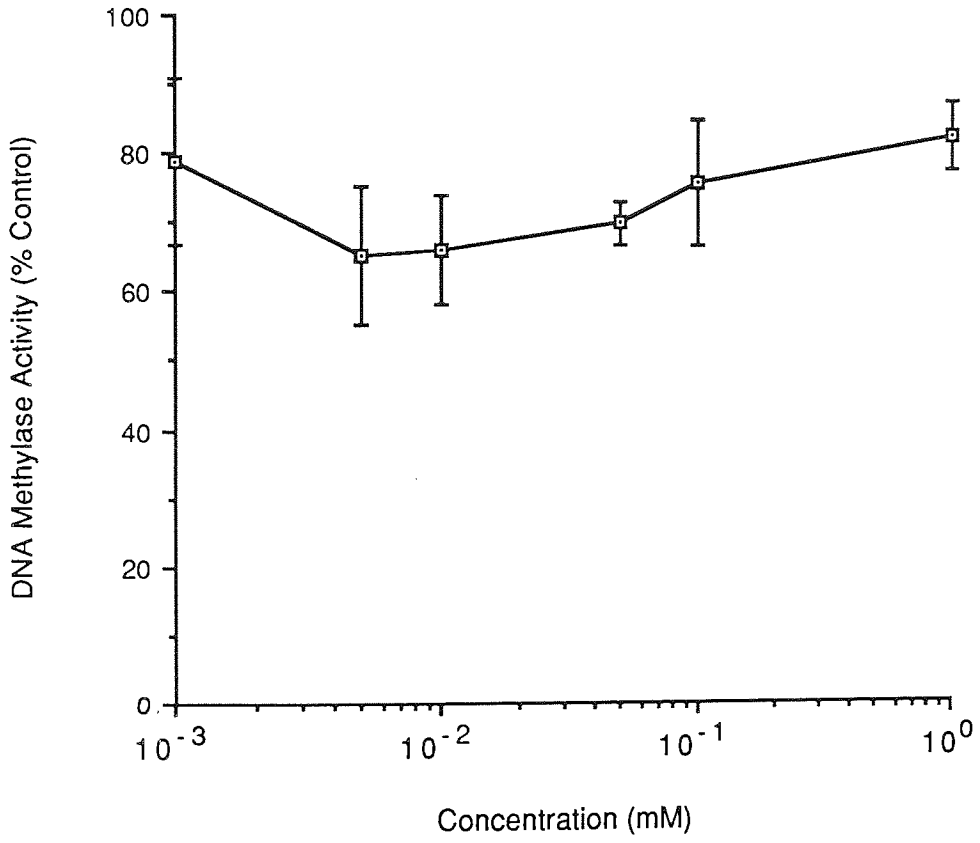


Figure 17: The effect of poly[dA].poly[dT] alkylated with MNU on the methylation of M.lysodeikticus DNA by DNA(cytosine-5) methyltransferase.
Poly[dA].poly[dT] was alkylated with MNU as described in section 4.2.2. 10 µg of the treated poly[dA].poly[dT] was added to the standard DNA methylase assay containing 20 µg M.lysodeikticus DNA, 1 µCi SAM and DNA(cytosine-5)methyltransferase capable of transferring 0.1 pmol of methyl groups under the conditions of the assay and incubated at 37°C for 4 h. In this graph the concentration on the x-axis represents the concentration of drug in the reaction with the poly[dA].poly[dT] and in this reaction the drugs were dissolved in DMSO which constituted less than 10% of the reaction volume. The results are expressed as a percentage of the methylation of M.lysodeikticus DNA observed with untreated poly[dA].poly[dT] and represent the mean of five experiments ± SEM.

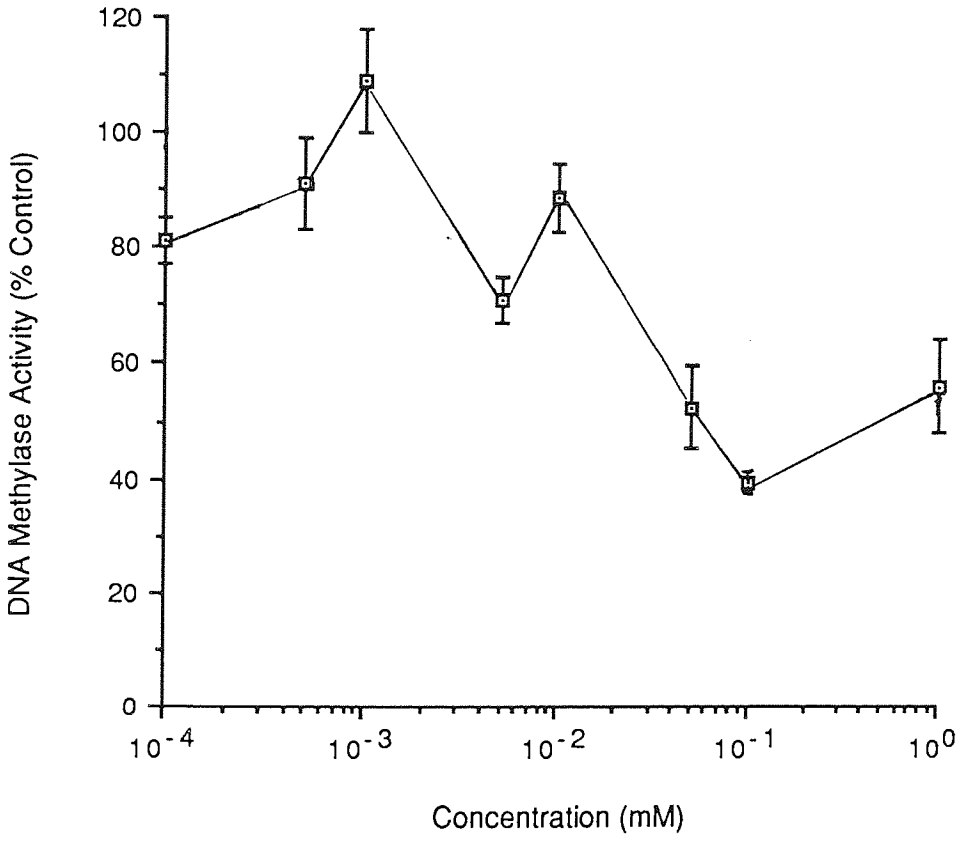


Figure 18: The effect of calf thymus DNA modified with mitozolomide on the methylation of M.lysodeikticus DNA by DNA(cytosine-5) methyltransferase.
Calf thymus DNA was treated with drug as described in section 4.2.2 and 10 µg of the treated-DNA was added to the standard DNA methylase assay which contained 20 µg M.lysodeikticus DNA, 1 µCi SAM and DNA(cytosine-5)methyltransferase capable of transferring 0.1 pmol of methyl groups under the conditions of the assay and incubated at 37°C for 4 h. In this graph the concentration on the x-axis represents the concentration of drug in the reaction with the calf thymus DNA and in this reaction the drugs were dissolved in DMSO which constituted less than 10% of the reaction volume.
The results are expressed as a percentage of the methylation of M.lysodeikticus observed with the untreated calf thymus DNA and represent the results of three experiments expressed as mean ± SEM.

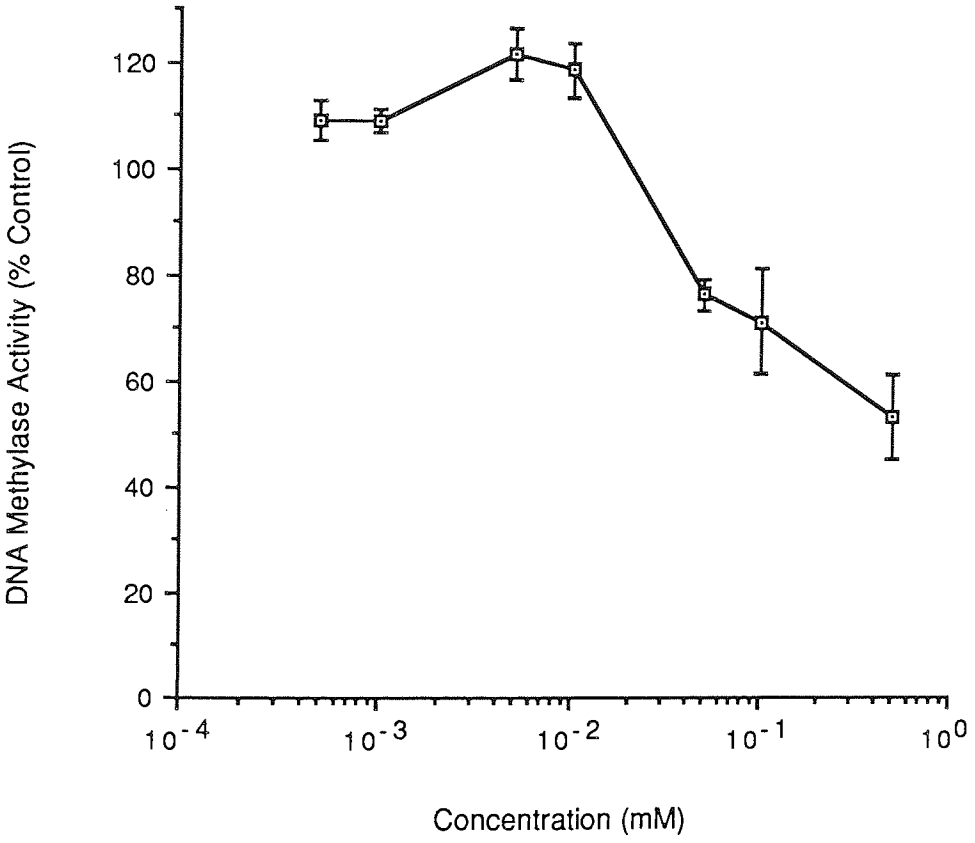
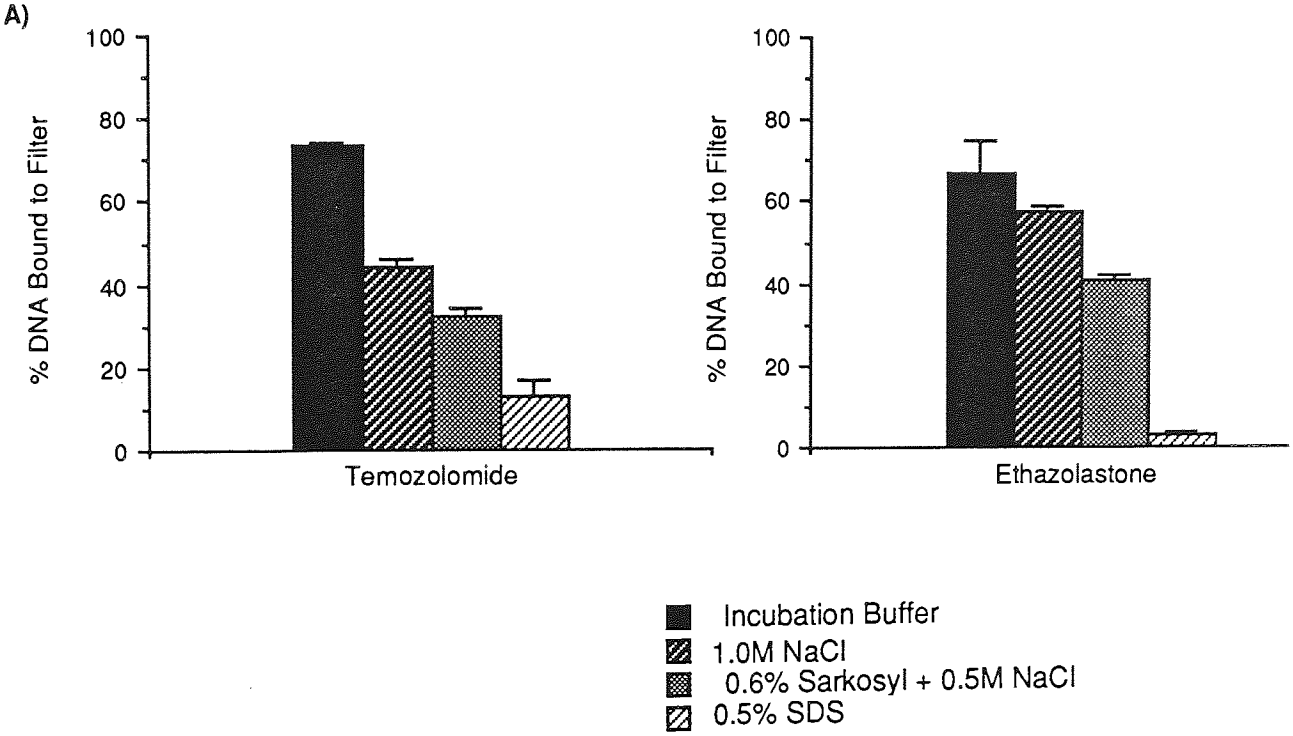
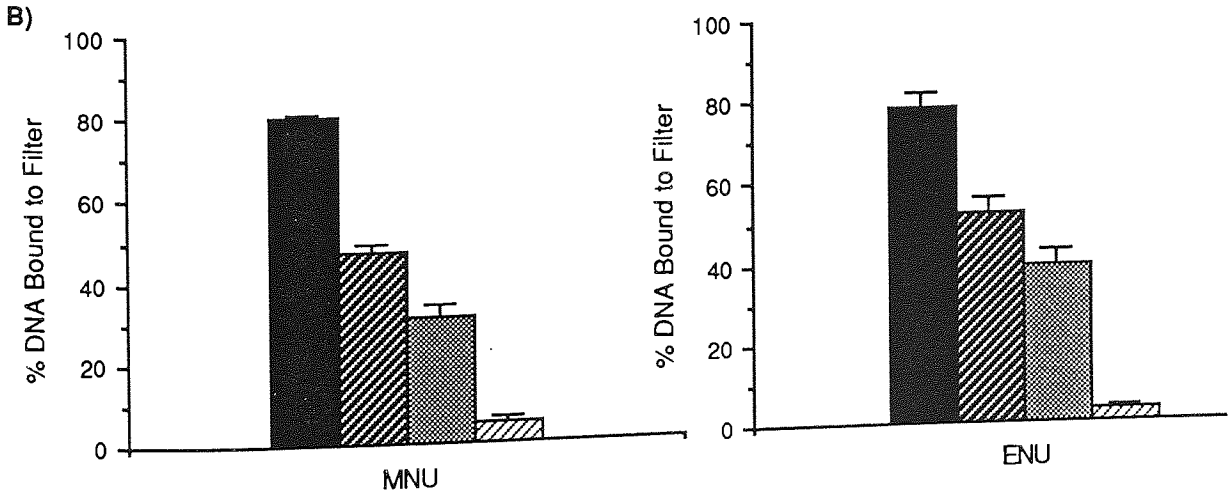


Figure 19: The effect of calf thymus DNA modified with chlorambucil on the methylation of M.lysodeikticus DNA by DNA(cytosine-5) methyltransferase.
Calf thymus DNA was treated with drug as described in section 4.2.2 and 10 µg of the treated-DNA was added to the standard DNA methylase assay which contained 20 µg M.lysodeikticus DNA. 1 µCi SAM and DNA(cytosine-5)methyltransferase capable of transferring 0.1 pmol of methyl groups under the conditions of the assay and incubated at 37°C for 4 h. In this graph the concentration on the x-axis represents the concentration of drug in the reaction with the calf thymus DNA and in this reaction the drugs were dissolved in ethanol which constituted less than 10% of the reaction volume.
The results are expressed as a percentage of the methylation of M.lysodeikticus observed with the untreated calf thymus DNA and represent the results of three experiments expressed as mean ± SEM.

A)


% DNA Bound to Filter
100
80
60
40
20
0
Temozolomide
Ethazolastone
Incubation Buffer
1.0M NaCl
0.6% Sarkosyl + 0.5M NaCl
0.5% SDS

B)


% DNA Bound to Filter
100
80
60
40
20
0
MNU
ENU

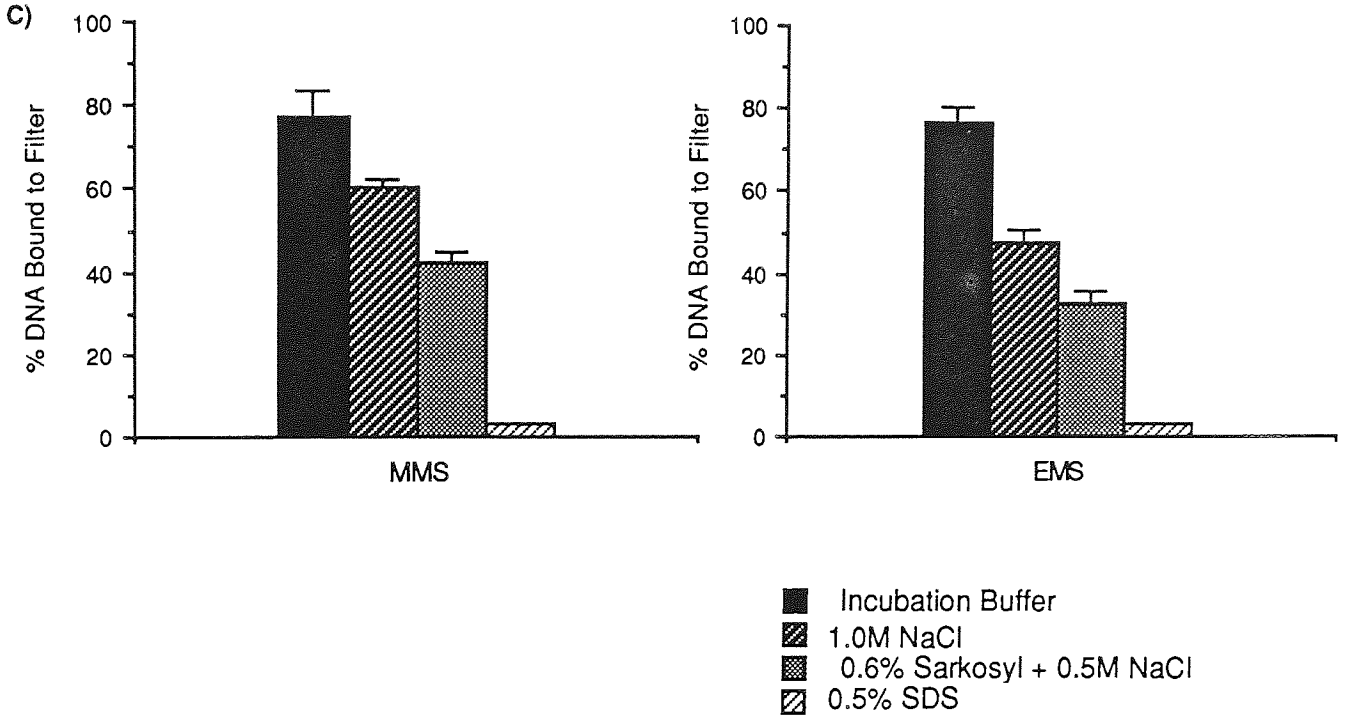


Figure 20: The stability of preformed alkylated L1210 [^{3}H]DNA: nuclear protein complexes to treatment with salt and detergents.
Labelled DNA [^{3}H] was reacted with the indicated drugs to give the same degree of chemical reaction in terms of the number of alkyl groups bound to the DNA, as described in section 4.2.10. The drug concentrations were temozolomide (1.5 mM), ethazolastone (11.0 mM) [A], MNU (0.5 mM), ENU (6.0 mM) [B], MMS (5.0 mM) and EMS (46.0 mM) [C]. The solvent for the drugs in all cases was DMSO.
Complexes were formed between 5 µg of the substituted DNA and the methyltransferase preparation during a 20 min incubation at 37°C. The complexes were then incubated for 10 min at 4°C prior to being washed onto a Millipore HA 0.45 µM filter, either with incubation buffer or the dissociating agents indicated.
The amount of DNA bound to the filter is expressed as a percentage of the total amount of DNA present in the assay and represents the mean ± SEM (n=8).

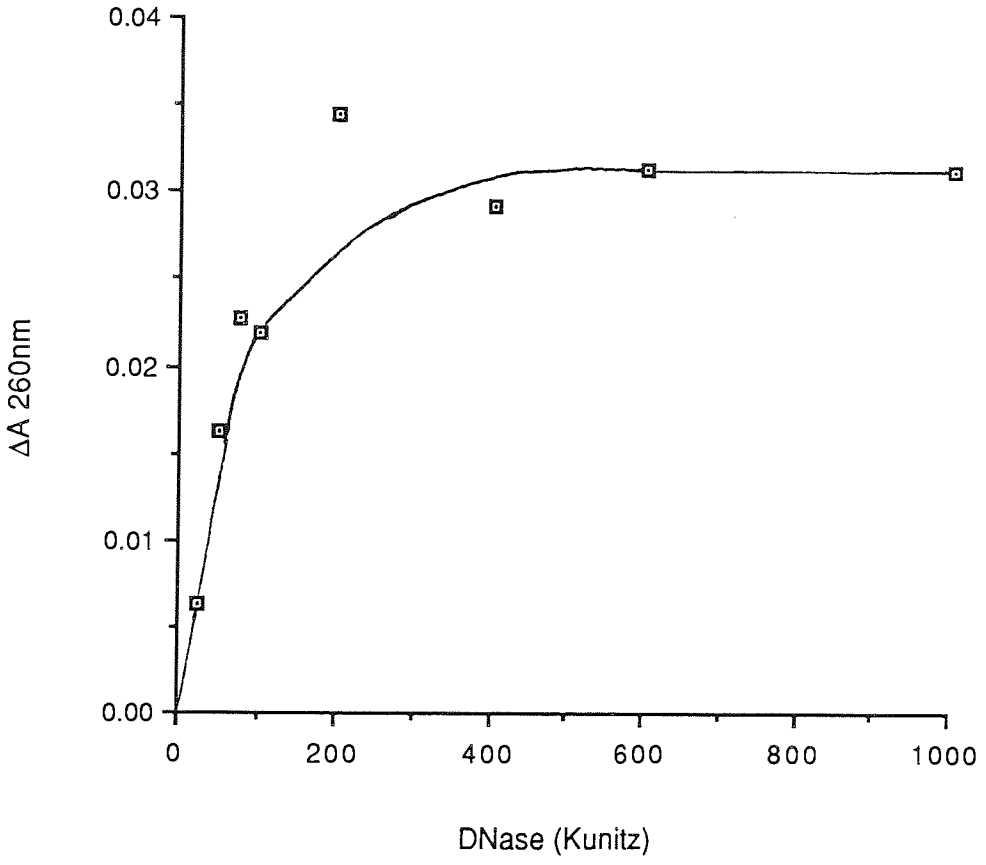


Figure 21: Monitoring the digestion of calf thymus DNA by deoxyribonuclease I.
Calf thymus DNA was treated with DNaseI as described in section 4.2.4. After 30 min, 10 µl (= 3.3 µg DNA) was mixed with 1 ml of water and the absorbance of the solution was read at 260 nm. The change in A_{260} was calculated by subtraction of the absorbance for untreated DNA from this value.

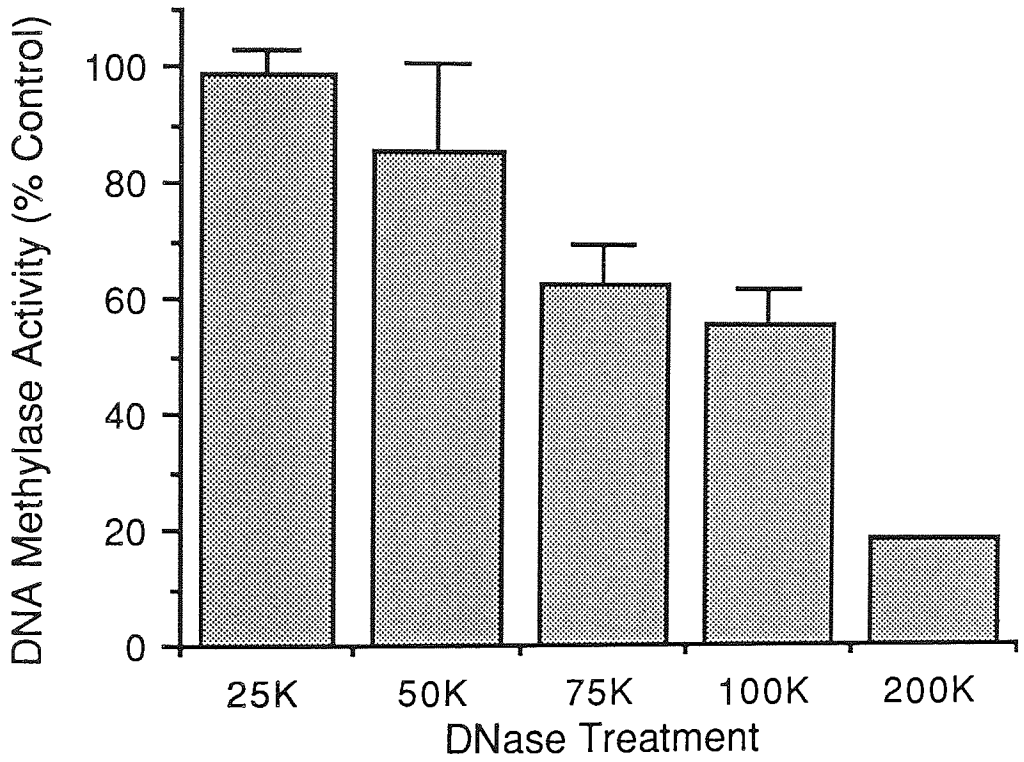


Figure 22: The effect of calf thymus DNA treated with deoxyribonuclease I on the methylation of M.lysodeikticus DNA by DNA(cytosine-5)methyltransferase.
Calf thymus DNA was treated with DNaseI as described in section 4.2.4 and 10 µg of the treated DNA (calculated according to the ethidium bromide/DNA fluorometric assay) was added to the standard DNA methylase assay containing 20 µg M.lysodeikticus DNA, 1 µCi SAM and DNA(cytosine-5)methyltransferase capable of transferring 0.4 pmol of methyl groups under the conditions of the assay.
Incubations were carried out at 37°C for 4 h.
The results are expressed as a percentage of the methylation of M.lysodeikticus DNA observed with untreated calf thymus DNA and are expressed as mean ± SEM (n=6).

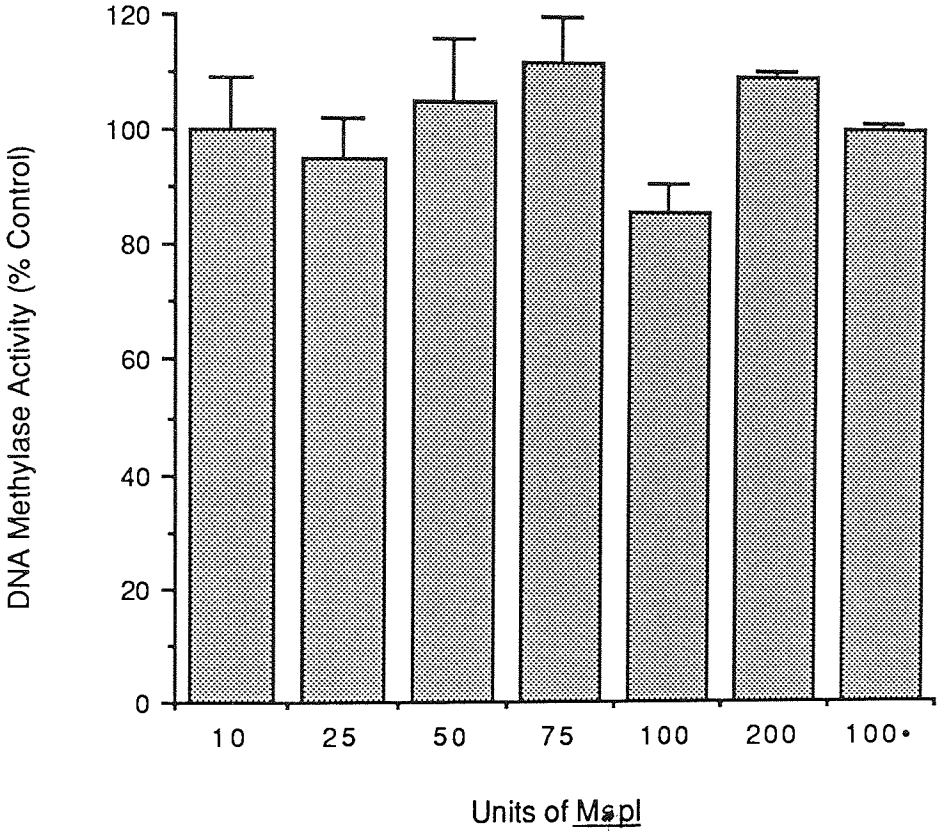


Figure 23: The effect of calf thymus DNA treated with the restriction endonuclease MspI on the methylation of M.lysodeikticus DNA by DNA(cytosine-5)methyltransferase.
Calf thymus DNA was treated with MspI as described in section 4.2.6 and 10 µg of the treated DNA (calculated according to the ethidium bromide/DNA fluorometric assay) was added to the standard DNA methylase assay containing 20 µg M.lysodeikticus DNA, 1 µCi SAM and DNA(cytosine-5)methyltransferase capable of transferring 0.4 pmol of methyl groups under the conditions of the assay. Incubations were carried out at 37°C for 4 h and the units on the x-axis represent units of MspI/mg DNA/2 h incubation.
The results are expressed as a percentage of the methylation of M.lysodeikticus DNA observed with untreated calf thymus DNA and are expressed as mean ± SEM (n=4).

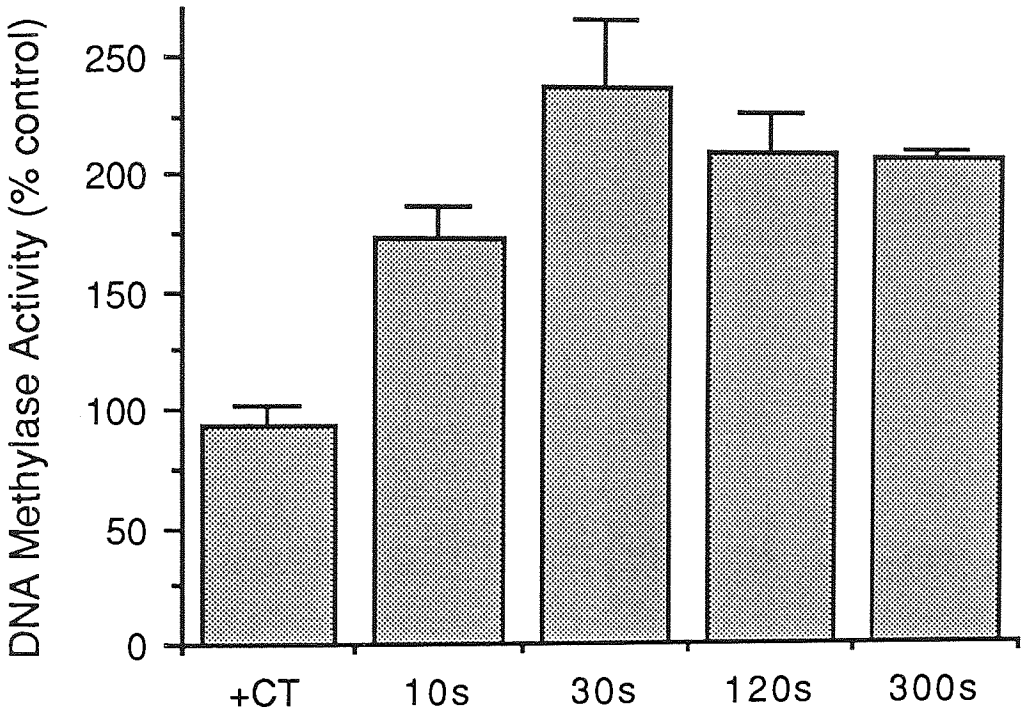


Figure 24: The effect of sonicated calf thymus DNA on the methylation of M.lysodeikticus DNA by DNA(cytosine-5) methyltransferase.
Calf thymus DNA at a concentration of 2.0 mg/ml was sonicated using a sonic probe for various periods of time and 10 µg of the treated DNA (based on the original concentration of DNA) was added to the standard DNA methylase assay. The standard assay contained 20 µg M.lysodeikticus DNA, 1 µCi SAM and DNA(cytosine-5)methyltransferase capable of transferring 0.4 pmol of methyl groups under the conditions of the assay. Incubations were carried out at 37°C for 4 h.
The results are expressed as a percentage of the methylation of the methylation of M.lysodeikticus DNA observed with untreated calf thymus DNA and are expressed as mean ± SEM (n=8).

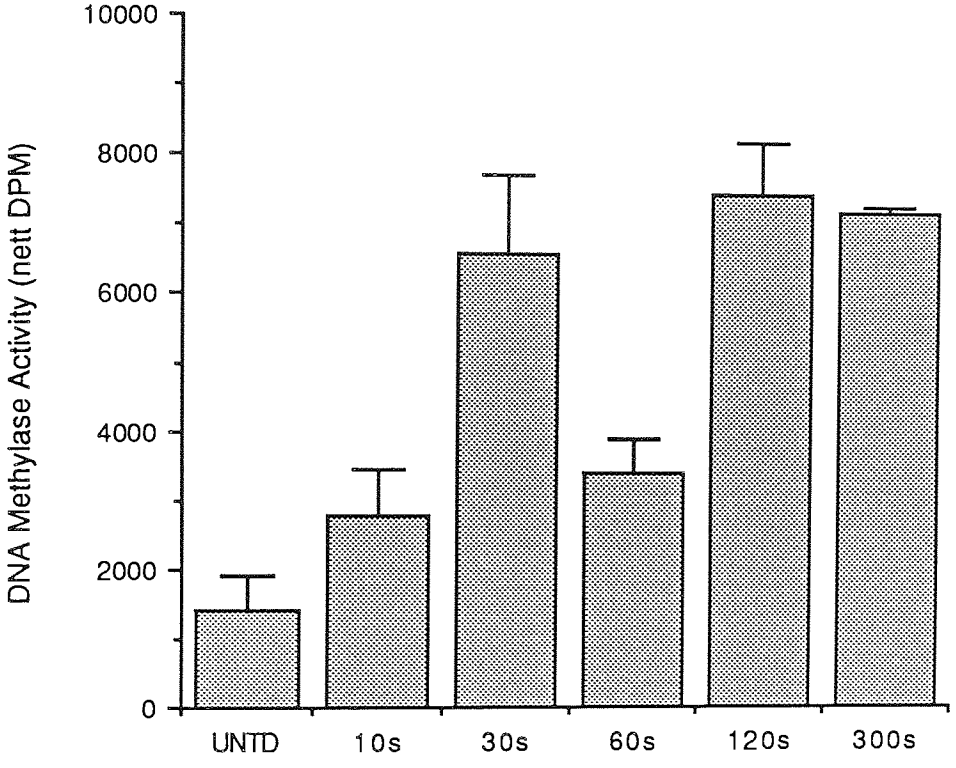


Figure 25: The ability of sonicated calf thymus DNA to behave as a substrate for DNA(cytosine-5)methyltransferase.
Calf thymus DNA was sonicated for various periods of time as described in section 4.2.5 and 20 µg of DNA was used as a substrate for DNA(cytosine-5)methyltransferase.
Incubations were carried out at 37°C for 4 h with 1 µCi SAM. The results are expressed as the mean nett DPM/assay ± SEM (n=3).

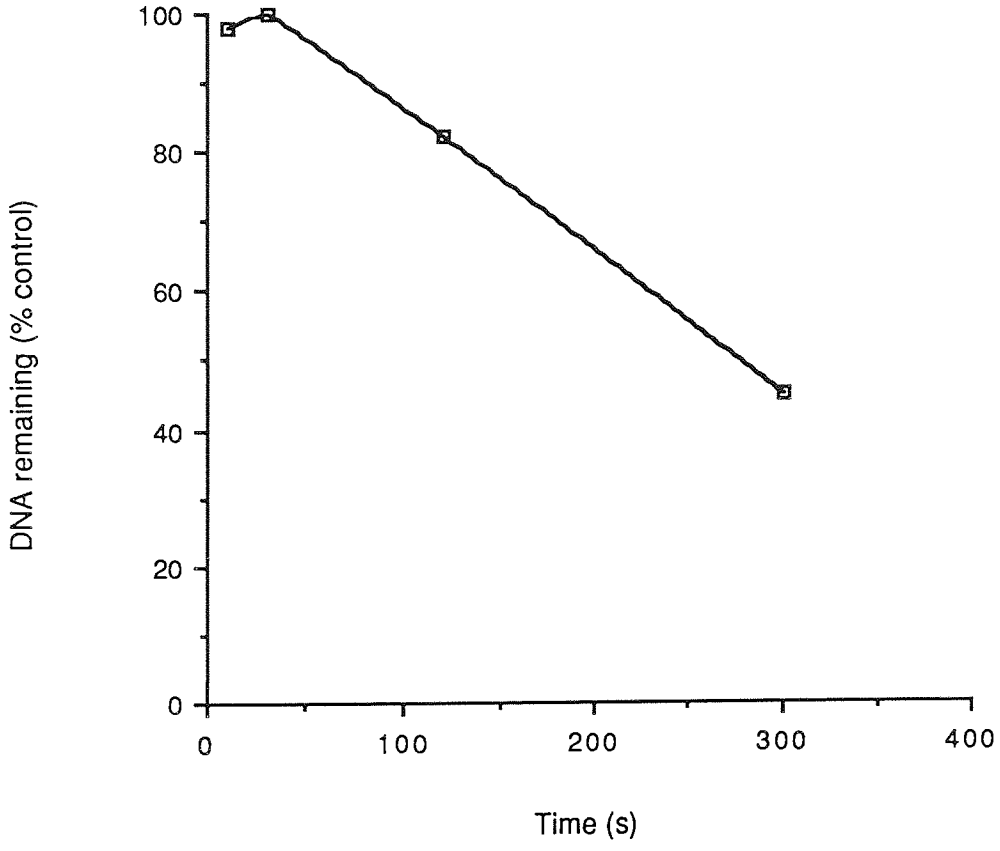


Figure 26: The amount of DNA remaining after various periods of sonication according to the DNA/ethidium bromide fluorometric assay. After sonication the equivalent of 20 µg of DNA was assessed for DNA by the ethidium bromide fluorometric assay.

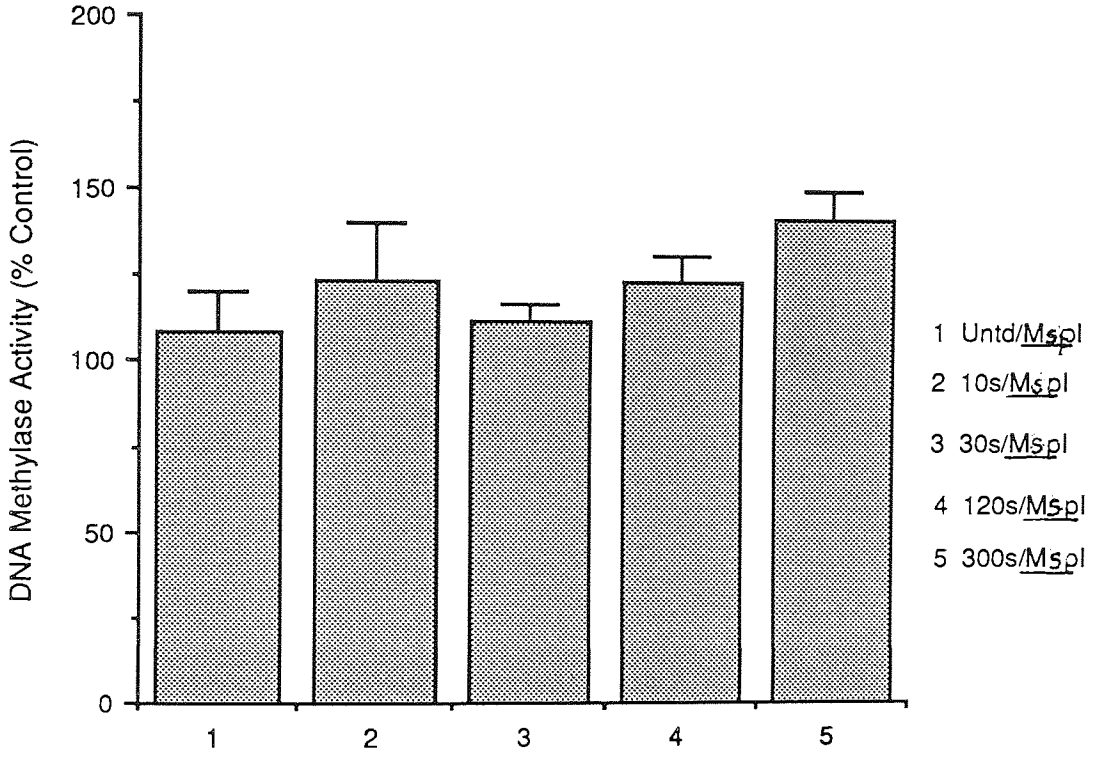


Figure 27: The effect of sonicated calf thymus DNA treated with the restriction endonuclease MspI on the methylation of M.lysodeikticus DNA by DNA(cytosine-5)methyltransferase.
Calf thymus DNA was sonicated for various periods of time as described in section 4.2.5 and then treated with an excess of MspI for 12 h. 10 µg of the treated-DNA (based on the original concentration) was added to the standard DNA methylase assay which was carried out at 37°C for 4 h containing 20 µg M.lysodeikticus DNA, 1 µCi SAM and DNA(cytosine-5)methyltransferase capable of transferring 0.4 pmol of methyl groups under the conditions of the assay.
The results are expressed as a percentage of the methylation of M.lysodeikticus DNA observed with the untreated calf thymus DNA and expressed as mean ± SEM (n=4) .

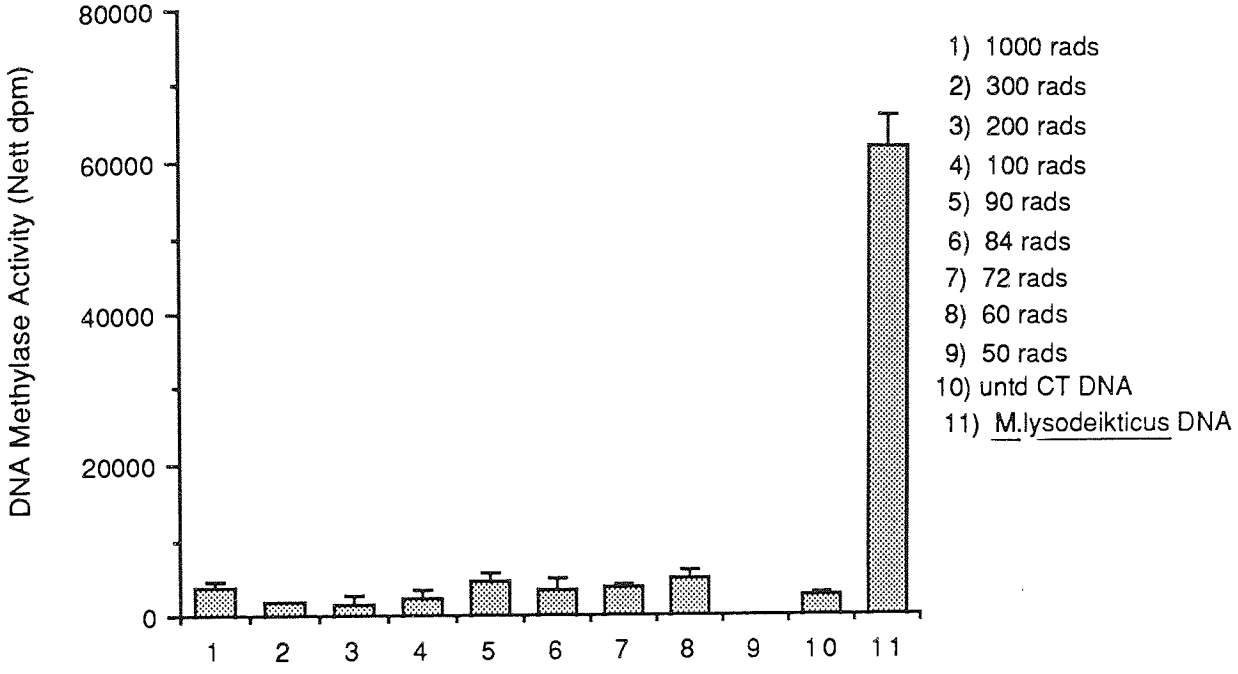


Figure 28: The ability of X-irradiated calf thymus DNA to behave as a substrate for DNA(cytosine-5)methyltransferase.
20 µg of the X-irradiated DNA was used as a substrate for DNA(cytosine-5)methyltransferase in which incubations were carried out at 37°C for 4 h with 1 µCi SAM.
The results are expressed as the mean nett DPM/assay ± SEM (n=4).

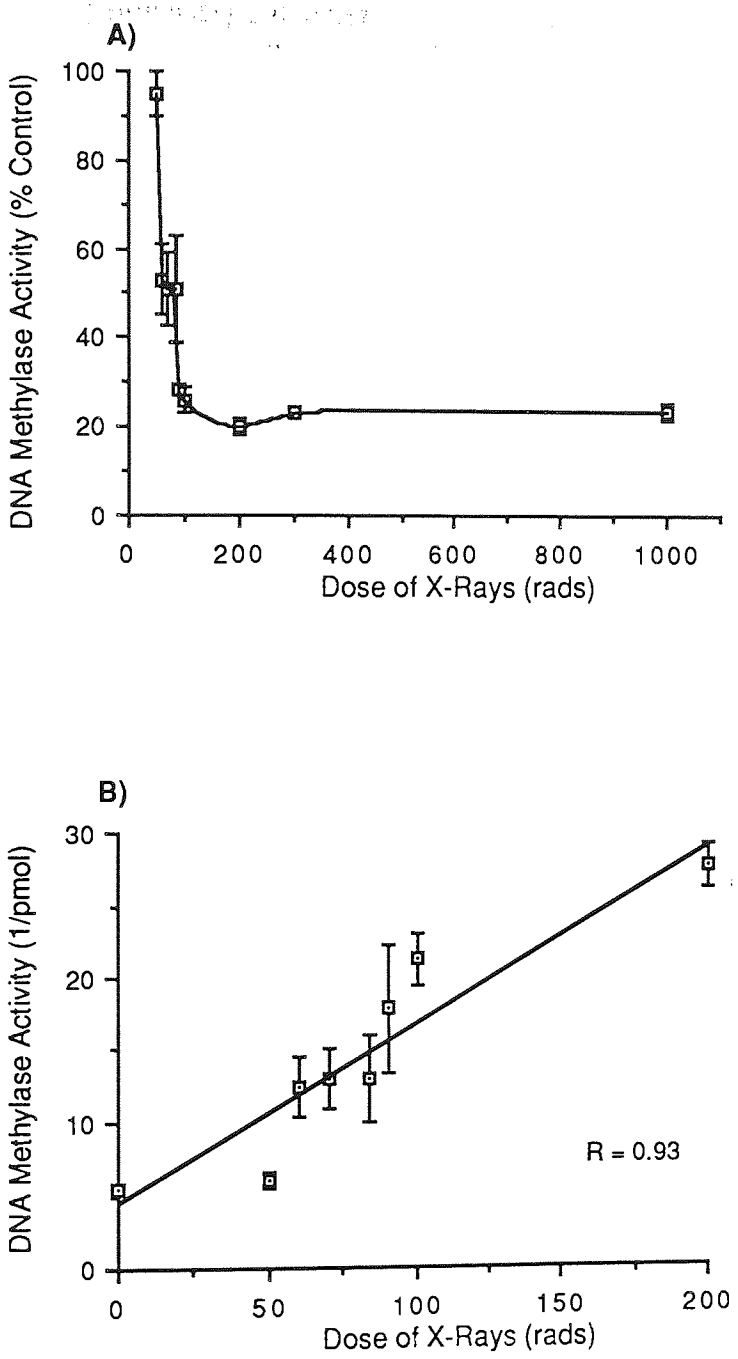


Figure 29: The effect of X-irradiated calf thymus DNA on the methylation of M.lysodeikticus by DNA(cytosine-5)methyltransferase. Calf thymus DNA at a concentration of 2 mg/ml in glass vials was exposed to ^{137}Cs γ-radiation at a dose of 7 rads s^{-1}. 10 µg of the irradiated DNA (based on the original concentration of DNA) was added to the standard DNA methylase assay, which was carried out at 37°C for 4 h containing 20 µg M.lysodeikticus DNA, 1 µCi SAM and DNA (cytosine-5)methyltransferase capable of transferring 0.4 pmol of methyl groups under the conditions of the assay.
a) The results are expressed as a percentage of the methylation of M.lysodeikticus DNA observed with untreated calf thymus DNA and are expressed as mean ± SEM (n=5).
b) The results in fig 29(a) are presented as a Dixon plot for the inhibition of DNA(cytosine-5)methyltransferase by X-irradiated calf thymus DNA. R = correlation coefficient.

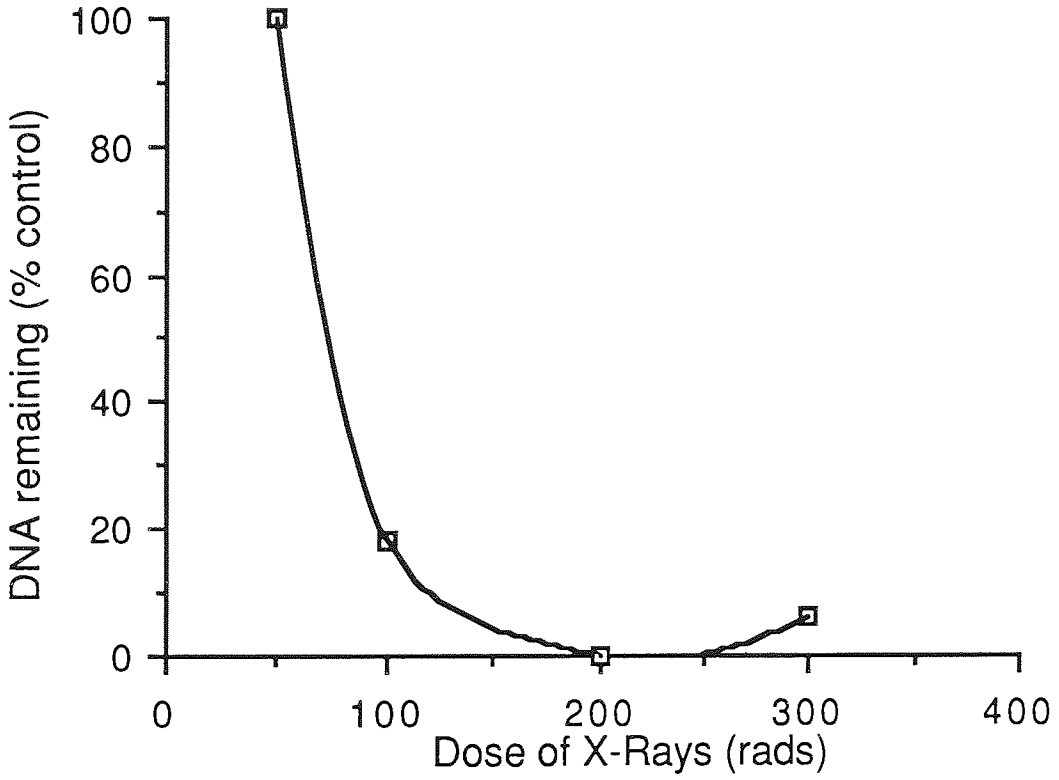


Figure 30: The amount of DNA remaining after exposure of calf thymus DNA to increasing doses of X-rays.
The equivalent of 20 µg of DNA was assayed for DNA by the DNA/ethidium bromide fluorometric assay and expressed as a percentage of the untreated DNA.

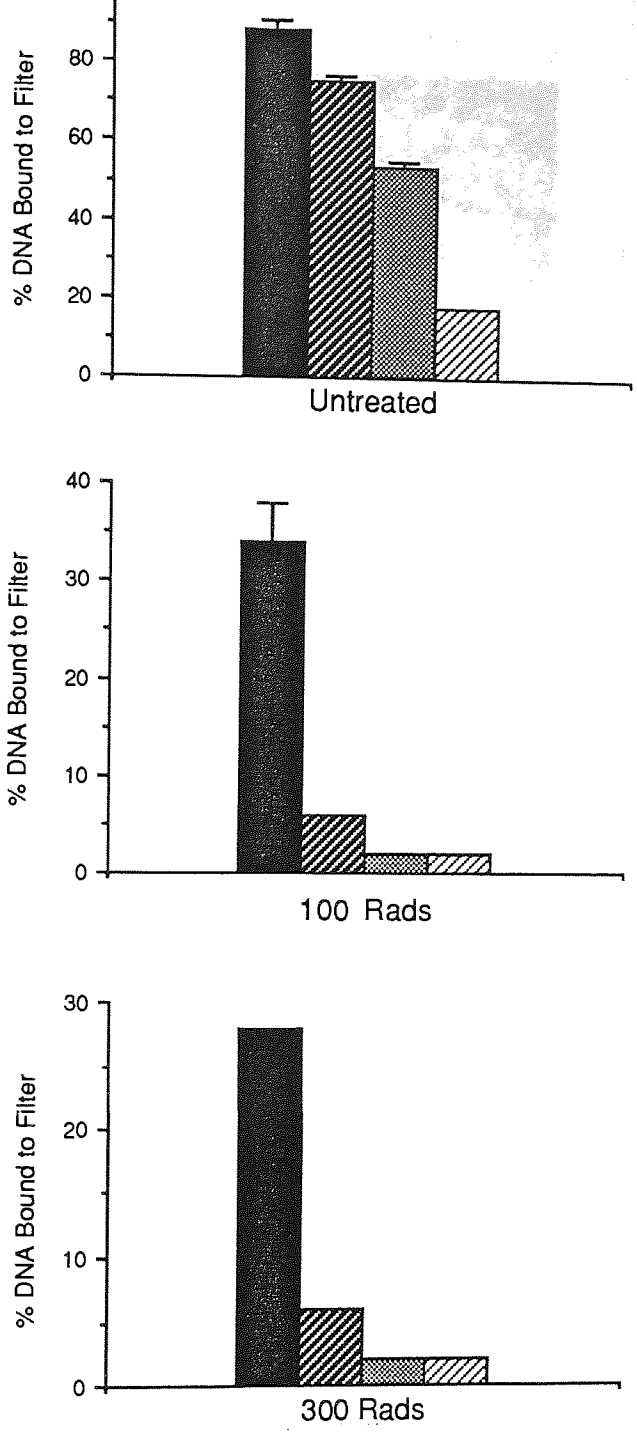


Figure 31: The stability of preformed X-irradiated [^{3}H] DNA : nuclear protein complexes to treatment with salt and detergents. Labelled [^{3}H]DNA was exposed to the indicated dose of X-rays and complexes were formed when 5 µg of the X-irradiated DNA was incubated with the DNA(cytosine-5)methyltransferase preparation for 20 min at 37°C. The complexes were then incubated for 10 min at 4°C prior to being washed onto a Millipore HA 0.45µm filter, either with the incubation buffer or the dissociating agents indicated. The amount of DNA bound to the filter is expressed as a percentage of the total amount of DNA present in the assay and represents the mean ± SEM (n=4).

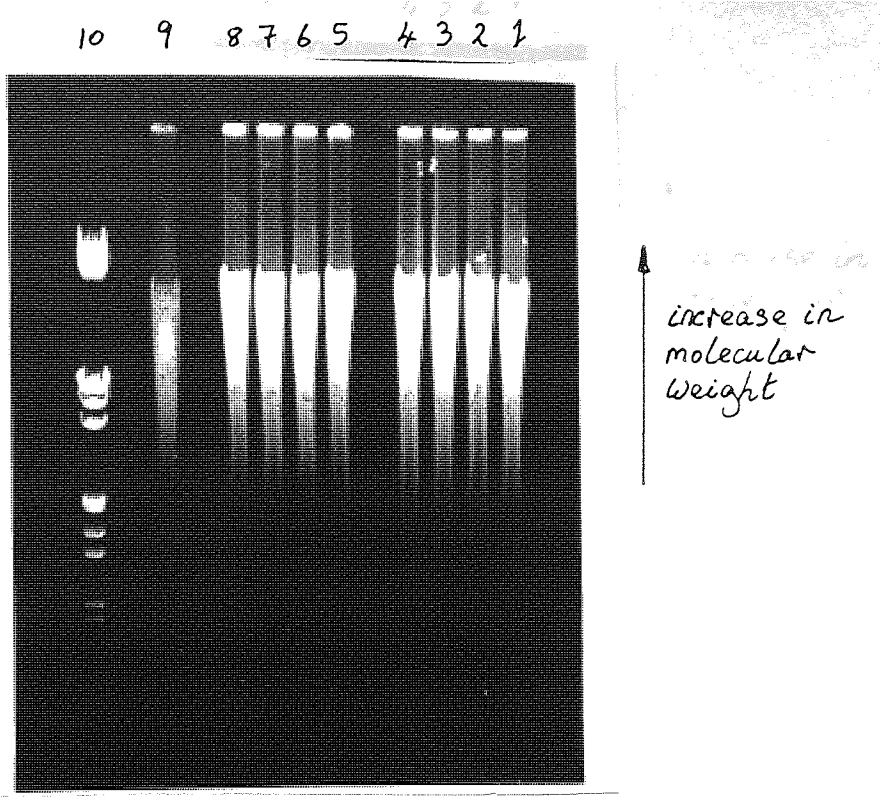


Figure 32: Agarose gel electrophoresis of temozolomide and ethazolastone-treated calf thymus DNA.
5 µg DNA/lane. 0.5% agarose. Temozolomide (lanes 1-4) and ethazolastone (lanes 5-8). The drug concentrations are 0.001 mM (lanes 1, 5), 0.01 mM (lanes (2, 6), 0.1 mM (lanes 3, 7) and 1.0 mM (lanes 4, 8). Untreated DNA (lane 9) and λ-phage Hind III/EcoRI digest (lane 10).

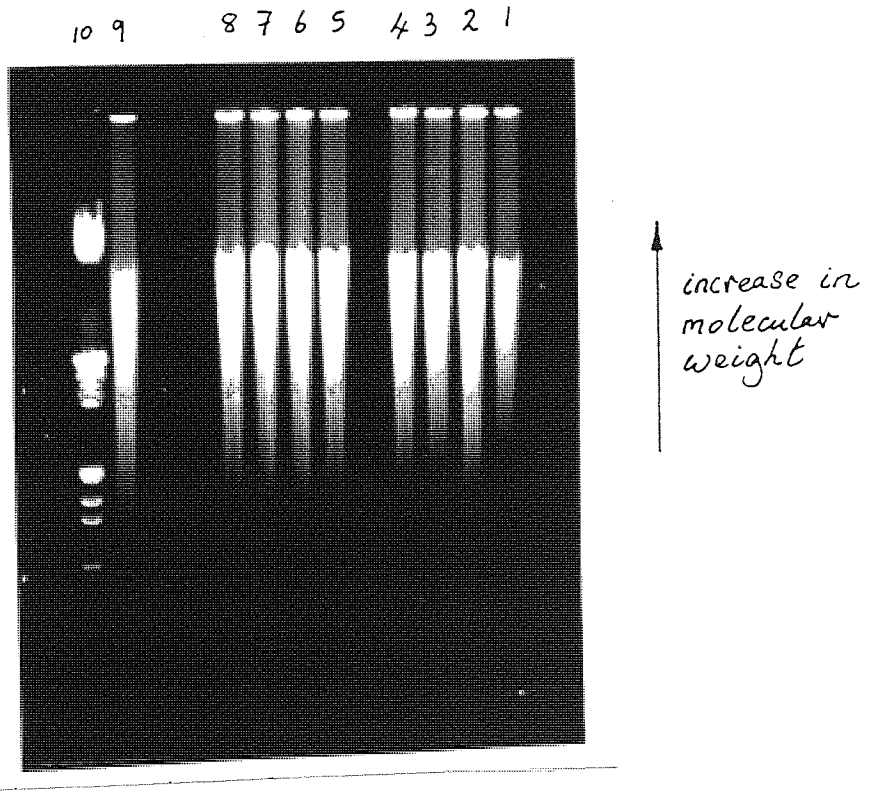


Figure 33: Agarose gel electrophoresis of MNU and ENU-treated calf thymus DNA.
5 µg DNA/lane. 0.5% agarose. MNU (lanes 1-4) and ENU (lanes 5-8). The drug concentrations are 0.001 mM (lanes 1, 5), 0.01 mM (lanes (2, 6), 0.1 mM (lanes 3, 7) and 1.0 mM (lanes 4, 8). Untreated DNA (lane 9) and λ-phage Hind III/EcoRI digest (lane 10).

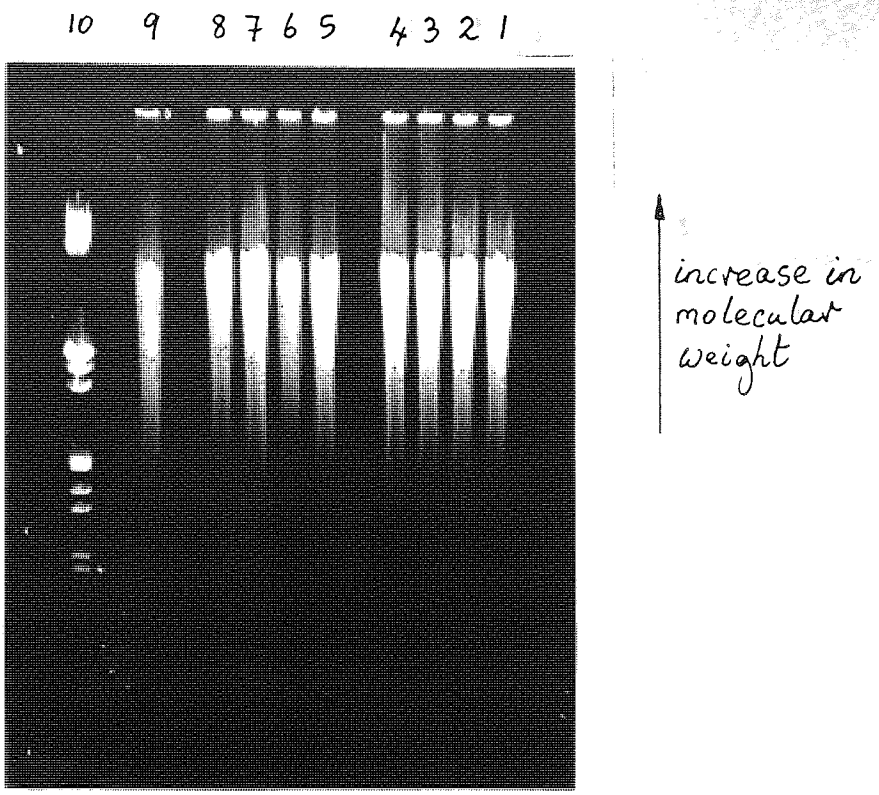


Figure 34: Agarose gel electrophoresis of MMS and EMS-treated calf thymus DNA.
5 µg DNA/lane. 0.5% agarose. MMS (lanes 1-4) and EMS (lanes 5-8). The drug concentrations are 0.001 mM (lanes 1, 5), 0.01 mM (lanes (2, 6), 0.1 mM (lanes 3, 7) and 1.0 mM (lanes 4, 8). Untreated DNA (lane 9) and λ-phage Hind III/EcoRI digest (lane 10).

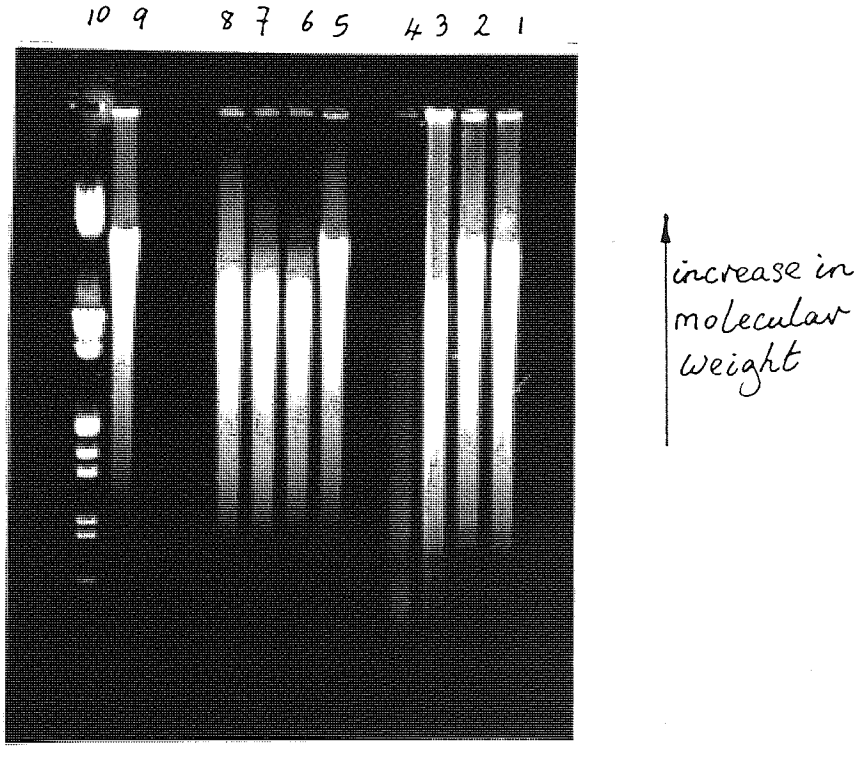


Figure 35: Agarose gel electrophoresis of chlorambucil and mitozolomide-treated calf thymus DNA.
5 µg DNA/lane. 0.5% agarose. Temozolomide (lanes 1-4) and ethazolastone (lanes 5-8). The drug concentrations are 0.001 mM (lanes 1, 5), 0.01 mM (lanes (2, 6), 0.1 mM (lanes 3, 7) and 1.0 mM (lanes 4, 8). Untreated DNA (lane 9) and λ-phage Hind III/EcoRI digest (lane 10).

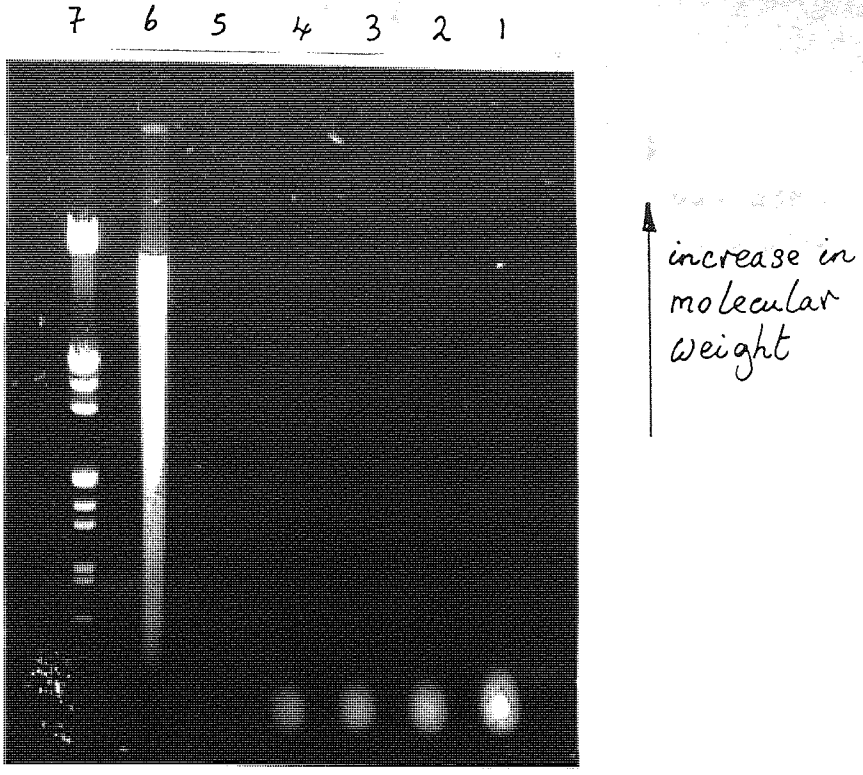


Figure 36: Agarose gel electrophoresis of deoxyribonuclease I -treated calf thymus DNA.
5 µg DNA/lane. 0.5% agarose. 25 Kunitz (lane 1), 50 Kunitz (lane 2), 75 Kunitz (lane 3), 100 Kunitz (lane 4), 200 Kunitz (lane 5), untreated DNA (lane 6) and λ-phage Hind III/EcoRI digest (lane 7).

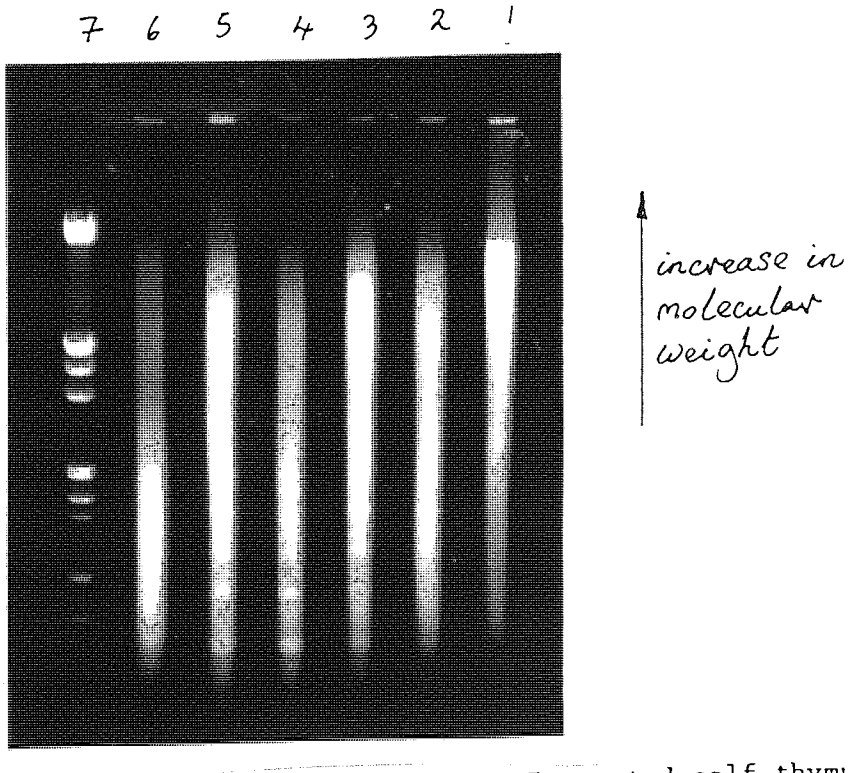


Figure 37: Agarose gel electrophoresis of MspI-treated calf thymus DNA.
5 µg DNA/lane. 0.5% agarose. 10 units/mg DNA/2 h (lane 2), 50 units/mg DNA/2 h (lane 3), 100 units/mg DNA/2 h (lane 4), 200 units/mg DNA/2 h (lane 5), 100 units/mg DNA/12 h (lane 6), untreated DNA (lane 1) and λ-phage Hind III/EcoRI digest (lane 7).

Figure 38: Agarose gel electrophoresis of sonicated calf thymus DNA. 5 µg DNA/lane. 0.5% agarose. 10 s (lane 1), 30 s (lane 2), 120 s (lane 3), 300 s (lane 4), untreated DNA (lane 5) and λ-phage Hind III digest (lane 6).

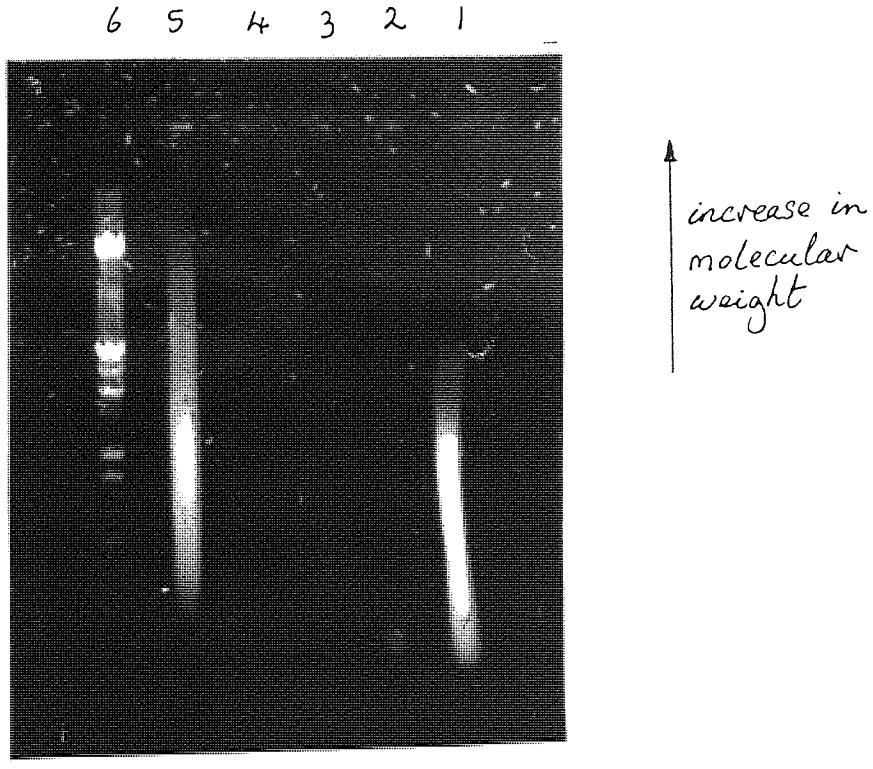


Figure 39: Agarose gel electrophoresis of X-irradiated calf thymus DNA.
5 µg DNA/lane. 0.5% agarose. 50 rads (lane 1), 100 rads (lane 2), 200 rads (lane 3), 300 rads (lane 4), untreated DNA (lane 5) and λ-phage Hind III/EcoRI digest (lane 6).

Drug	Concentration		
	50mM	10mM	1mM
Temozolomide		10±1	75±12
Ethazolastone	1±1	28±3	75±17
Mitozolomide		89±11	119±13
MNU	0±0	104±12	144±11
ENU	11±4	83±20	93±19
EMS	3±2	68±9	76±12
MMS		0±0	63±11

Table 11: The effect of free alkylating agent on DNA(cytosine-5)methyltransferase activity.
The drugs were dissolved in DMSO so that the final concentration of DMSO did not exceed 10% of the assay volume (100 µl). The drugs were then added to the standard DNA(cytosine-5)methyltransferase assay which was carried out at 37°C for 4 h and contained 20 µg of M.lysodeikticus DNA, 1 µCi SAM and DNA(cytosine-5)methyltransferase capable of transferring 1 pmol of methyl groups to DNA under the conditions of the assay. The results are expressed as a percentage of the control (DMSO alone) and are expressed as the mean ± SEM of three experiments.

Base (1mM)	Enzyme Activity (% Control)
C-5 Methylcytosine	101±9
N-3 Methyladenine	90±5
N-7 Methylguanine	108±17
N-1 Methyladenine	89±6
N-1 Methylguanine	113±13
O^6- Methylguanine	96±4
Cytosine	94±21

Table 12: The effect of certain bases on the activity of DNA(cytosine-5)methyltransferase.
Drugs were dissolved in 0.01 N HCl so that the final concentration in the assay was 1.0 mM and the HCl did not exceed 10% of the assay volume (100 µl). Incubations were carried out at 37°C for 4 h with 20 µg M.lysodeikticus DNA, 1 µCi SAM and DNA(cytosine-5)methyltransferase capable of transferring 1 pmol of methyl groups under the conditions of the assay.
The results are expressed as the mean ± SEM of at least three experiments.

CHAPTER FIVE

EXPERIMENTS TO DETERMINE THE ABILITY OF DNA(CYTOSINE-5)METHYLTRANSFERASE TO REPAIR O^6-METHYLGUANINE LESIONS IN DNA

5.1 INTRODUCTION

O^6-Alkylguanine is the major mutagenic and carcinogenic lesion in DNA induced by simple alkylating agents (reviewed by Yarosh, 1985; Saffhill et al., 1985). A repair activity for O^6-methylguanine was first observed in E.coli (Lawley and Orr, 1970) and has now been described in other prokaryotes and eukaryotes including mammals (Yarosh, 1985; Lindahl et al., 1988). The repair of O^6-alkylguanine in DNA is performed by the enzyme, O^6-alkylguanine DNA-alkyltransferase (O^6AT). O^6AT transfers alkyl groups from the O^6 position of guanine in DNA to a cysteine thiol residue within the protein itself and produces S-methylcysteine. Each alkyltransferase molecule can stoichometrically accept only a single alkyl group and the alkylated enzyme is inactive and not regenerated (Foote et al., 1980; Pegg et al., 1980).

The O^6AT in E.coli has been purified to physical homogeneity (Nakabeppu et al., 1985). The purification of mammalian O^6AT has proved to be more difficult as the methyltransferase has a relatively low abundance in mammalian tissues and is unstable upon purification. Partial purifications of O^6AT have been made from human tissues (Harris et al., 1983; Yarosh et al., 1983; Brent, 1986; Myrnes and Wittwer, 1988) and rat tissues (Pegg et al., 1980; Hora et al., 1983). Fragments of the methylated form

of human O^6AT have recently been purified to homogeneity for amino acid sequence investigations (Major et al., 1990).

The E.coli ada gene, which encodes a methyltransferase of molecular weight 39000 Da has been cloned and sequenced by Demple et al. (1985). Amino acid sequence determination showed that the active site for O^6AT has a rare -Pro-Cys-His- sequence, the cysteine of which becomes alkylated after repair of O^6-alkylguanine lesions in DNA. The same cysteine is an acceptor site for alkyl groups from O^4-alkylthymine in DNA and another cysteine residue also present within a -Pro-Cys-His- sequence accepts alkyl groups from the S-stereoisomer of alkylphosphotriesters in DNA (McCarthy and Lindahl, 1985; Teo et al., 1986; Takano et al., 1988).

Recently the cloning of complementary DNA for a human O^6AT from HeLa cells has been achieved (Tano et al., 1990). From the determined amino acid sequence it was predicted that Cys-145, once again in the sequence-Pro-Cys-His-accepts the alkyl group from O^6-alkylguanine in DNA. This has since been confirmed by Hayakawa et al. (1990) who cloned and determined the base sequence of O^6AT from human Raji cells.

The cloning of complementary DNA from a murine DNA(cytosine-5)methyltransferase from MEL cells has also been achieved (Bestor et al., 1988). This

revealed the presence of three -Pro-Cys- sequences within the enzyme; one of which was in the proposed active site of the methyltransferase.

All bacterial cytosine methyltransferases sequenced to date contain a -Pro-Cys- dipeptide which has been proposed to form a transient covalent bond between the cysteine thiol and DNA through the 6 position of cytosine (Wu and Santi, 1987). Carbanion formation at the 5 position would then allow attack of the methyl moiety of SAM.

Comparison of the sequence of bacterial O^6AT with bacterial thymidylate synthase (Demple et al., 1985) has revealed that the sequence -Pro-Cys-His- is conserved and suggests that this is important in methyltransferase reactions. In the reaction catalysed by thymidylate synthase an intermediate covalent bond is formed between the cysteine at the active site and the 6-carbon position of dUMP (Maley et al., 1984).

In all these methyltransferase reactions, the cysteine is of some importance and it has been suggested by Demple et al. (1985) that the proline is of conformational importance for cysteine activation by causing the cysteine to protrude, or by bringing the nearby hydrophobic residues into close proximity.

Since the rare -Pro-Cys- sequence was found in both DNA(cytosine-5)methyltransferase and O^6-alkylguanine-DNA alkyltransferase, an investigation

was carried out to determine whether DNA(cytosine-5)methyltransferase was able to transfer methyl groups from methylated moieties in DNA to its own molecule, by a so far unknown repair activity of the enzyme. It was previously shown in chapter four that various alkylated DNAs could inhibit DNA(cytosine-5)methyltransferase. The aim of this section was to determine whether the observed inhibition of DNA(cytosine-5)methyltransferase could be accounted for by 'suicide' alkyltransferase activity.

5.2 METHODS

DNA(cytosine-5)methyltransferase was prepared from murine L1210 cells as described in section 3.1.1.

5.2.1 Preparation of [^{3}H]MNU-labelled DNA

Labelled ([^{3}H-methyl)] M.lysodeikticus DNA was prepared by reaction with N-[^{3}H]methyl-N-nitrosourea as described by Demple et al. (1983) and as outlined below.

20 mg of M.lysodeikticus DNA (3.0 mg/ml) in water was mixed with 1.6 ml of 1 M EPPS buffer (pH 8.6) and 200 μl (1.0 mCi) of N-[^{3}H]methyl-N-nitrosourea (15 Ci/mmol) so that the final concentration of buffer was 0.2 M. After the mixture had been incubated at 37^{o}C for 2 h, the reaction vessel was placed on ice and 0.1 volumes of 5 M NaCl was added. Two volumes of cold absolute ethanol were then added and the DNA was wound onto a glass rod. The DNA was then washed twice with 80% ethanol, and then dissolved in 6.0 ml of 10 mM Tris-HCl (pH 7.5), 1 mM EDTA. This material was then dialysed for 24 h against 0.1 M NaCl, 1 mM sodium citrate and 10 mM potassium phosphate (pH 7.1) and was then suitable for use.

5.2.2 Assay to measure the loss of O^6-methylguanine or 7-methylguanine from [^{3}H]MNU-labelled DNA. Extraction of nuclear proteins.

L1210 cells in exponential growth (1.60×10^8) were sedimented by centrifugation at 1500 rpm for 5 min using an Heraeus bench top centrifuge and washed twice by resuspension and resedimentation in 10 ml of 0.9% saline. The nuclei were prepared by treating the cell pellet with 2 ml of cell lysis buffer and vortexing for 10 min. The nuclei were then sedimented by centrifugation for 5 min at 3000 rpm and resuspended in 0.5 ml of buffer M containing 0.4 M NaCl, vortexed and left to stand on ice for 1 h. The suspension was then centrifuged at 3000 rpm for 10 min to pellet the DNA and the supernatant was then assayed.

Alkyltransferase assay

The reactions were carried out in 1.5 ml Eppendorf tubes and each reaction contained 40 μg [^{3}H]MNU alkylated M.lysodeikticus DNA and various amounts of nuclear extract, made up to 200 μl with HEPES (DNA repair) buffer. After incubation at 37^{o}C for 4 h the tubes were placed on ice and the polymers (protein and DNA) were precipitated by adding 200 μl of ice cold 0.8 M TCA. After 10 min at 0^{o}C they were centrifuged in a microfuge for 10 min at 4^{o}C. The supernatant was removed avoiding disturbance of the protein/DNA pellet. Purine bases were then released

by adding 100 μl of 0.1 M HCl, vortexing and heating for 30 min at 70°C. The mixtures were then centrifuged in a microfuge for 10 min at 4°C and the supernatant was then carefully removed.

Analysis by paper chromatography

The entire acid hydrolysate and 10 μl of 10 mg/ml carrier $\underline{O}^6$-methylguanine was applied to 2 cm lanes on Whatman 3MM paper. The paper was then air dried and developed for approximately 8 h using the following solvent conditions [isopropanol: conc NH_3:water (7:1:2)]. After drying and observation under a UV lamp the spots co-eluting with marker $\underline{O}^6$-methylguanine were cut out, shredded and extracted three times with 1 ml of 0.1 M HCl. These extracts were neutralised with 100 μl of 1.0 M NaOH and then mixed with 10 ml of optiphase MP scintillation fluid and counted on a Packard Tri-Carb 2000CA liquid scintillation counter. Methyltransferase activity was determined directly from the loss of radioactivity.

Analysis by HPLC

HPLC separations were performed on a system consisting of a Waters Maxima 820 chromatography workstation, two waters model 510 pumps, a Waters Lambda-Max model 480 spectrophotometer (at 254 nm), a Waters automatic sample injector WISP 710B and a 25 cm Whatman Partisil 10SCX column. All buffers were degassed under vacuum immediately prior to use. A flow rate of 2.0 ml/min was used in which the compo-

sition of the mobile phase changed from buffer A (6% methanol adjusted to pH 4.0 with formic acid) to buffer B (0.2 M ammonium formate, 8% methanol adjusted to pH 4.0 with formic acid) over 25 min using Waters programme number 9. Fractions were collected every 0.5 min directly into liquid scintillation vials using a Pharmacia FRAC-100 fraction collector. To each fraction was added 3.0 ml of Optiphase MP scintillation fluid and the amount of radioactivity was determined using a Packard Tri-Carb 2000CA liquid scintillation counter. The amount of each product formed was determined from the radioactivity which co-eluted with marker compounds. The marker compounds used were O^6-methylguanine and 7-methylguanine dissolved in 0.1 M HCl at a concentration of 0.1 mg/ml.

5.2.3 O^6-Alkyltransferase assay to determine the level of protein methylation.

This method provides a means of assessing the level of protein methylation which occurs by the enzyme O^6-alkyltransferase in nuclear extracts using [^{3}H]MNU-labelled DNA as the substrate.

Preparation of nuclear extracts

GM892, Raji or L1210 cells (1.0 x 10^7/assay) were sedimented by low speed centrifugation at 1500 rpm for 5 min in an Heraeus bench top centrifuge and washed twice by resuspension and resedimentation in 5

ml of PBS. The nuclei were prepared by treating the cell pellet with 1.0 ml of cell lysis buffer and vortexing for 10 min. The nuclei were sedimented by centrifugation for 5 min at 3000 rpm and the pellet was resuspended in 100 µl/assay of buffer M containing 0.4 M NaCl, vortexed and left to stand on ice for 1 h. The suspension was then resedimented by centrifugation at 3000 rpm for 5 min.

Alkyltransferase assay

Each reaction consisted of 100 µl nuclear extract and 200 µg [^{3}H]MNU labelled M.lysodeikticus DNA, made up to 1.0 ml with buffer I. Incubations were carried out at 37°C for 4 h in 3.0 ml plastic scintillation vials. The sample volume was made up to 1.1 ml with water and 100 µl of BSA (10 mg/ml) was added as carrier protein. The mixture was then made to 1.0 M $HClO_4$ by adding 400 µl of 4.0 M $HClO_4$ and 2.0 ml of 1.0 M $HClO_4$. The samples were then hydrolysed for 45 min at 70°C in a water-bath which completely rendered the DNA acid soluble but did not hydrolyse the protein. The samples were then centrifuged at 3000 rpm for 10 min and the supernatant was discarded. The precipitate was then washed by resuspending in 1.0 M $HClO_4$ and sedimented by centrifugation at 3000 rpm for 10 min in an Heraeus bench top centrifuge, the supernatant was discarded and to the precipitate was added 300 µl of 10 mM NaOH followed by 3.0 ml of optiphase MP

scintillation fluid. Radioactivity was then determined using a Packard Tri-Carb 2000CA liquid scintillation counter.

5.2.4 Determination of S-methylcysteine as a product of DNA repair activity.

The determination of S-methylcysteine formed in acceptor protein was based on the method described by Ro et al. (1984).

100 µl of DNA(cytosine-5)methyltransferase preparation was incubated with 100 µg [^{3}H] DNA made up to a total volume of 1.0 ml with HEPES (DNA repair) buffer. After incubation at 37°C for 4 h, the reactions were terminated by adding 0.1 ml of 2.5 M sodium acetate and 2.0 ml of cold absolute ethanol. The precipitates were collected by centrifugation at 5000 rpm for 20 min in an Heraeus bench top centrifuge, and then suspended in 5.0 ml of 0.1 M HCl and hydrolysed at 70°C for 45 min, to release the alkylated purine bases from the substrate DNA. The mixtures were then precipitated by adding 5.0 ml of 15% TCA and collected by centrifugation at 5000 rpm for 20 min. The precipitates were resuspended in 20% formic acid-2 M HCl and transferred to pyrex glass tubes, which were then sealed in an atmosphere of N_2(g) to reduce amino acid oxidation. The proteins were then completely hydrolysed by heating at 110°C for 4 h in a stirred oil-bath. The hydrolysate was

concentrated to dryness under a stream of $N_2(g)$ whilst maintaining the temperature at 60°C in a water-bath. The hydrolysate was then dissolved in 1.0 ml of 0.1 M sodium citrate buffer (pH 2.7) ready for analysis on a Dowex 50(H^+) column.

Cation exchange chromatography

The Dowex 50 material was soaked in distilled water for approximately 6 h, changing the water every hour. The water was then replaced by 0.1 M HCl. The column (0.5x5.0 cm) was then loaded with the Dowex material which was washed with 10 volumes of 0.1 M HCl. The column was then equilibrated with 20 volumes of 0.1 M sodium citrate buffer, pH 2.7.

The hydrolysate was analysed using cation exchange chromatography. A Dowex 50 (H^+) column which had been equilibrated with 0.1 M sodium citrate buffer (pH 2.7), was loaded with the sample, which had been redissolved in 1.0 ml of the same buffer and also contained 10 μg of S-methylcysteine as a marker. The column was then washed with 10 ml of the citrate buffer. The S-methylcysteine was eluted with 0.1 M citrate buffer containing 0.4 M NaCl (pH 3.0), whilst collecting 1.0 ml fractions. A 0.05 ml aliquot of each 1.0 ml fraction was assayed by the ninhydrin reaction and the remainder was counted for radioactivity by adding 10 ml of optiphase MP scintillation fluid and counted on a Packard Tri-Carb 2000CA liquid scintillation counter.

Ninhydrin reaction for the determination of amino acids.

The ninhydrin reaction was performed in a similar manner to that described by Moore and Stein (1948).

To each 0.05 ml aliquot, 1.0 ml of ninhydrin reagent was added and whirlimixed. The tubes were then sealed and heated for 20 min in a boiling water-bath. After leaving for 15 min they were read on a spectrophotometer at 580 nm against a reference tube of ninhydrin reagent.

5.2.5 Alkyltransferase assay by SDS-PAGE and fluorography.

100 μl of DNA(cytosine-5)methyltransferase preparation was incubated with 100 μg [^{3}H]DNA made up to a total volume of 1.0 ml with HEPES (DNA repair) buffer and incubated at 37^{o}C for 4 h.

The protein concentration of the samples was calculated by the Bradford method (section 3.1.6) and 40 μg of protein was concentrated before loading into the wells. The protein solution was concentrated by first of all adding 5.0 N perchloric acid to give a final molarity of 0.25 M, to precipitate the protein. Samples were then centrifuged at 3000 rpm for 10 min using an Heraeus bench top centrifuge to pellet the protein. The samples were then washed by resuspension and resedimentation by centrifugation at 3000

rpm for 10 min in 70% ethanol:0.35 M Tris, pH 6.8. It was very important that the samples were washed until the pH of the supernatant was 6.8, and the ethanol was then removed by evaporation.

The appropriate volume of loading buffer was mixed with 40 μg of the protein sample and then heated for 10 min at 100^{o}C in a boiling water-bath to denature the proteins, and then centrifuged for 30 s using a microfuge to produce an homogenous sample.

The preparation of SDS-polyacrylamide gels for electrophoresis was carried out as described in section 3.1.4. The samples were then loaded onto the gel as well as labelled molecular weight markers for comparison, and electrophoresed at a constant voltage of 150 V through the stacking gel, and 250 V through the resolving gel, until the blue dye front was 1cm from the bottom of the gel.

Transfer of proteins to nitrocellulose paper and fluorography.

An unstained gel was placed onto nitrocellulose paper, on either side of which was placed Whatman 3MM filter paper and a layer of plastic mesh. This was then 'sandwiched' together in a Bio-Rad Western Electroblot apparatus and submerged in electroblot buffer and then blotted overnight using a constant current of 30 mA. The nitrocellulose paper was then air dried and coated with HiSafe scintillant and placed against photographic film and left for three

days to expose. The autoradiograph was then developed in Kodak D-19 developer and then fixed in Kodafix solution and washed in distilled water.

5.2.6 Comparison of the amino acid sequence of murine DNA(cytosine-5)methyltransferase with E.coli O^6-alkylguanine DNA-alkyltransferase.

This work was carried out in collaboration with S. Gardner, Department of Crystallography, Birkbeck College, University of London. The murine DNA(cytosine-5)methyltransferase amino acid sequence determined by Bestor et al. (1988) was compared with the E.coli O^6AT sequence determined by Demple et al. (1985), using the DOTPLOT programme written in standard C on a micro VAX II microcomputer. The programme compares all possible 15 amino acid residue segments between the two sequences and returns a hit (x) when the sum of the match scores between the two 15 residue segments is greater than the specified cut-off (140). The match scores are based on the mutational distance matrix MD78 of Dayhoff (1972) and it scores the observed probability of one residue being replaced by another.

5.2.7 Statistical analysis

All results are expressed as mean ± standard error of the mean (SEM) for at least three separate determinations.

5.3 RESULTS

Assay of methyltransferase activity.

[^{3}H]MNU-labelled DNA was used as a substrate for DNA(cytosine-5)methyltransferase to determine whether this enzyme has any repair activity. Paper chromatography and HPLC analysis of the hydrolysate after treatment with DNA(cytosine-5)methyltransferase, showed that there was no disappearance of radioactivity from either O^6-methylguanine or 7-methylguanine (tables 13 and 14). However these methods were not very sensitive and do not consider methylphosphotriesters or any of the minor alkylated bases.

Alkyltransferase assay to measure the level of protein methylation.

Table 15 shows the level of methyl transfer to protein that occurs when the nuclear extracts from GM892, Raji or L1210 cells were incubated with [^{3}H]MNU-labelled DNA. As expected, Raji which is classified as proficient in the repair of O^6-alkyl guanine lesions (Mer+), has the highest level of methyl transfer, whilst GM892 which is classified as Mer- has poor activity. However, L1210 cells which have been used in this study for the extraction of DNA(cytosine-5)methyltransferase have methyltransferase activity midway between the two. As this activity may be due to O^{6}AT, it was important then to

assess the level of S-[^{3}H]methylcysteine formed in the partially purified DNA(cytosine-5)methyltransferase after incubation with the labelled substrate.

Determination of S-methylcysteine as a product of DNA repair activity.

An accurate analysis of the level of S-[^{3}H]methylcysteine was performed and fig 40 shows that for the partially purified DNA(cytosine-5)methyltransferase, there was no difference between the control which consists of DNA alone and the DNA(cytosine-5)methyltransferase. As a positive control, the methylated bases were separated on a Dowex column and as can be seen from fig 41, the S-methylcysteine peak does not co-elute with any of these bases. One possibility is that the background peak which co-elutes with the S-methylcysteine corresponds to methylphosphotriesters. When the peak of radioactivity co-eluting with S-methylcysteine was separated and oxidised with 5% H_2O_2 and reanalysed on a Dowex column the profiles for the treated and the control are similar, once again suggesting no difference between the two (fig 42).

Alkyltransferase assay by SDS-PAGE and fluorography

An attempt was made to confirm that there was no transfer of methyl groups to the DNA(cytosine-5)-methyltransferase using the technique of SDS-PAGE and fluorography. However, [^{3}H]DNA migrated with the

protein producing a confusing autoradiograph, from which it was impossible to make any conclusions. There would potentially be very low levels of radioactivity present and bearing in mind that [^{3}H] is difficult to detect by this technique, it was not a suitable method to use.

Comparison of the amino acid sequence of DNA(cytosine-5)methyltransferase with O^6-alkylguanine DNA-alkyltransferase.

There is some similarity between DNA(cytosine-5)methyltransferase and O^6-alkylguanine DNA-alkyltransferase at the active site, where they both contain the rare-Pro-Cys-sequence. Using the DOTPLOT programme the murine DNA(cytosine-5)methyltransferase sequence (Bestor et al., 1988) was compared with the E.coli O^{6}AT sequence (Demple et al., 1985).

The DOTPLOT matrix in fig 43 shows that there is little similarity between the two amino acid sequences. An ideal plot of two proteins that are structurally similar would produce a diagonal line from left to right and the greater the similarity the longer and more complete this line would be.

Recently the sequence of human O^{6}AT has been deduced (Tano et al., 1990; Hayakawa et al., 1990). Therefore, it would be more appropriate now to compare the amino acid sequences of the two mammalian enzymes i.e. murine DNA(cytosine-5)methyltransferase with human O^6-alkylguanine DNA-alkyltransferase.

5.4 DISCUSSION

Alkylations in DNA are generally repaired by the conventional enzymes, glycosylases (reviewed by Sancar and Sancar, 1988). However, the O^6-alkylguanine lesion is corrected by a unique self destructive system in which the enzyme O^6AT removes the alkyl group from the O^6 atom of guanine, thereby restoring native guanine in DNA and transferring it to an internal cysteine residue. The amino acid sequence of O^6AT from E.coli (Demple et al., 1985) has been shown to contain 3 Pro-Cys sequences, two of which at least are involved in the suicide repair mechanism of O^6-alkylguanine, alkylphosphotriesters and O^4-alkylthymine lesions (Demple et al., 1985; McCarthy and Lindahl, 1985). Murine DNA(cytosine-5)methyltransferase has also been shown to contain three Pro-Cys sequences, one of which is at the active site of the methyltransferase (Bestor et al., 1988).

Therefore, the large size of DNA(cytosine-5)methyltransferase (1573 amino acid residues) (Bestor et al., 1988) and the presence of three Pro-Cys sequences suggested that the enzyme may have another function. In order to explain the observed inhibition of DNA(cytosine-5)methyltransferase with alkylated DNA (chapter 4), it was important to determine whether DNA(cytosine-5)methyltransferase

apart from catalysing the transfer of methyl groups from SAM to cytosine in DNA also had any 'suicide' methyltransferase DNA repair activity.

There was no loss of radioactivity from [^{3}H]MNU-treated DNA after treatment with DNA(cytosine-5)-methyltransferase. The crucial experiment was the measurement of S-methylcysteine formation, since this alone would be the end-point of the predicted 'suicide' repair activity. Such an action of DNA(cytosine-5)methyltransferase was an exciting and novel mechanism, but as yet seem unlikely to be true as no evidence arose to indicate the formation of S-methylcysteine in DNA(cytosine-5)methyltransferase. The large size of the methyltransferase may be explained by the complex structural requirements that the enzyme has in recognising regions of DNA and regulating DNA methylation.

In conclusion, the inhibition of DNA(cytosine-5)methyltransferase by alkylated DNA (chapter 4) was not due to a 'suicide' methyltransferase DNA repair activity.

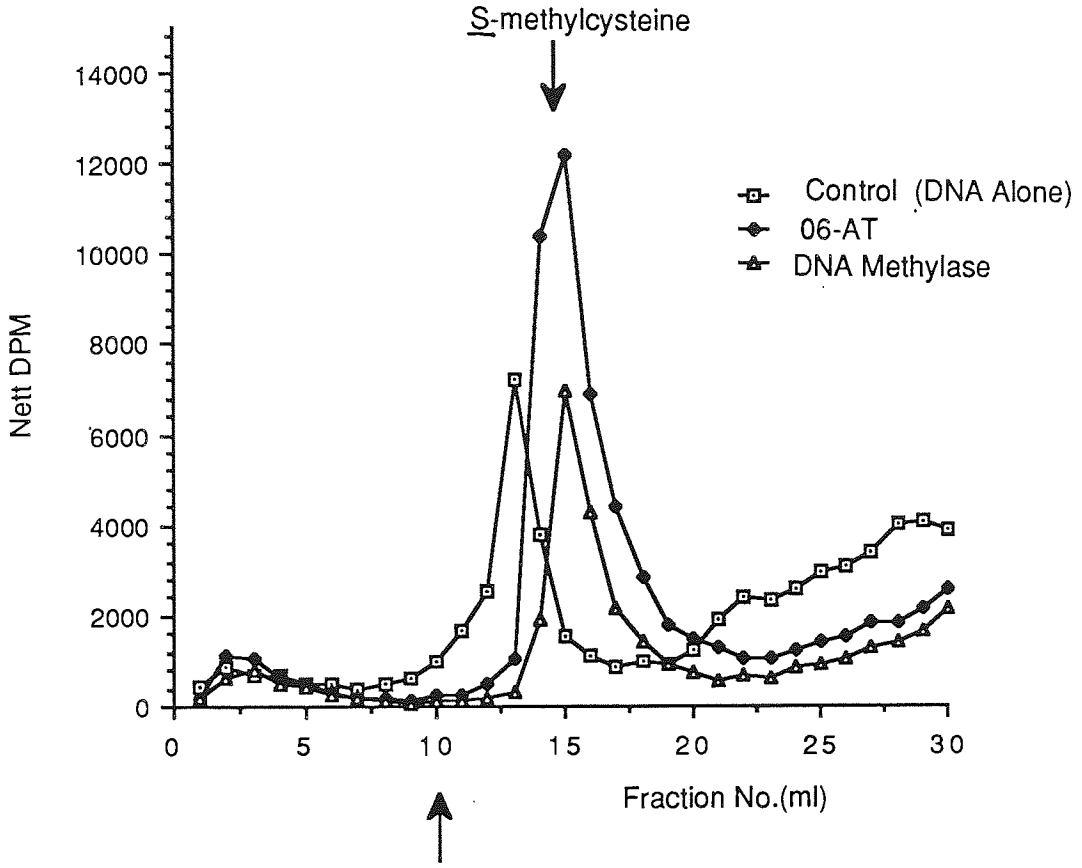


Figure 40: Chromatographic separation of DNA(cytosine-5) methyltransferase and O^{6}AT hydrolysates on a Dowex 50(H^+) column. The enzymes were incubated with 100 µg [^{3}H]MNU-labelled DNA for 4 h at 37°C. The protein was then hydrolysed with 20% formic acid:2 M HCl at 110°C for 4 h. The hydrolysate was dissolved in 0.1 M sodium citrate (pH 2.7) and 10 µg of S-methylcysteine was added before being applied to a column of Dowex 50(H+) which had been previously equilibrated with the same buffer. After elution of 10 ml of the 0.1 M sodium citrate buffer (pH 2.7), the buffer was changed to 0.1 M sodium citrate (pH 3.0) containing 0.4 M NaCl and the elution was continued for a further 20 ml. A 0.05 ml aliquot of each 1.0 ml fraction was assayed for absorption at 580 nm after reaction with ninhydrin reagent and the radioactivity in the remaining fraction was determined.

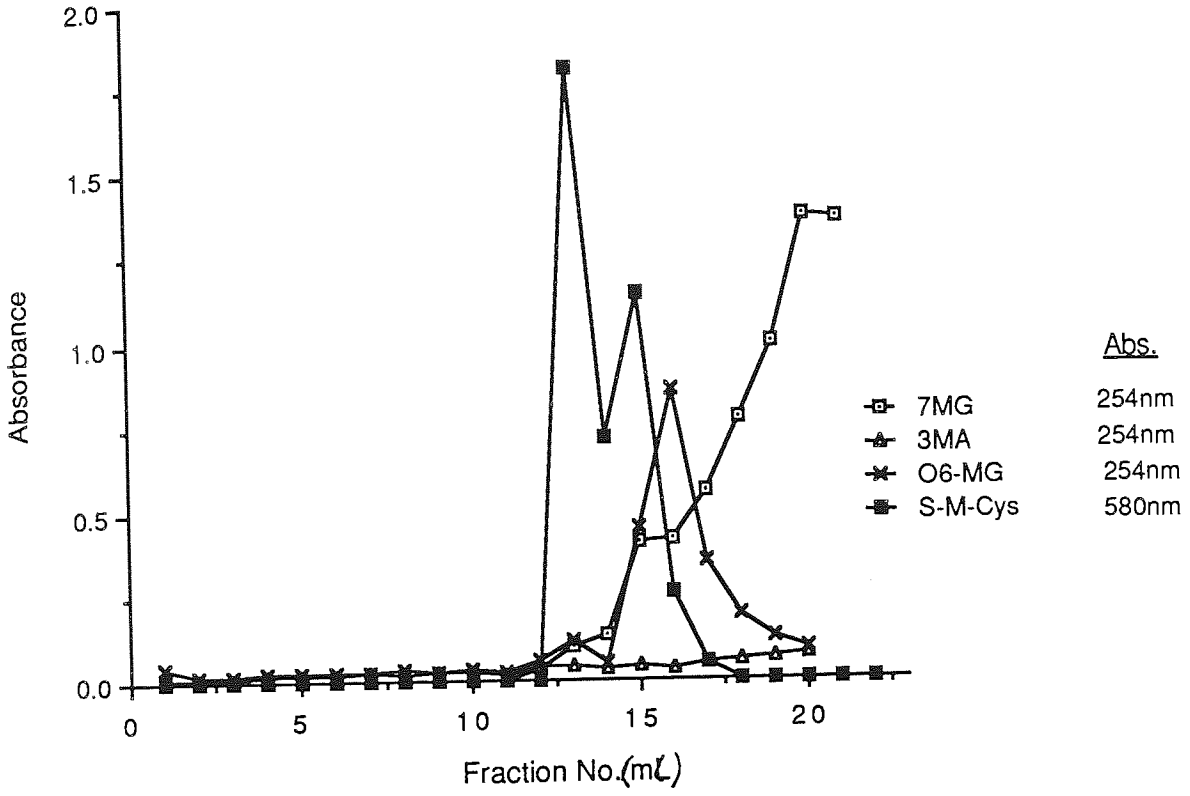


Figure 41: The separation of methylated bases on a Dowex 50(H^+) column.
The column had previously been equilibrated with 0.1 M sodium citrate buffer (pH 2.7) and 0.1 mg of the appropriate base was applied. The column was washed with 0.1 M sodium citrate buffer (pH 2.7) and 1.0 ml fractions were collected. After elution of 10 ml the buffer was changed to 0.1 M sodium citrate (pH 3.0) containing 0.4 M NaCl and the elution was continued for a further 15 ml. The absorbance of each fraction was measured at 254 nm. The same procedure was performed for S-methylcysteine, except the absorbance was measured at 580 nm after reaction with the ninhydrin reagent.

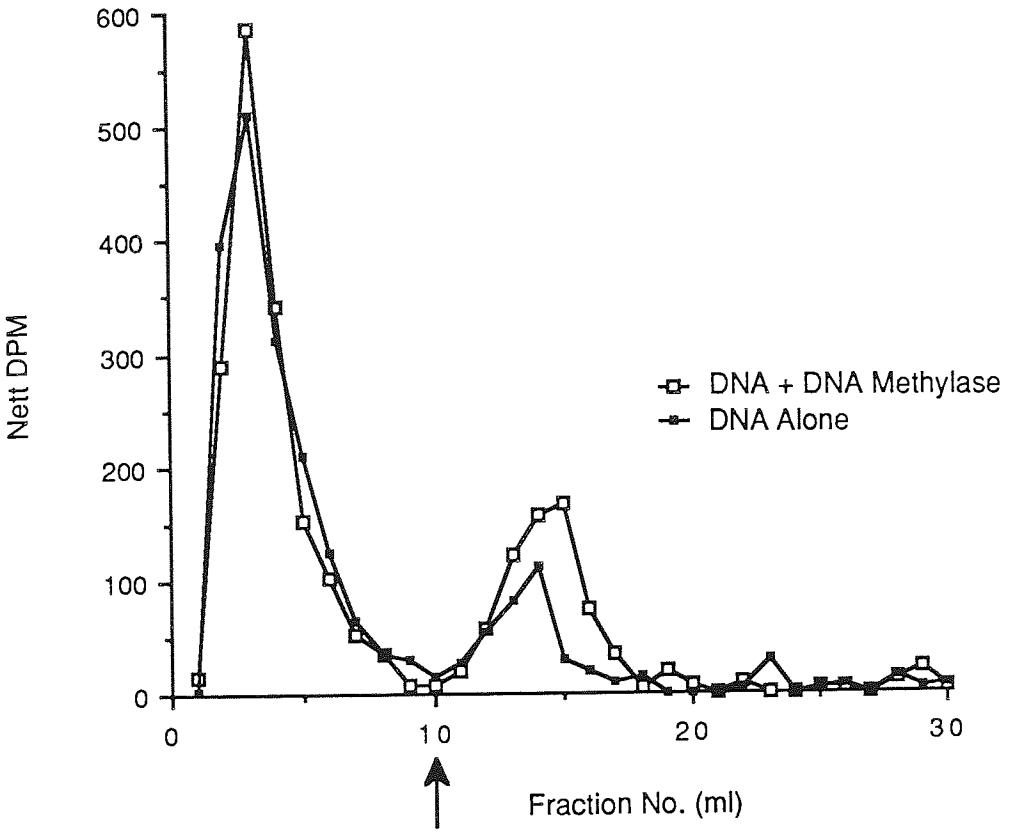


Figure 42: Analysis of the oxidation products of DNA(cytosine-5) methyltransferase hydrolysates on a Dowex 50(H^+) column.
The peak of radioactivity which co-elutes with S-methylcysteine illustrated in fig 40, was oxidised with 5% H_2O_2 at room temperature for 2 h. To the freeze dried fraction which had been redissolved in 0.1 M sodium citrate (pH 2.7) was added 10 µg of S-methylcysteine. This was then applied to a column of Dowex 50(H^+), which had been previously equilibrated with the same buffer. After elution of 10 ml, the buffer was changed to 0.1 M sodium citrate (pH 3.0) containing 0.4 M NaCl and the elution was continued for a further 20 ml. A 0.05 ml aliquot of each 1.0 ml fraction was assayed for absorption at 580 nm after reaction with the ninhydrin reagent and the radioactivity in the remaining fraction was determined.

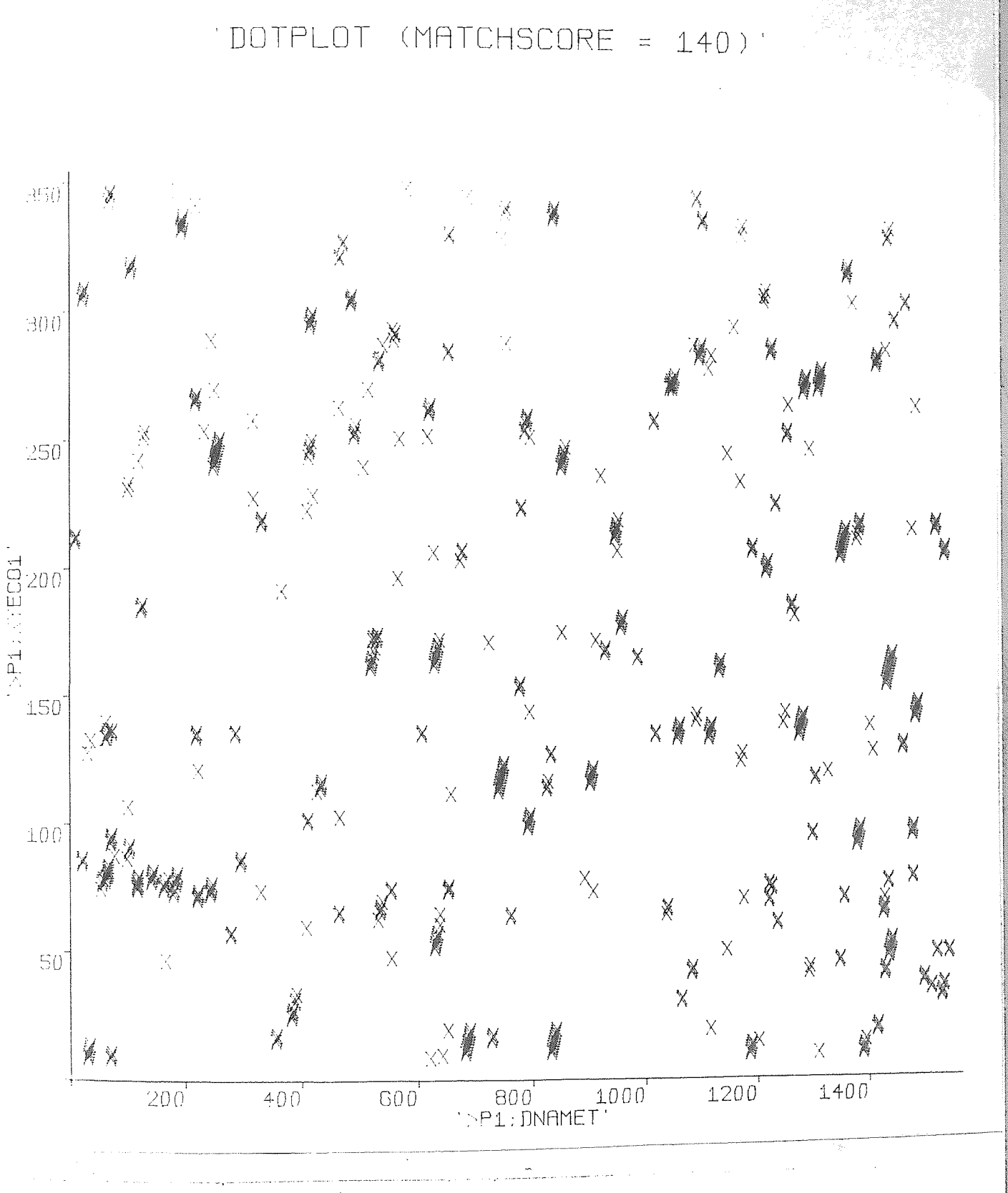


Figure 43: DOTPLOT matrix comparing the amino acid sequence of O^6AT (E.coli) with DNA(cytosine-5)methyltransferase (murine).
The graph is the output from a DIAGON type comparison, using a window size of 15 residues and the Dayhoff MD78 mutational distance matrix as the source of the comparative scores. The match score is the cut-off above which the score between two 15 residue segments must be to be included as an X on the plot.

	O^6-methylguanine (pmol)
No Enzyme	0.065±0.003
10µl methylase preparation	0.067±0.004
20µl methylase preparation	0.067±0.008
30µl methylase preparation	0.064±0.0002

Paper chromatography conditions.

Isopropanol:conc.NH_3:water (7:1:2)

rf values O^6-methylguanine = 0.75

N-7 methylguanine = 0.32

N-3 methyladenine = 0.61

Table 13: Experiment to determine whether DNA(cytosine-5) methyltransferase has any repair activity of O^6-methylguanine lesions - by measuring the loss of radioactivity from [^{3}H]MNU labelled DNA by paper chromatography.
Each assay contained 40 µg [^{3}H] MNU labelled DNA and incubations were carried out at 37°C for 4 h as described in section 5.2.2. 10 µl of the DNA(cytosine-5)methyltransferase preparation in the standard DNA methylase assay transfers 0.32 pmol of methyl groups to 20 µg M.lysodeikticus DNA in 4 h. The results are expressed as the mean number of pmol remaining in the DNA (O^6-position of guanine) after treatment with the DNA(cytosine-5)methyltransferase preparation ± SEM (n=6).

Base	Control (pmol)	10µl methylase (pmol)	30µl methylase (pmol)
O^6 MG	0.084±0.010	0.095±0.002	0.095±0.006
N-7 MG	0.640±0.093	0.666±0.048	0.595±0.080

Table 14: Experiment to determine whether DNA(cytosine-5) methyltransferase has the ability to repair O^6-methylguanine in DNA by measuring the loss of radioactivity from specific bases in [^{3}H]MNU labelled DNA by HPLC.
Each assay contained 40 µg [3H]MNU labelled DNA and incubations were carried out at 37°C for 4 h as described in section 5.2.2.
10 µl of the DNA(cytosine-5)methyltransferase preparation in the standard DNA methylase assay transfers 0.32 pmol of methyl groups to 20 µg M.lysodeikticus DNA in 4 h.
The results are expressed as the mean number of pmol ± SEM (n=3) remaining in the DNA after treatment with DNA(cytosine-5) methyltransferase.

Nuclear Extract	(pmol/mg prot.)
GM892 (mer-)	0.069±0.035
Raji (mer+)	3.882±0.246
L1210 (mer-)	0.242±0.162

Table 15: Experiment to determine the level of protein methylation (O^6AT), when nuclear extracts from various cell lines were incubated with [^{3}H]MNU-labelled DNA.
A nuclear extract was prepared from 1.0 x 10^7 cells/assay and incubated with 20 µg [^{3}H]MNU-labelled DNA and the level of protein methylation was determined as described in section 5.2.3.
The results are expressed as the mean number of pmol/mg protein ± SEM (n=3).

CHAPTER SIX

GROWTH SUPPRESSION BY DNA FROM CELLS TREATED WITH IMIDAZOTETRAZINONES

6.1 INTRODUCTION

There are strict structural requirements for the alkyl group in the series of 3-alkyl substituted imidazotetrazinones, in order for them to exert antitumour activity. Thus, while 3-(2-chloroethyl) (mitozolomide) and 3-methyl (temozolomide) imidazotetrazinones are effective antitumour agents, the presence of a 3-ethyl (ethazolastone) leads to a loss of cytotoxic potency *in vitro* (Bull and Tisdale, 1987) and antitumour activity *in vivo* (Stevens *et al.*, 1987). Connors *et al.* (1976) have shown a similar structure-activity relationship in the series of antitumour triazenes *in vivo*.

Gibson *et al.* (1986) have investigated the cytotoxicity of a range of structurally related triazenes to two human colon carcinoma cell lines, BE and HT-29 and two human embryonic cell lines, IMR-90 and VA-13. The BE and VA-13 cell lines are deficient in the ability to repair O^6-alkylguanine lesions (Mer-) whereas the HT-29 and IMR-90 cell lines are proficient in the ability to repair O^6-alkylguanine lesions (Mer+). In this study the antitumour activity of the triazenes investigated was linked to alkylation of the O^6-position of guanine in DNA and the role of DNA inter-strand cross-linking as a mechanism for cell killing by chloroethylating agents was questioned. Thus, Mer- cells show an increased sensitivity to both a monomethyl or monochloroethyl-

triazene, compared with a Mer+ cell line. However, no difference in toxicity between a Mer- and a Mer+ cell line was observed with an ethyltriazene, suggesting that $\underline{O}^6$-alkylation of guanine may not be important for the cytotoxicity of the ethyl derivative.

The role of $\underline{O}^6$-guanine alkylation in the mechanism of cytotoxicity of imidazotetrazinones has been investigated by Tisdale (1987). He showed that cell lines in culture, proficient in the ability to repair $\underline{O}^6$-alkylguanine lesions (Mer+) were less sensitive to the cytotoxic effects of temozolomide and mitozolomide than Mer- cell lines. In contrast to this, ethazolastone did not show any differential toxicity between Mer+ and Mer- cell lines. Incubation of Mer+ cell lines with the free base $\underline{O}^6$-methylguanine for 16 h caused a depletion of $\underline{O}^6$-alkylguanine DNA-alkyltransferase activity and this loss of activity was thought to be due to the free base acting as a very weak substrate for the enzyme (Dolan et al., 1985). Tisdale showed that depletion of the repair activity of a range of Mer+ cell lines (not Raji) with $\underline{O}^6$-methylguanine, resulted in an increased sensitivity towards both mitozolomide and temozolomide but not ethazolastone.

These results suggest that mitozolomide and temozolomide are similar to the nitrosoureas (Scudiero et al., 1984) and triazenes (Gibson et al., 1986) in that cytotoxicity correlates with alkylation at the O^6-position of guanine.

Mitozolomide is thought to exert its cytotoxicity by a similar mechanism to BCNU (Tong et al., 1982). Alkylation of guanine with a chloroethyl group occurs at the O^6-position of guanine followed by internal rearrangement to the N-1 position of guanine followed by the completion of the interstrand link to the N-3 position of the complementary cytosine residue. Cross-linking is chemically impossible with temozolomide or ethazolastone and therefore it is difficult to see how a methyl group and not an ethyl group at the O^6-position of guanine could lead to cytotoxicity.

Alkylation at the O^6-position of guanine correlates well with the induction of mutations. It was originally proposed by Loveless (1969) that O^6-methylguanine in DNA is mutagenic because of its potential mispairing properties. This was demonstrated by experiments in vitro where O^6-methylguanine in templates for E.coli DNA polymerase I caused the misincorporation of cytosine (Abbott and Saffhill, 1979) and in templates for M.luteus RNA polymerase leads to the misincorporation of uridine (Gerchman and Ludlum, 1973). Further, in vitro replication of

poly(dT.O^{6}MG) and poly(dC.O^{6}MG) by T4 and T5 phage DNA polymerases and E.coli DNA polymerase I demonstrated that O^6-methylguanine paired mainly with thymine (Snow et al., 1984).

A good correlation was demonstrated between the level of O^6-alkylguanine in mouse thymus DNA and the yield of induced thymic lymphomas by the alkylating agents EMS, ENU and MNU (Frei et al., 1978). The mutagenic activity of dimethyl sulphate, MMS and MNU in V79 Chinese hamster cells in culture correlated with the level of methylation of the O^6-position of guanine and there was no correlation between O^6-methylguanine production and cytotoxicity (Newbold et al., 1980).

Thus, the replication of O^6-methylguanine can result in mutations as a consequence of mispairing. This has been demonstrated in E.coli using a single-stranded M13mp8 vector in which a single O^6-methylguanine residue was positioned in the unique site for the restriction endonuclease PstI (Loechler et al., 1984). The transfection of E.coli cells with this vector gave progeny phage in which 0.4% were mutated at their PstI site. The depletion of O^{6}AT with MNNG prior to viral uptake increased the mutation frequency to 20%. DNA sequence analysis revealed that O^6-methylguanine mainly induced G-to-A transitions.

Further evidence that O^6-methylguanine is a mutagenic lesion came with the work of Zarbl *et al*. (1985) in which they showed that activation of the Ha-*ras* oncogene during the initiation of mammary carcinogenesis in rats by MNU was due to an MNU-induced GC to AT transition in codon-12.

The study of mutagenesis at the molecular level in mammalian systems has been made possible with the availability of recombinant shuttle plasmids, which can replicate in both eukaryotic and prokaryotic cells. The analysis of mutations in human cells using an Epstein-Barr virus (EBV) shuttle system has been studied by DuBridge *et al*. (1987). EBV vectors carrying the *lac*I bacterial gene as the target for mutation were established in human embryonic kidney cells and after exposure to MNU were returned to *E.coli* for analysis of *lac*I mutations. The majority of the mutations in the *lac*I gene induced by exposure of the mammalian cells to MNU were GC to AT transitions.

Sikpi *et al*. (1990) have used an SV40-based recombinant shuttle plasmid pZ190 to characterise mutations induced by MNU. A 250bp fragment (containing the entire target tyrosine tRNA gene *E.coli supF*) was exposed to MNU, reconstituted to form the original plasmid and then transfected into the human lymphoblastoid cell lines GM606 or L33. There was a ten-fold increase in mutational frequency

when replicated in O^6AT deficient cells (L33) and an eighty-fold increase when replicated in cells containing normal levels of O^6AT (GM606). The majority of the mutations were GC to AT transitions which supports the theory that $\underline{O}^6$-methylguanine is the main mutagenic adduct produced by MNU.

Therefore, while the induction of mutations correlates well with the production of $\underline{O}^6$ -methyl-guanine in DNA, the contribution of $\underline{O}^6$-methylguanine in DNA to the cytotoxic effects of methylating agents is less apparent. Whilst O^6AT activity levels have been directly correlated with resistance to alkylating agents (Shiloh and Becker, 1981; Tsujimura *et al*., 1987; Lunn and Harris, 1988; Yarosh, 1985), other workers have suggested that adducts at the $\underline{O}^6$ atom of guanine in DNA are not potentially cytotoxic lesions and that defects other than lack of O^6AT are responsible for the sensitivity of Mer^- cells to killing by alkylating agents (Karran and Williams, 1985). The Burkitts lymphoma cell line Raji (Mer^+) is more resistant to the alkylating agents, MNNG, BCNU and CNU than the sub-line Raji TK^-(Mer^-). Treatment of the Raji cells with $\underline{O}^6$-methylguanine which reduces the level of O^6AT, does not sensitise the cells to killing with these alkylating agents (Karran and Williams, 1985). This implies that adducts at the $\underline{O}^6$ atom of guanine in DNA are not the major cytotoxic lesions.

Recently it has been shown by a number of workers that cell lines of the Mer$^-$ phenotype can be made resistant to MNNG by selection. From the treatment of the Chinese hamster ovary cell line (CHO-9) with 3 μg/ml MNNG for 1 h, was isolated a resistant variant Cl3 (Goth-Goldstein and Hughes, 1987). The Cl3 cells were more resistant to MNNG and other methylating N-nitroso compounds, which was not due to a more efficient repair of methylated purine bases.

Similarly, Ishida and Takahashi (1987) produced a clone of MNNG resistant HeLa S3 cells which also had very low levels of O^6AT. Some cell lines have been made resistant to MNNG after transfection with human DNA. In particular, Kaina et al. (1987) transfected Chinese hamster ovary cells with human DNA ligated to the bacterial gpt (xanthine-guanine-phosphoribosyltransferase) gene and these cells could acquire MNNG resistance upon transfection with DNA from human fibroblasts. This was not caused by an increased ability to remove methylated purines from DNA. Thus it has been shown that the resistance to alkylating agents can be dissociated from the repair of O^6-methylguanine.

Therefore, in order to further investigate the potential mechanisms responsible for cytotoxicity, a novel approach has been adopted. An investigation has been carried out on the ability of alkylated DNA to

influence directly the growth of a cell line after transfer into the cell by co-precipitation with calcium phosphate. It was hoped that the use of DNA obtained from the Burkitt's lymphoma cell line, Raji (Mer^+) and the human lymphoblastoid cell line, GM892 (Mer^-) after treatment with temozolomide or ethazolastone, would provide further clues to the role of $\underline{O}^6$-methylguanine in cell killing.

6.2 METHODS

6.2.1 Cell lines used and their maintenance.

GM892A

This is a human lymphoblastoid cell line, derived from normal individuals, which has very low levels of O^6-alkylguanine-DNA alkyltransferase and has been classified as Mer^- (Harris et al., 1983).

It was maintained as a suspension culture in exponential growth at a density between 0.8-8.0 x 10^5 cells/ml. Cells were grown in RPMI 1640 medium (with 25 mM HEPES and L-glutamine), supplemented with 10% foetal calf serum, under an atmosphere of 5% CO_2 in air. Under these conditions the cells were found to have a doubling time of 22-26 h.

Raji

This is a human lymphoma cell line derived from a patient with Burkitt's lymphoma, which is proficient in the repair of O^6-alkylguanine lesions in DNA and has been classified as Mer^+ (Harris et al., 1983).

It was maintained as a suspension culture in exponential growth at a density between 0.8-8.0 x 10^5 cells/ml. Cells were grown in RPMI 1640 medium (with 25 mM HEPES and L-glutamine), supplemented with 10% foetal calf serum, under an atmosphere of 5% CO_2 in air. Under these conditions the cells were found to have a doubling time of 22-26 h.

MAC13

This is a cell line derived from a solid murine adenocarcinoma which was induced by dimethylhydrazine (Double and Ball, 1975). MAC13 cells were maintained as a monolayer in exponential growth attached to plastic. Cells were grown in RPMI 1640 medium (with 25 mM HEPES and L-glutamine), supplemented with 10% foetal calf serum, under an atmosphere of 5% CO_2 in air. Under these conditions the cells were found to have a doubling time of approximately 24 h.

MAC13 cells were detached from the plastic by trypsinisation. The medium was aspirated from cultures and the cells were washed with 10 ml of Mg^{2+}/Ca^{2+} free PBS and then rinsed quickly with 5.0 ml of trypsin solution, which was then removed by aspiration. Trypsin solution was added again, but this time cells were incubated at 37°C for 5-20 min until detached. Ten volumes of medium were then added to inactivate the trypsin.

For routine subculturing, 1.0×10^4 cells were reseeded into a T50 flask to which was added 10 ml of culture medium.

Cell counting.

All cell counting was carried out using a Coulter Counter (model ZM) (Coulter Electronics, Bedfordshire), using the following settings:-

	GM892A	Raji	MAC13
Current	200	200	120
Attenuation	16	8	8
Lower threshold	10	12	15
Upper threshold	99.9	99.9	99.9

Cytotoxicity assay

Cells in exponential phase were used. 2×10^4 cells were seeded into Nunc 24 well plates and the appropriate concentration of drug was added. The drugs were dissolved in DMSO so that the final concentration in the culture medium did not exceed 1%. The cells were counted 72 h after drug addition. The results were expressed as the growth of the treated cell population relative to the growth of the untreated cell population.

Storage of cultures in liquid nitrogen

Cells in logarithmic growth were pelleted using an Heraeus benchtop centrifuge at a speed of 1500 rpm for 5 min. The supernatant was carefully removed by aspiration and the pellet resuspended at a density of $1-2 \times 10^6$ cells/ml in nutrient medium containing 10% DMSO as a cryogenic preservative. The cell suspension was then tranferred to cryogenic vials (1 ml/vial), cooled to -80°C and then immersed into

liquid nitrogen. When the cells were used from the cell bank they were thawed rapidly and pelleted using an Heraeus bench top centrifuge at a speed of 1500 rpm for 5 min. The medium containing DMSO was carefully removed by aspiration and the pellet was resuspended in 1 ml of medium. The suspension was then transferred to a T50 flask and grown for two days in medium containing 20% serum.

6.2.2 Treatment of cells with drugs and extraction of DNA.

Temozolomide and ethazolastone were dissolved in DMSO at a concentration of 20 mg/ml so that when added to the cell suspension the final concentration of DMSO in the culture medium did not exceed 0.4%.

The cell lines GM892 and Raji show a differential response to the cytotoxic effects of the drugs. The concentrations required to give a 50% inhibition of cell growth as determined by Bull and Tisdale (1987) were used and are illustrated below.

	GM892	Raji
Temozolomide	10±7μM	206±20μM
Ethazolastone	229±20 M	360±30μM

Cells were exposed to equitoxic concentrations of the drugs at the ID_{50} for either 6,12 or 24 h.

The method for isolation of DNA from the cells was based on that described by Warren (1984). The cells were pelleted by low speed centrifugation (1500 rpm) using an Heraeus bench top centrifuge, and washed by resuspending in 0.9% saline. To the cell pellet in a Universal container was added between 1-2 ml of 6% p-aminosalicylic acid (sodium salt) and vortexed for 5 min. 0.1 Volumes of 10% SDS were then added and the mixture was incubated at 37°C for 5 min. The solution clarified and became more viscous as lysis proceeded. After transferring to polypropylene tubes, an equal volume of phenol reagent was added and the mixture was vortexed for 5 min. The mixture was then centrifuged at 2000 rpm for 30 min in an Heraeus benchtop centrifuge. The upper aqueous layer was removed using a Pasteur pipette taking care not to disrupt the interfacial material. The DNA was then precipitated from the aqueous phase by adding 1.5 volumes of ethoxyethanol and gently agitating. The DNA was then either removed using a glass spatula or separated by centrifugation at 3000 rpm in an Heraeus benchtop centrifuge. The DNA was then washed by resuspending and then resedimentation at 3000 rpm using an Heraeus benchtop centrifuge, three times with 70% (v/v) ethanol 2% sodium acetate(w/v) and two times with absolute ethanol and then air dried.

6.2.3 The transfer of DNA into cells by calcium phosphate co-precipitation with DNA.

A precipitate containing calcium phosphate and DNA was formed by slowly mixing HEPES-buffered saline solution with a solution containing calcium chloride and DNA. The precipitate adheres to the cells which take up the DNA precipitate by an unknown mechanism. Therefore, it is essential to use cells which adhere to plastic T50 flasks. MAC13 cells were used in this procedure as they adhere to plastic and are frequently used in this laboratory.

The day before the precipitation process was to take place, 5.0×10^4 cells were placed into T50 flasks with 9 ml of RPMI 1640 medium (with 25 mM HEPES and L-glutamine) supplemented with 10% foetal calf serum (complete medium) and allowed to adhere overnight. The next day the cells were checked to be evenly distributed on the bottom of the flask.

Unless otherwise stated 30 μg of DNA were used in each precipitation process, which was determined according to the ethidium bromide-fluorometric assay (section 4.2.1). The DNA was sterilised by precipitation with two volumes of absolute ethanol and then air dried by inverting the microfuge tube in a tissue culture hood. The DNA was then resuspended in 450 μl of sterile water and 50 μl of 2.5 M $CaCl_2$ was added. Into a sterile Universal container was placed 500 μl of sterile 2 x HeBS and using a mechanical pipettor

attached to a plugged 5 ml pipette, air was bubbled through the 2 x HeBS whilst the DNA/$CaCl_2$ solution was added slowly using a Pasteur pipette. The solution was then vortexed for 5 s, and allowed to precipitate for 20 min at room temperature. The DNA precipitate was then distributed evenly over the surface of the flask of cells using a Pasteur pipette, gently agitating to mix the precipitate and medium. The cells were then incubated for 5 h, after which the medium was removed and the cells were washed twice with 5 ml of 1 x PBS. The cells were then fed with 10.0 ml of complete medium and gassed. The cells were then incubated for 4 days and then counted.

6.2.4 Experiment to determine the amount of [^{3}H]DNA transferred into MAC13 cells after co-precipitation with calcium phosphate.

The labelled DNA was prepared from murine L1210 cells grown in culture by the method described in section 4.2.9.

Into each plastic T50 flask was placed 5.0 x 10^4 cells and 10 ml of RPMI 1640 medium (with 25 mM HEPES and L-glutamine) supplemented with 10% foetal calf serum. The cells were allowed to attach overnight and the next day they were treated with the calcium phosphate/DNA precipitate as described in section 6.2.3 which contained various amounts of [^{3}H]DNA from

L1210 cells. After 12 h the cells were removed by trypsinisation, pelleted and extensively washed with PBS. The cells were then lysed with 0.5 ml cell lysing buffer and the amount of radioactivity associated with the cells was determined by adding 10 ml of optiphase 'Hisafe' scintillant and counting on a Packard Tri-Carb 2000CA liquid scintillation counter. Non-specific binding of labelled DNA to the cell membrane was excluded by carrying out a control in which [^{3}H]DNA was added to the medium in which the cells were growing without calcium phosphate.

6.2.5 Experiment to determine the methylation status of MAC13 cells after treatment with calcium phosphate/DNA precipitate.

This method was carried out essentially as described by Parker et al. (1986).

5.0 x 10^4 cells were treated with the calcium phosphate/DNA precipitate for 5 h after which the cells were washed with PBS. The cells were then fed with RPMI 1640 medium (with 25 mM HEPES and L-glutamine) supplemented with 10% foetal calf serum (complete medium) containing 10 μM deoxycytidine and thymidine, in order to reduce the incorporation of the label into DNA via the salvage pathways. Labelling of DNA was carried out by adding [6-^{3}H] uridine (1.0 μCi/ml; 17 Ci/mmol) to the culture medium and incubating the cells for 24 h. The cells

were then harvested by trypsinisation and washed twice in PBS by resuspension and resedimentation at 1500 rpm using an Heraeus benchtop centrifuge. The DNA was then isolated from the MAC13 cells by the method described in section 6.2.2.

The DNA was placed in a pyrex glass tube (150 x 18 mm) to which was added 1-2 ml of 90% formic acid and the tube was then sealed using an oxygen/natural gas flame torch. The tube was then placed within a metal cage and immersed into a stirring oil bath at 180°C. Hydrolysis of the DNA was carried out for 25 min, after which the tubes were removed and allowed to cool for at least 30 min. The seal was then broken and the formic acid was removed under a stream of N_2(g) whilst warming in a 60°C water-bath.

HPLC separations were performed on a system consisting of a Waters Maxima 820 Chromatography Workstation, a Waters model 510 pump, a Waters Lambda-Max model 480 spectrophotometer (at 254 nm) and a Waters automatic sample injector WISP 710B. A Whatman Partisil 10SCX column was used with a isocratic mobile phase of 0.04 M ammonium formate pH 2.0. The flow rate was 1.0 ml/min with a run time of 10 min. All buffers were degassed under vacuum immediately prior to use.

The residue from the DNA hydrolysis was redissolved in 100 μl of 0.1 M HCl and 30 μl was injected onto the HPLC column. Fractions were

collected every 30 s directly into liquid scintillation glass vials using a Pharmacia FRAC-100 programmable fraction collector and the amounts were determined in Optiphase MP scintillation fluid.

The percentage of cytosines (C) that were converted to 5-methylcytosine (5MC) was calculated as follows:

$$\%5MC = \frac{[dpm \text{ in } 5MC]}{[dpm \text{ in } 5MC] + [dpm \text{ in } C]} \times 100$$

6.2.6 Statistical analysis.

All results are expressed as mean ± standard error of the mean (SEM) for at least three determinations. Differences have been analysed statistically using the Students t-test.

6.3 RESULTS

The transfer of DNA into cells by calcium phosphate co-precipitation with DNA.

In order to try to understand the cellular reactions responsible for cytotoxicity after treatment with the imidazotetrazinones, cellular DNA extracted from GM892 or Raji cells has been transferred into MAC13 cells using the calcium phosphate co-precipitation technique. The calcium phosphate transfection technique was first used to introduce adenovirus DNA into mammalian cells (Graham and Van der Eb, 1973). The Burkitts lymphoma cell line, Raji and the human lymphoblastoid cell line, GM892, differ in their level of the DNA repair protein, O^6-alkylguanine-DNA alkyltransferase. GM892 has very low levels of this enzyme (Harris et al., 1983) and is 25-fold more sensitive to temozolomide than Raji cells (Bull, 1988) which are proficient in the repair enzyme (Harris et al., 1983). Ethazolastone showed relatively little differential cytotoxicity between the two cell lines and towards GM892 was about 15-fold less potent than temozolomide (Bull, 1988). The concentration of drugs used in this series of experiments was that concentration required to give 50% inhibition of cell growth as determined by Bull and Tisdale (1987) and is shown in table 16.

DNA from cells treated in this way was then transferred into the murine adenocarcinoma cell line MAC13, using the calcium phosphate precipitation technique. MAC13 cells were used, due to their ability to adhere to plastic which is essential in order for the procedure to be carried out. The sensitivity of the MAC13 cell line to the imidazotetrazinones is illustrated in fig 44.

When cells were treated with $Ca_3(PO_4)_2$ without any DNA present, an inhibition of cellular proliferation was observed compared with untreated cells (key # 1 fig 45, key # 1 fig 46, key # 1 fig 47). This is opposite to the results observed by Rubin and Sanui (1977), who observed that water insoluble complexes of PPi, HPO_4^{2-} and Ca^{2+} stimulated the growth of BALB/c3T3 cells. In all these experiments the cells were counted four days after the calcium phosphate precipitation and cells which had not received any treatment were confluent at this stage and were therefore not counted. An arbitrary time point of four days was used as the point at which the cells were counted and no staining procedure was used to check the viability of the cells.

The treatment of MAC13 cells with DNA from GM892 or Raji cells exposed to the DMSO solvent, caused a reduction in cellular proliferation compared with control cells treated with the $Ca_3(PO_4)_2$ precipitate (key # 2 fig 45 and key # 2 fig 46). Therefore, all

the results obtained with DNA obtained from drug-treated cells have been compared with the results for DNA obtained from solvent control-treated cells.

The DNA obtained from Raji or GM892 cells treated with temozolomide or ethazolastone at equitoxic concentrations caused an inhibition of cellular proliferation when transferred into MAC13 cells by calcium phosphate precipitation (fig 45 and fig 46). There was no effect on cellular proliferation when MAC13 cells were incubated with DNA from either cell line in the absence of calcium phosphate.

The inhibition was seen to be dependent on the length of time the cells were incubated with drug prior to extraction. Thus, the effect was most pronounced for DNA extracted 6 h after drug addition and became less pronounced by 12 h and 24 h after drug addition, for both GM892 (fig 45) and Raji (fig 46) cell lines. The transfer of DNA from GM892 cells treated with either temozolomide or ethazolastone into MAC13 cells had a more pronounced effect on the subsequent growth than that from Raji cells. This occurred despite the lower concentrations of drugs to which GM892 cells were exposed.

For temozolomide-treated GM892 and Raji cells at 6 h, significant inhibition of the growth of MAC13 cells was produced ($P<0.001$ and $P<0.005$ respectively). However, at 12 h and 24 h only GM892 DNA

still significantly inhibited the growth of MAC13 cells ($P<0.005$ and $P<0.05$ respectively). Similarly, for ethazolastone-treated GM892 and Raji cells at 6 h, significant inhibition of MAC13 cell growth was produced ($P<0.001$ and $P<0.05$ respectively), while at 12 h and 24 h only GM892 DNA still significantly inhibited the growth of MAC13 cells ($P<0.005$ and $P<0.05$ respectively).

DNA extracted from cells treated with temozolomide was a more potent inhibitor of the growth of MAC13 cells than that extracted from cells treated with ethazolastone. Therefore, at 6 h DNA from GM892 and Raji cells exposed to temozolomide was a more potent inhibitor of the growth of MAC13 cells than DNA from cells exposed to ethazolastone ($P<0.05$ and $P<0.005$ respectively).

The concentration of temozolomide used to treat GM892 cells was 10 μM. If one assumes that the extent of alkylation of cellular DNA would be proportional to the dose administered, the extent of methylation of cellular DNA can be calculated. The data of Bull and Tisdale (1987) shows that after 6 h, 12 h and 24 h exposure of GM892 cells to 100 μM temozolomide the amount of drug bound to DNA was 0.3, 0.35 and 0.5 pmol per μg DNA respectively. Therefore, for 10 μM temozolomide this would be approximately 0.03, 0.035 and 0.05 pmol per μg DNA or 10, 12 and 17 μmol methyl per mol DNA-P (based on the

assumption that the average molecular weight of a DNA nucleotide is 330). These values correspond to between 50,000 and 85,000 methylations in the total cellular genome of approximately 5 x 10^9 DNA bases (Davidson, 1965). If one assumes that the molecular weight of DNA from GM892 cells is similar to that of calf thymus DNA, then from fig 32 to fig 35 (section four) it can be seen that the largest DNA is approximately 20,000 base pairs i.e. 40,000 bases. Therefore, each DNA molecule on average would have 0.4 methyl groups per DNA molecule. This is a very low level of methylation and the observation that this may have an effect on the growth of the MAC13 cell line after calcium phosphate co-precipitation is remarkable. However, the nature of the neighbouring base can influence the extent of alkylation of guanine residues (Briscoe and Cotter, 1984) and it has been proposed that GC rich regions in DNA may be preferred sites of damage by such alkylating agents (Mattes et al., 1988). Therefore, it remains possible that the DNA damage may be 'clustered' within certain regions of the DNA.

In order to investigate the possibility that the inhibition of cellular proliferation was not due to a non-specific effect produced with damaged DNA, the effect of X-irradiated calf thymus DNA on the subsequent growth of MAC13 cells was investigated. The transfer of calf thymus DNA irradiated with up to

400 rads of X-rays had no effect on the subsequent growth of MAC13 cells (fig 47). Although the comparison was complicated by using calf thymus DNA as opposed to cellular DNA, it would appear that the inhibition of cell growth was not due to a non-specific effect from damaged DNA.

An important control was to determine whether any DNA during the calcium phosphate precipitation technique was actually getting into the MAC13 cells or whether the DNA simply remained attached to the cell membrane. The results in fig 48 show a dose related increase in the amount of DNA incorporated into the cells, which was proportional to the amount of DNA used. Non-specific binding of the labelled DNA to the cell membrane was excluded by carrying out a control in which the [^{3}H]labelled DNA was added to the medium in which the cells were growing without using calcium phosphate. In this case, the radio-activity associated with the cells was similar to background levels. These experiments were conducted using labelled DNA extracted from murine L1210 cells grown in the presence of [^{3}H]thymidine rather than labelled GM892 or Raji DNA. Whilst it is unlikely that this would have any effect on its transfer into the cells, this difference should be borne in mind.

Although these results suggest that the DNA is crossing the cell membrane, it does not necessarily imply that the DNA is actually crossing the nuclear

membrane and is being integrated into the murine cellular DNA. Since the results in fig 45 and fig 46 are for human DNA being transferred into murine cells, an important experiment would be to determine whether the human DNA has been integrated into the murine DNA, by carrying out Southern blots using a range of suitable specific human DNA probes. At the moment it cannot be determined whether the growth inhibitory effect of the alkylated DNA was exerting its effect by integration into the cellular DNA or through another mechanism within the cytoplasm.

Experiment to determine the methylation status of MAC13 cells after treatment with calcium phosphate/-DNA precipitate.

The previous experiments suggest that DNA from Raji or GM892 cells treated with imidazotetrazinones can cause the suppression of MAC13 cell growth. A possible mechanism for this suppression of cell growth is through the inhibition of DNA(cytosine-5)-methyltransferase, since it has been shown by Tisdale (1989) that the drugs temozolomide and ethazolastone cause a reduction in the enzymatic methylation of DNA in GM892 cells. Therefore, it was proposed to investigate whether DNA from GM892 or Raji cells treated with the imidazotetrazinones was capable of

causing inhibition of DNA(cytosine-5)methyl-transferase in MAC13 cells when transferred into these cells by calcium phosphate co-precipitation.

The amount of 5-methylcytosine in newly synthesised MAC13 cellular DNA was determined by treating the cells with [6-^{3}H] uridine immediately after the calcium phosphate precipitation procedure was complete. The extent of enzymatic methylation 24 h after treatment with the alkylated DNA was determined by HPLC analysis of the bases after formic acid hydrolysis and was calculated according to the formula:

$$\% \text{ 5MC} = \frac{[\text{dpm in 5MC}]}{[\text{dpm in cytosine}] + [\text{dpm in 5MC}]}$$

Formic acid was used to hydrolyse the DNA to its constitutive bases and this causes some deamination of cytosine to produce thymine, which results in a degree of inaccuracy. A more precise method to determine the level of 5-methylcytosine is to carry out base analysis after enzyme (venom phospho-diesterase, pancreatic DNase I) digestion. A comparison of formic acid hydrolysis and enzymatic digestion of [^{3}H]labelled DNA (Ford et al., 1980) was reported to result in variable deamination of 5-methylcytosine. An alternative to formic acid for hydrolysis of DNA is hydrofluoric acid. The deoxy-ribonucleotides of cytosine and 5-methylcytosine were shown by Catania et al. (1987) not to undergo any

detectable levels of deamination with 48% hydrofluoric acid at 80°C for up to 24h. The hazardous nature of this material prevented its use in these experiments.

M.lysodeikticus DNA was treated with DNA(cytosine-5)methyltransferase and S-adenosyl-L-[methyl-^{3}H]methionine and then hydrolysed with formic acid. The hydrolysate was analysed by HPLC and the thymine was shown to represent approximately 6% of the total radioactivity (fig. 11, section 3). Although a value of 6% is significant, it is not great enough to affect the conclusions which are to be made. The extent of enzymatic methylation of MAC13 cells is shown in table 17 (APPENDIX 4).

The extent of enzymatic methylation of newly synthesised DNA in MAC13 cells 24 h after labelling with [6-^{3}H]uridine was 2.4±0.0%. There is no value in the literature for the level of enzymatic methylation of newly synthesised DNA in the murine adenocarcinoma cell line (MAC13), but this value is similar to values obtained for other cell lines, for example 2.94±0.46% in WI-38 human embryonic lung fibroblasts (Parker et al., 1986), 2.50±0.18% in hamster fibrosarcoma cells A(TI)C1-3, 2.80±0.14% in human fibroblasts T_1 and 2.17±0.10% in human fibrosarcoma cells, HT-1080 (Nyce et al., 1986). However, this value is lower than certain other cell lines, in particular 3.40±0.21% to 3.55±0.11% in the human lymphoblastoid

cell line, GM892 (Tisdale, 1989), 3.4±0.1% for the human erythroleukemia cell line K562 (Tisdale, 1986), 3.6% for the Burkitt's lymphoma cell line Raji, 3.6% for the murine thymic lymphoma cell line S49 and 3.2% for human diploid fibroblasts (Krawisz and Lieberman, 1984). The values for the level of enzymatic methylation were determined after enzymatic digestion (DNaseI, alkaline phosphatase and nuclease P1 or snake venom phosphodiesterase) for those results obtained by Krawisz and Lieberman (1984) and all the other results including those presented in this thesis were determined after formic acid hydrolysis.

The amount of 5-methylcytosine in newly synthesised DNA from MAC13 cells was 2.4±0.0% (table 17). A value of 2.4±0.0% was also obtained after the MAC13 cells had been treated with $Ca_3(PO_4)_2$ which did not contain any DNA. However, treatment with $Ca_3(PO_4)_2$ when co-precipitated with DNA from untreated Raji or GM892 cells caused an increase in the proportion of 5-methylcytosine to 3.1±0.2% and 2.6±0.6% respectively. The use of DNA from GM892 or Raji cells treated with temozolomide or ethazolastone produced an increase in the overall level of 5-methylcytosine in newly synthesised DNA which was dependent on the length of time that the GM892 or Raji cells were incubated with the drug.

The DNA from Raji cells treated with temozolomide for 6 h, 12 h or 24 h when transferred by $Ca_3(PO_4)_2$ precipitation into MAC13 cells caused an increase in the level of 5-methylcytosine in newly synthesised DNA from MAC13 cells to 3.5±0.6%, 2.8±0.2% and 3.4±0.4% respectively. The DNA from Raji cells treated with ethazolastone for 6 h and 24 h when transferred by $Ca_3(PO_4)_2$ precipitation into MAC13 cells caused an increase in the level of 5-methylcytosine in newly synthesised DNA from MAC13 cells to 3.5±0.1% and 2.9±0.1% respectively.

The DNA from GM892 cells treated with temozolomide for 12 h or 24 h when transferred by $Ca_3(PO_4)_2$ precipitation into MAC13 cells caused an increase in the level of 5-methylcytosine in newly synthesised DNA from MAC13 cells to 6.7±0.6% and 8.3±0.3% respectively. The radioactivity in the fractions corresponding to cytosine and 5-methylcytosine for 6 h was too low for an accurate estimation of the level of 5-methylcytosine to be made. The DNA from GM892 cells treated with ethazolastone for 6 h, 12 h or 24 h when transferred by $Ca_3(PO_4)_2$ precipitation into MAC13 cells caused an increase in the level of 5-methylcytosine in newly synthesised DNA in MAC13 cells to 5.5±0.8%, 4.7±2.2% and 4.8±0.4% respectively. These results indicate that DNA from GM892 cells treated with imidazotetrazinones causes a more pronounced hypermethylation of newly synthesised DNA

in MAC13 cells than DNA from Raji cells. Further, the DNA from GM892 cells treated with temozolomide caused a more pronounced hypermethylation of newly synthesised DNA in MAC13 cells than DNA from GM892 cells treated with ethazolastone.

Therefore, the transfer of DNA (obtained from GM892 or Raji cells treated with the imidazotetrazinones) into MAC13 cells by co-precipitation with calcium phosphate causes hypermethylation of the cellular DNA. Previously, Tisdale (1989) has shown that the free drugs, temozolomide or ethazolastone, cause hypomethylation of DNA in GM892 cells. Therefore, any effects on DNA(cytosine-5)methyltransferase are clearly different in the two cases. The observed hypermethylation after the transfer of alkylated DNA into the cells by calcium phosphate co-precipitation, may be occurring as a consequence of the slowing down of DNA synthesis.

A similar effect was produced with drugs which inhibit DNA synthesis, for example hypermethylation in DNA of hamster fibrosarcoma cells was induced by the DNA synthesis inhibitors, cytosine arabinoside, hydroxyurea and aphidicolin (Nyce et al., 1986). De Haan and Parker (1988) demonstrated hypermethylation in various cell lines using the same drugs. Cytosine arabinoside has also been shown to cause hyper-

methylation of P815 mouse mastocytoma cells in culture with an increase from 3% to 15% methylated cytosines (Boehm and Drahovský, 1982).

6.4 DISCUSSION

The technique of calcium phosphate transfection was first used to introduce adenovirus DNA into mammalian cells by Graham and Van der Eb (1973). Since then this technique has been used widely to incorporate isolated genes of interest into cellular DNA, which has been used to study factors such as DNA methylation which may affect the expression of that gene (for example Yisraeli et al., 1988). The data presented is another approach to the technique of calcium phosphate co-precipitation which has not been previously reported.

The cell lines GM892 and Raji differ in their constitutive level of the DNA repair protein O^6AT (Harris et al., 1983) and they were treated with the methylating agent temozolomide or the ethylating agent ethazolastone at their respective ID_{50}. Cellular DNA extracted from these cells at various times was transferred into another cell line (MAC13) and the ability of this DNA to influence the growth of these cells was studied. It was hoped that this would provide further information on the potential mechanisms responsible for the cytotoxicity of the imidazotetrazinones, in particular to the importance of O^6-methylguanine in DNA.

The possible effect of calcium phosphate on the growth properties of the MAC13 cells was investigated. The calcium phosphate precipitate caused a

suppression of cell growth which was reflected in an inhibition of DNA synthesis. The effect was not as pronounced as that produced when DNA or in particular alkylated DNA was coprecipitated with the calcium phosphate. These observations were opposite to those reported by Rubin and Sanui (1977), in which they report that water insoluble complexes of PPi, $HPO_4 2-$ and Ca^{2+} actually stimulate the growth of BALB/c3T3 cells. The process of calcium phosphate-mediated DNA transfection has been associated with alterations in the tumorigenic or metastatic behaviour of the CBA/J mouse mammary adenocarcinoma SPI (Kerbel et al., 1987).

In order to draw any conclusion from these results, it was important to know the specific base modifications formed after drug treatment. An investigation was made by Bull (1988) of the specific base alkylations formed after in vitro treatment of the calf thymus DNA with temozolomide and ethazolastone. The greatest proportion of the adducts formed after temozolomide treatment was 7-methylguanine (70%), whilst ethazolastone produced only 24% 7-ethylguanine. The amount of 3-alkyl-adenine formed after temozolomide treatment was 9.2% 3-methyladenine and for ethazolastone was 4.9% 3-ethyladenine. The relative proportions of O^6-alkylguanine formed after temozolomide or ethazolastone treatment was almost identical with O^6-

methylguanine and O^6-ethylguanine representing 5.3% and 5.4% of the total alkylated bases respectively. These values are similar to those determined for the nitrosoureas MNU and ENU by Beranek et al. (1980). As Bull (1988) did not determine the level of alkylphosphotriester formation it was assumed to be similar to that reported for the nitrosoureas, since they alkylate DNA by a similar mechanism. Alkylphosphotriesters were therefore thought to represent 50-60% of the total DNA alkylated by ethazolastone and approximately 12% for temozolomide. Using these values for the percent of total DNA alkylated, the overall alkylation of particular bases in DNA has been calculated for GM892 and Raji cells treated with temozolomide or ethazolastone at the ID_{50} concentration (table 18 and table 19). This was based on the assumption that the overall level of alkylation at the particular drug concentration used (ID_{50} for the cell line), was directly proportional to the level produced with a drug concentration of 0.1 mM as determined by Bull and Tisdale (1987). Although it is realised that repair of certain bases may have occurred over time, this was not considered in the calculation.

The growth inhibitory effect of the alkylated DNA in MAC13 cells was most pronounced for DNA extracted from GM892 or Raji cells 6 h after addition of temozolomide or ethazolastone and became less

pronounced by 12 h and 24 h after drug addition. This time course for the inhibition of growth suggests that this is due to a repairable lesion present in the DNA of both GM892 and Raji cells.

The alkylphosphotriester lesion is not removed from DNA at an appreciable rate in eukaryotes (Frei et al., 1978), whilst in E.coli it is removed by part of the ada gene product (McCarthy and Lindahl, 1985). There was also no correlation between the extent of the alkylphosphotriester formation in the alkylated DNA from GM892 or Raji cells (table 18 and 19) and the suppression of MAC13 cell growth. Therefore, it was unlikely that this lesion was responsible for the growth inhibition. The work of Hall et al. (1988) in which CHO cells expressing E.coli Ada protein with or without alkylphosphotriester activity, demonstrated that methylphosphotriesters do not contribute substantially to the lethal effects of MNNG.

The possibility that O^6-alkylguanine lesions in the DNA from GM892 or Raji cells was responsible for the inhibition will now be considered. The greatest inhibition of MAC13 cell growth occurred with DNA extracted from GM892 or Raji cells 6 h after treatment with temozolomide at the LD_{50} concentration for the particular cell line. GM892 cells were treated with 10 μM temozolomide and in the absence of repair of O^6-methylguanine (GM892 is a Mer- cell line and has a poor capacity to repair this lesion), it

has been approximated that the amount of $\underline{O}^6$-methylguanine formed will be 0.5 μmol/mol DNA-P (table 18). However, Raji cells were treated with 206 μM temozolomide and in the absence of DNA repair it has been approximated that the amount of $\underline{O}^6$-methylguanine formed will be 7.2 μmol/mol DNA-P (table 19). Raji cells are proficient in the repair of $\underline{O}^6$-methylguanine in DNA and whilst some repair of $\underline{O}^6$-methylguanine will have occurred, it is likely that at the LD_{50} concentration of drug used, O^6AT would be titrated out so that there is little removal over a 24 h period (Bull, 1988). Further, DNA extracted from GM892 cells 6 h after treatment with temozolomide at the LD_{50} drug concentration (10 μM) causes a greater suppression of MAC13 cell growth than ethazolastone at the LD_{50} drug concentration (229 μM). The approximate extent of alkylation at the $\underline{O}^6$ atom of guanine in the absence of any DNA repair is 0.5 μmol/mol DNA-P and 12.3 μmol/mol DNA-P respectively (table 18). There is clearly no correlation between the extent of alkylation at the $\underline{O}^6$ atom of guanine in GM892 or Raji DNA and the ability of this DNA to suppress the growth of MAC13 cells.

Two other lesions which are repairable in both cell lines are 7-alkylguanine and 3-alkyladenine. There are no data in the literature on the ability of GM892 or Raji cells to repair these lesions but,

Medcalf and Lawley (1981) have studied the rate of removal of alkylated bases from the DNA of MNU-treated human fibroblasts and they found that the removal of both of these bases occurred. However, 3-methyladenine was removed more rapidly with a time course similar to that for the ability of alkylated DNA to suppress the growth of MAC13 cells. This suggests that the formation of 3-alkyladenine may be the lesion responsible for the growth inhibitory effect of the alkylated DNA. This theory does not stand up when the extent of alkylation at the N-3 atom of adenine is considered. DNA extracted from GM892 cells 6 h after treatment with temozolomide (10 μM) produced a greater inhibition of MAC13 cell growth than with DNA extracted from GM892 cells 6 h after treatment with ethazolastone (229 μM). However, it has been approximated that in the absence of any DNA repair the amount of 3-alkyladenine formed was 0.9 and 11.2 μmol/mol DNA-P respectively. Further, DNA extracted from Raji cells 6 h after treatment with temozolomide (206 μM) produced a greater inhibition of MAC13 cell growth than with DNA extracted from Raji cells 6 h after treatment with ethazolastone (360 μM). In this case the approximate amounts of 3-alkyladenine formed in the absence of any DNA repair were similar, 12.4 and 14.6 μmol/mol DNA-P respectively. Clearly, these values do not correlate with the growth inhibitory effect of the

alkylated DNA.

3-Methyladenine has been implicated in the lethal effects of alkylating agents to bacteria (Karran et al., 1980; Karran et al., 1982), but the effect in eukaryotes is less clear. Alkylation at the N-3 atom of adenine is lethal to the cell because the alkyl group protrudes into the minor groove of DNA and is a block to DNA polymerase and DNA replication (Lawley and Warren, 1976; Boiteaux et al., 1984). However, it is difficult to imagine how DNA bearing such a lesion could suppress the growth of MAC13 cells after transfer into the cells by co-precipitation with calcium phosphate. The inhibition of DNA polymerase is only relevant when the 3-alkyladenine is part of the genomic DNA and it is likely that very little of the alkylated DNA is actually incorporated into the cellular DNA of the MAC13 cells.

DNA extracted from GM892 cells treated with temozolomide at the LD_{50} concentration for these cells (10 μM) produced the greatest suppression of cell growth and it has been calculated this represents approximately 0.4 methyl groups per DNA molecule. One may be forgiven a degree of scepticism, that this very low level of methylation was capable of suppressing the growth of the MAC13 cells when they were treated with the DNA co-precipitated with calcium phosphate. However, this potent effect has

been verified in the study of the level of DNA(cytosine-5)methylation in the MAC13 cells which was run in parallel with the study of growth suppression. An increase in the level of cytosine methylation was observed in newly replicated DNA, which was thought to occur as a result of inhibition of DNA synthesis.

X-Irradiated calf thymus DNA does not cause significant inhibition of the proliferation of MAC13 cells after co-precipitation with calcium phosphate. Therefore, the effect appears not to be due to a non-specific inhibition of proliferation by damaged DNA. However, the use of calf thymus DNA as opposed to DNA extracted from human cells in these experiments casts doubts on the interpretation. In hindsight more experiments should have been done to investigate further the possibility that strand breaks or abasic sites in the DNA were responsible for the inhibition.

It remains a possibility that the production of apurinic sites in the alkylated DNA by spontaneous release or as a result of the action of DNA N-glycosylases may be responsible for the suppression of MAC13 cell growth. Apurinic sites have been shown to be toxic in E.coli and in mammalian cells grown in culture (reviewed by Strauss, 1985 and Loeb and Preston, 1986).

Recently, Dunn et al. (1991) transfected Chinese hamster ovary cells (no O^6AT activity) with human cell DNA and obtained two stable transformant clones

which had high activities of O^6AT. The ability to repair N-methylpurines did not change after transfection. The sensitivity of these new cell lines and the parent cell line to the alkylating agents CNU, MNU, MNNG and MMS varied widely and they concluded that N-alkylpurines and abasic sites may be lethal lesions.

Further support for this idea comes from the work of Waters et al. (1990), in which they treated four mammalian cell lines, Chinese hamster ovary, mouse lymphoma L5178Y, human lymphoblastoid TK6 and Chinese hamster V79 with EMS. Although they had different sensitivities to the drug, there were no significant differences in ethylation of DNA in the cells per unit does or time at O^6 or N-7 of guanine or N-3 of adenine. It was suggested by these authors that the greater cytotoxicity of EMS towards TK6 cells may be due to apurinic sites created during the repair of N-alkylations and the TK6 cells may have a defect in the processing of N-alkylations.

The earlier work of Kalamegham et al (1988) further supports the theory that strand breaks may be responsible for cytotoxicity. These authors carried out studies on the HeLa cell line A2-8, which was defective in the repair of O^6-methylguanine. They suggest that unrepaired O^6-methylguanine after treatment with MNNG caused an excess of strand breaks which were responsible for cytotoxicity.

In K562 cells (Zucchetti et al., 1989) and L1210 cells (Catapano et al., 1987), temozolomide has been reported to form apurinic sites in DNA. In comparison using the technique of alkaline elution, ethazolastone has been shown to be very poor at producing this form of DNA damage in K562 cells (Zucchetti et al., 1989). This correlates with the fact that DNA from temozolomide-treated Raji or GM892 cells was a better inhibitor of cell growth than DNA from ethazolastone-treated cells. Since DNA from GM892 cells treated with temozolomide or ethazolastone produced a greater suppression of cell growth than DNA from Raji cells, I propose that GM892 cells in comparison to Raji cells are poorer at the repair of this form of damage.

It is likely that very little of the alkylated DNA is actually incorporated into the cellular DNA. Therefore, if strand breaks and apurinic sites in the alkylated GM892 or Raji DNA are responsible for the suppression of MAC13 cell growth, one is still left asking the question how does this occur?

The results suggest that alkylation of DNA by the imidazotetrazinones is not the ultimate reaction, but that the alkylated DNA is capable of exerting other actions in the cell which are ultimately responsible for the inhibition of cellular proliferation. I can make no suggestion at this stage what the ultimate reaction might be.

These experiments are clearly incomplete at this stage and open up an area of work which warrants further investigation. The ability of alkylated DNA to suppress the growth of a cell line after co-precipitation with calcium phosphate has not been described before and is of particular interest when a very low level of alkylation is apparently capable of causing the inhibition of cell growth. Therefore it is proposed that attention should be directed in the following areas. Firstly, a more precise identification of the lesion responsible. This should include an examination of other methods to produce strand breaks and apurinic sites. The use of DNA repair enzymes and synthetic oligonucleotides may be useful. Secondly, calf thymus DNA treated in vitro with the drugs should be investigated. This would provide further information regarding the lesion responsible in the absence of any repair. Thirdly, to determine whether the suppression of cell growth was dependent on the drug concentration, DNA from cells exposed to a range of drug concentrations should be used. Fourthly, to determine whether the suppression of cell growth was dependent on the amount of DNA, various amounts of DNA from cells exposed to the same concentration of drug should be used.

Note added in proof

This work was continued by M J Tisdale and the results have recently been reported (Hepburn, P.A. and Tisdale, M.J. (1991). Biochem. Pharmacol. 41 (3) 339-343).

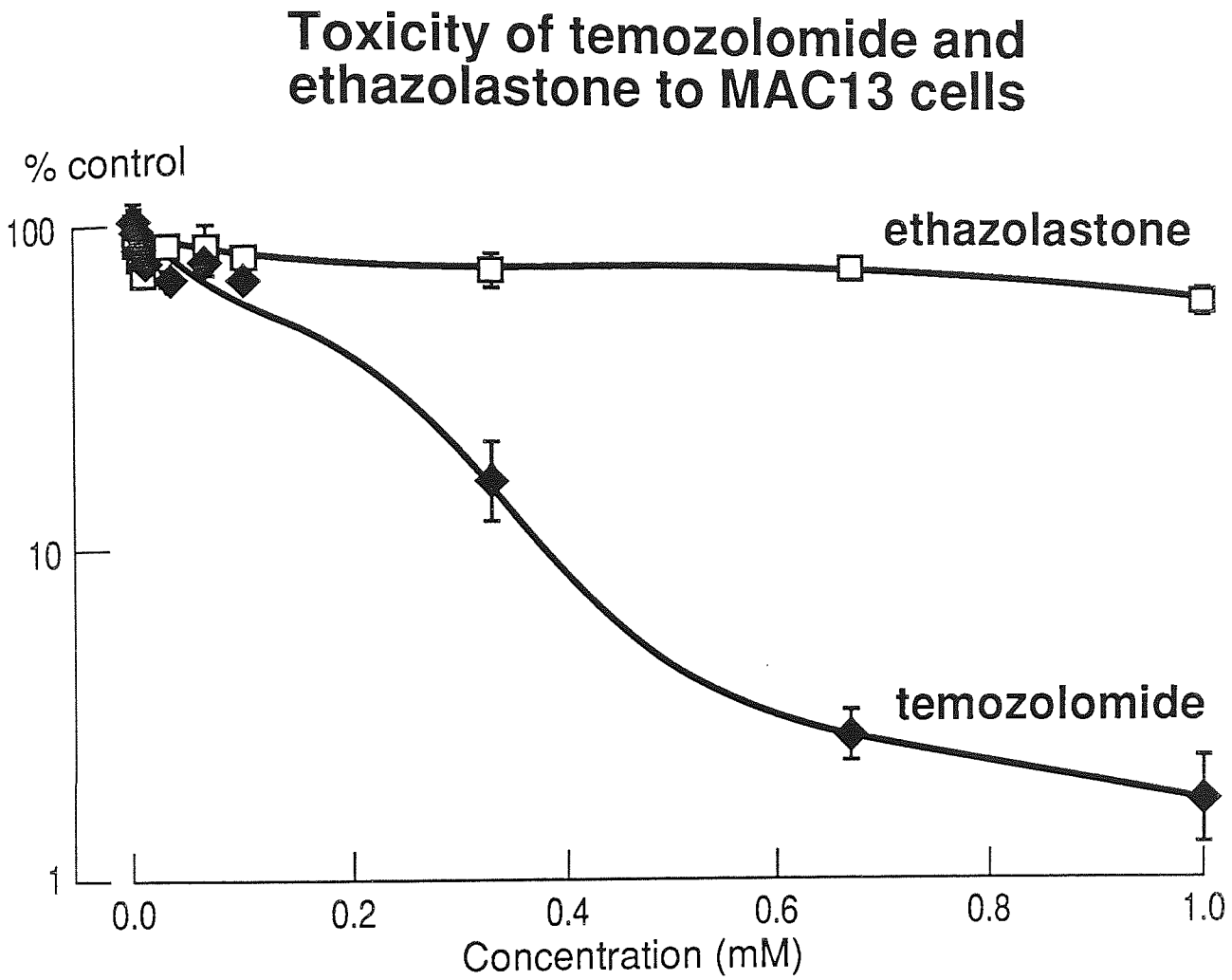


Figure 44: Sensitivity of the MAC13 cell line to the imidazotetrazinones.
2×10^4 cells were set up in a Nunc 24 well plate and were dosed with the drugs which were dissolved in DMSO, so that the final concentration in the culture medium did not exceed 1%. The cells were then counted 72 h after drug addition.
The results are expressed as the growth of the treated cell population relative to the growth of the untreated cell population and represent the mean of three experiments.

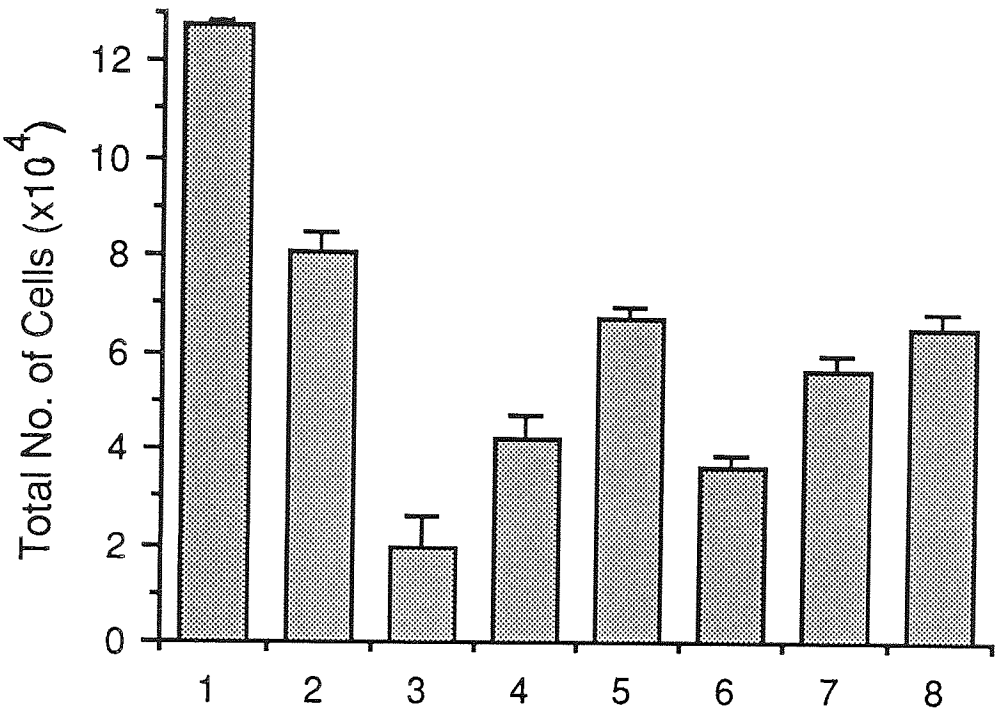


Figure 45: MAC13 cells treated with DNA extracted from GM892 cells alkylated with imidazotetrazinones.
5.0×10^4 cells were allowed to attach overnight and were then treated with 30 µg GM892 DNA co-precipitated with calcium phosphate. The cells were counted three days after treatment. The results are expressed as the mean ± SEM of three experiments.
Key: 1) Treated with the calcium phosphate precipitate but containing no DNA, 2) DNA from untreated GM892 cells, 3) DNA from GM892 cells 6 h after treatment with temozolomide, 4) DNA from GM892 cells 12 h after treatment with temozolomide, 5) DNA from GM892 cells 24 h after treatment with temozolomide, 6) DNA from GM892 cells 6 h after treatment with ethazolastone, 7) DNA from GM892 cells 12 h after treatment with ethazolastone and 8) DNA from GM892 cells 24 h after treatment with ethazolastone.

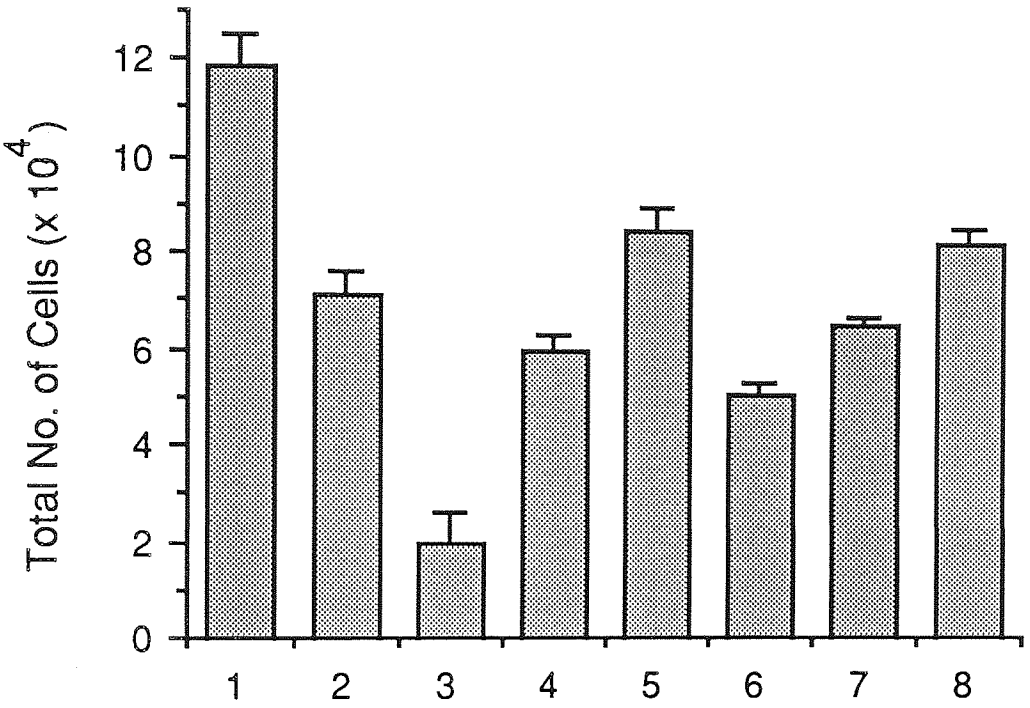


Figure 46: MAC13 cells treated with DNA extracted from Raji cells alkylated with imidazotetrazinones.
5.0×10^4 cells were allowed to attach overnight and were then treated with 30 µg Raji DNA co-precipitated with calcium phosphate. The cells were counted three days after treatment. The results are expressed as the mean ± SEM of three experiments.
Key: 1) Treated with the calcium phosphate precipitate but containing no DNA, 2) DNA from untreated Raji cells, 3) DNA from Raji cells 6 h after treatment with temozolomide, 4) DNA from Raji cells 12 h after treatment with temozolomide, 5) DNA from Raji cells 24 h after treatment with temozolomide, 6) DNA from Raji cells 6 h after treatment with ethazolastone, 7) DNA from Raji cells 12 h after treatment with ethazolastone and 8) DNA from Raji cells 24 h after treatment with ethazolastone.

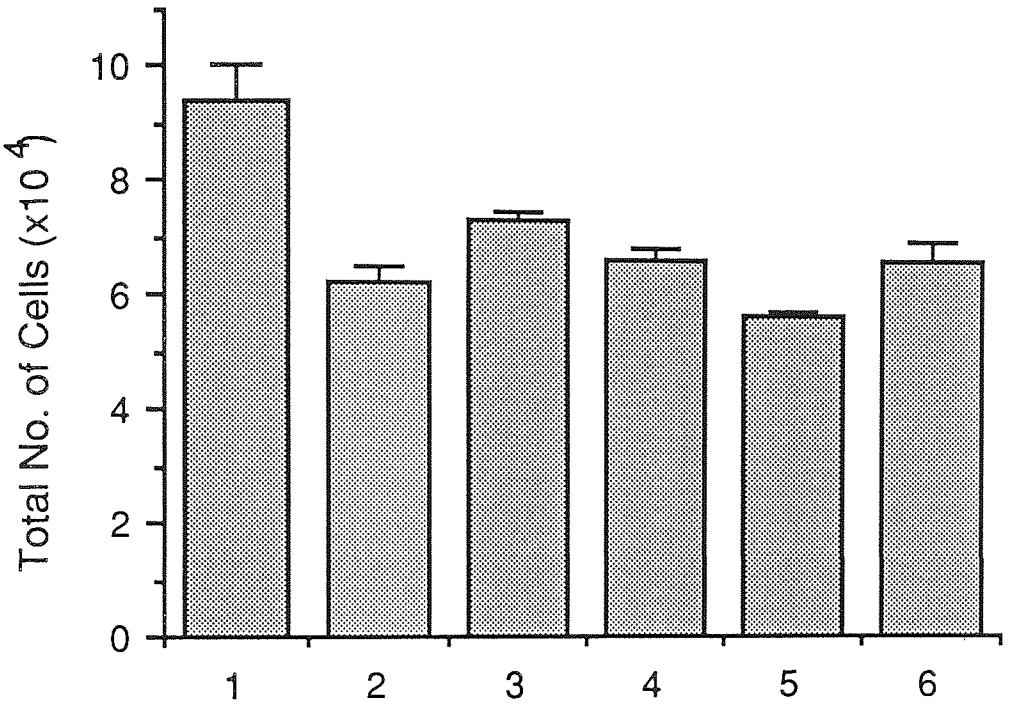


Figure 47: MAC13 cells treated with X-irradiated calf thymus DNA. 5.0×10^4 cells were allowed to attach overnight and were then treated with 30 µg X-irradiated calf thymus DNA co-precipitated with calcium phosphate. The cells were counted three days after treatment. The results are expressed as the mean ± SEM of three experiments.
Key: 1) Treated with the calcium phosphate precipitate but containing no DNA, 2) untreated calf thymus DNA, 3) 72 rads, 4) 100 rads, 5) 200 rads and 6) 400 rads.

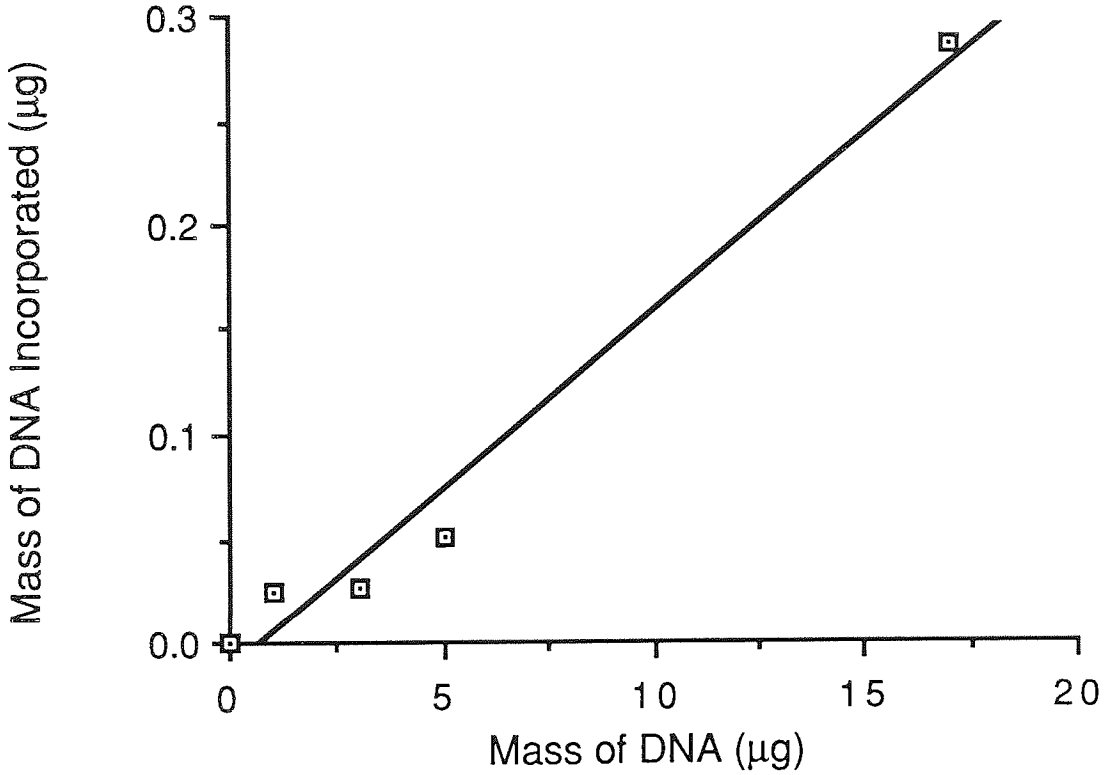


Figure 48: Determination of the level of [^{3}H] DNA incorporation into MAC13 cells when transferred by co-precipitation with calcium phosphate.
5.0×10^4 cells were allowed to attach overnight and were then treated with increasing amounts of [^{3}H] DNA by co-precipitation with calcium phosphate. After 12 h the cells were removed by trypsinisation, pelleted and extensively washed with PBS. The cells were then lysed with 0.5 ml cell lysing buffer and the amount of radioactivity was determined. Non-specific binding of the labelled DNA to the cell membrane was excluded by carrying out a control in which [^{3}H] DNA was added to the medium in which the cells were growing in the absence of calcium phosphate.
The values are the result of an experiment carried out in duplicate.

Cell Line	ID_{50} µM* (±SEM)	
	Temozolomide	Ethazolastone
Raji	206 ± 20	360 ± 30
GM892A	10 ± 7	229 ± 20

* Concentration required to give 50% inhibition of cell growth. Cells were plated at an initial density of $8X10^4$ ml^{-1} and growth inhibition was calculated from the linear part of the growth curves. Drugs were dissolved in DMSO at 10^3 times their required concentration such that the final concentration of DMSO in the culture medium did not exceed 0.1%. Clonogenic assays on either cell line were unsuccessful.

From: Bull and Tisdale (1987).

Table 16: Sensitivity of the Raji and GM892A cell lines to the imidazotetrazinones.

Treatment	$\frac{[5MC]}{[C] + [5MC]} \times 100$
No DNA (calcium phosphate only)	2.4 ± 0.0
No treatment	2.4 ± 0.0
GM892 DNA	
Temozolomide 6 h	counts too low to give an accurate estimation
Temozolomide 12 h	6.7 ± 0.6
Temozolomide 24 h	8.3 ± 0.3
Ethazolastone 6 h	5.5 ± 0.8
Ethazolastone 12 h	4.7 ± 0.2
Ethazolastone 24 h	4.8 ± 0.4
Untreated DNA	2.6 ± 0.6
Raji DNA	
Temozolomide 6 h	3.5 ± 0.6
Temozolomide 12 h	2.8 ± 0.2
Temozolomide 24 h	3.4 ± 0.4
Ethazolastone 6 h	3.5 ± 0.1
Ethazolastone 12 h	not determined
Ethazolastone 24 h	2.9 ± 0.1
Untreated DNA	3.1 ± 0.2

Table 17: The effect of temozolomide and ethazolastone-treated Raji and GM892 DNA on the enzymatic methylation of DNA when transferred by coprecipitation with calcium phosphate into MAC13 cells. Cells were labelled with [6-^{3}H]uridine immediately after treatment with the DNA/calcium phosphate precipitate. The cells were labelled for 24 h after which they were harvested for DNA isolation. The results are the mean of at least three determinations ± SEM.

Table 18. The extent of alkylation of DNA from GM892 cells treated with temozolomide or ethazolastone.

	TEMOZOLOMIDE (10μM)				ETHAZOLASTONE (229μM)			
		Overall alkylation[2] (μmol/mol DNA-P)				Overall alkylation[2] (μmol/mol DNA-P)		
	% of total DNA alkylated[1]	6h	12h	24h	% of total DNA alkylated[1]	6h	12h	24h
7-alkylguanine	70.0	6.9	8.1	11.6	24.0	54.7	63.4	73.0
O^6-alkylguanine	5.3	0.5	0.6	0.9	5.4	12.3	14.3	16.4
3-alkyladenine	9.2	0.9	1.1	1.5	4.9	11.2	12.9	14.9
Total alkyl-phosphotriester	12.0	1.2	1.4	2.0	55.0	125.4	145.2	167.2
TOTAL	100	9.9	11.6	16.5	100	228	264	304

Note: These values are based on there being no repair of these lesions. These lesions are subject to repair and therefore the true values are likely to be lower.

1 Determined by Bull (1988)

2 The overall alkylation was determined from the data of Bull and Tisdale (1987)

Table 19. The extent of alkylation of DNA from Raji cells treated with temozolomide or ethazolastone.

	TEMOZOLOMIDE (206μM)				ETHAZOLASTONE (360μM)			
		Overall alkylation[2] (μmol/mol DNA-P)				Overall alkylation[2] (μmol/mol DNA-P)		
	% of total DNA alkylated[1]	6h	12h	24h	% of total DNA alkylated[1]	6h	12h	24h
7-alkylguanine	70.0	94.5	120	94.5	24.0	71.3	71.3	71.3
O^6-alkylguanine	5.3	7.2	9.1	7.2	5.4	16.0	16.0	16.0
3-alkyladenine	9.2	12.4	15.8	12.4	4.9	14.6	14.6	14.6
Total alkyl-phosphotriester	12.0	16.2	20.6	16.2	55.0	163	163	163
TOTAL	100	135	172	135	100	297	297	297

Note: These values are based on there being no repair of these lesions. These lesions are subject to repair and therefore the true values are likely to be lower.

1 Determined by Bull (1988)

2 The overall alkylation was determined from the data of Bull and Tisdale (1987)

CHAPTER SEVEN

THE IMPORTANCE OF THE $\underline{O}^6$-POSITION OF GUANINE RESIDUES IN THE RECOGNITION AND METHYLATION OF DNA BY DNA(CYTOSINE-5)METHYLTRANSFERASE

7.1 INTRODUCTION

In eukaryotes the site of methylation of DNA by DNA(cytosine-5)methyltransferase is the 5-position of cytosine residues and the main dinucleotide in which cytosine is methylated is 5'-CG-3'. However, it has been shown that other dinucleotides in eukaryotes apart from 5'-CG-3' also contain 5-methylcytosine (Harbers et al., 1975; Wagner et al., 1985; Grafstrom et al., 1985; Nyce et al., 1986). Methylation at non-CG sequences has been regarded as minor and unimportant until the report by Woodcock et al. (1987) which showed that only 45.5% of total 5-methylcytosine was present in 5'-mCG-3' of DNA from human spleen, with 54.5% in 5'-mCA-3', 5'-mCT-3' and 5'-mCC-3'.

The sequence specificity of rat liver DNA(cytosine-5)methyltransferase has been investigated by Hubrich-Kühner et al. (1989), who showed that besides 5'-CG-3' the enzyme methylates DNA cytosines in 5'-CA-3', 5'-CT-3' and 5'-CC-3' sequences. A comparison of natural and synthetic substrates by these authors demonstrated that the enzyme favours the methylation of the dinucleotides in the order: C-I>C-G>C-A>C-T>C-C. Since C-G is the major natural dinucleotide that is methylated by DNA(cytosine-5)methyltransferase, a comparison of the possible interactions in the major groove of DNA between guanine and this enzyme were made with dinucleotides. An O^6-carbonyl group and an

N-7 group are both present in the major groove of DNA for inosine and guanine. An N-7 group is present in both adenine and guanine and the O^4-carbonyl group of thymine is very close to the position that the O^6 group of guanine would take up. Cytosine shares the least similarity between the other bases.

Nitrogen and oxygen atoms in DNA are reactive sites for a number of chemical alkylating agents, such as environmental N-nitroso compounds and others which are used in antitumour chemotherapy. Of the four major DNA bases, guanine is generally the most reactive to the alkylating carcinogens (Singer and Grunberger, 1983). The N-7 and O^6 position of guanine generally receive the greatest proportions of alkylations. This is certainly true *in vitro* for MMS, EMS, MNU and ENU (Beranek *et al.*, 1980; Lawley, 1984) and the imidazotetrazinones, temozolomide and ethazolastone (Bull, 1988). It was suggested by the work of Hubrich-Kühner *et al.* (1989) that the N-7 and the O^6 position of guanine are important points of interaction between DNA(cytosine-5)methyltransferase and DNA in determining whether the adjacent cytosine will be methylated. Therefore, modification of guanine by alkylating agents at these positions is likely to impair their ability to behave as substrates for DNA(cytosine-5)methyltransferase.

Potent inhibition of DNA(cytosine-5)methyltransferase *in vitro* has been demonstrated with poly[G] and poly[dG].poly[dC] (Drahovský and Morris, 1972); Bolden *et al*., 1984) which illustrates the importance of guanine as a binding site for DNA(cytosine-5)methyltransferase.

Studies have been conducted to investigate the features of DNA responsible for the recognition of specific cytosines in 5'-CG-3' sequences. In particular, the effect of specific polynucleotides, which are not substrates for DNA(cytosine-5)methyltransferase, on the enzymatic methylation of *M.lysodeikticus* DNA has been investigated. The aim of this approach was to identify functional groups responsible for enzyme recognition and binding and then to investigate whether modification of these functional groups would affect methyltransferase recognition.

7.2 METHODS

7.2.1 DNA(cytosine-5)methyltransferase assay

a) For the study of the inhibition of *de novo* methylation *in vitro* by synthetic polynucleotides.

Each assay contained 20 μg of *M.lysodeikticus* DNA (2.0 mg/ml in water), 1.0 μCi *S*-adenosyl-L-[methyl-^{3}H]methionine (83 Ci/mmol) and 10 μl of DNA(cytosine-5)methyltransferase preparation made up to 100 μl with DNA methylase assay buffer. After incubation at 37°C for 4 h in a shaking water-bath, the reaction was stopped by adding 40 μg calf thymus DNA (2.0 mg/ml) to act as a carrier and 200 μl of 1.0 M NaOH. The mixtures were then heated to 65°C for 20 min in a water-bath, after which they were put on ice and the DNA was precipitated by adding 80 μl of 5.0 N $HClO_4$. After at least 1 h the DNA was filtered onto a Whatman GF/C glass microfibre filter and washed with cold 5% TCA (w/v) and absolute ethanol. After air drying the filters overnight, the radioactivity was determined by adding 8 ml of toluene/PPO/POPOP scintillant and counting on a Packard Tri-Carb 2000CA liquid scintillation counter.

Unless otherwise stated, 10 μg of polynucleotide (dissolved in water) was added to the assay.

b) For the study of enzymatic methylation of cytosine in synthetic polydeoxyribonucleotides.

This procedure was based upon the method described by Bolden *et al*. (1985).

Each assay contained 20 μg of polydeoxyribonucleotide (2.0 mg/ml in water), 1.0 μCi S-adenosyl-L-[methyl-^{3}H] methionine (83 Ci/mmol) and 10 μl of DNA(cytosine-5)methyltransferase preparation made up to 100 μl with DNA methylase buffer. After incubation at 37°C for 4 h in a shaking water-bath the samples were cooled to 0°C and unlabelled SAM was added to a final concentration of 1.5 mM. The entire reaction mixture was then spotted onto 2.1 cm discs of Whatman DE81 (DEAE-cellulose) paper and air dried. The discs were washed 5 times in 20 mM Na_2HPO_4, pH 8.4, twice in water and once in ethanol:ether (95:5) (v/v). All washes were carried out at room temperature for 5 min. After air drying the filters overnight, the radioactivity was determined by adding 8 ml of the toluene/PPO/POPOP scintillant and counting on a Packard Tri-Carb 2000CA liquid scintillation counter.

7.2.2 Synthesis of oligonucleotides

The oligonucleotides were synthesised by Dr G Margison and Dr M Mackett of the Paterson Institute for Cancer Research, Manchester by solid state methodology using β-cyanoethyl-N,N-diisopropyl phosphoramidite intermediates (Cruachem, Glasgow), (American Bionetics Inc., Hayward, California, USA) as described by Borowy-Borowski and Chambers (1987)

and Atkinson and Smith (1985). The oligonucleotide synthesiser used was a Du Pont Coder 3000. They were synthesised on a 1 μmol scale.

The oligonucleotides were released from the solid support and deprotection was carried out by treating the oligonucleotide in a sealed tube with 10% DBU (1,8-diazabicyclo [5.4.0] undec-7-ene) in methanol at room temperature for 3 days or 5 days if containing O^6-methylguanine. The oligonucleotides were then precipitated with diethyl ether and then reprecipitated with 0.1 volumes of 2.5 M sodium acetate and 3 volumes of absolute ethanol. The oligonucleotides were then washed three times with 80% ethanol. The oligonucleotide was then redissolved in water and reprecipitated a further time.

The oligonucleotides were shown to be pure by labelling with [^{32}P]ATP using T4 polynucleotide kinase (Boehringer Corporation Ltd., Lewes, Sussex), and then running on a 20% urea:acrylamide gel, which was then autoradiographed.

7.2.3 Annealing of oligonucleotides

Equimolar concentrations of the appropriate oligonucleotides were mixed together in a 1.5 ml microfuge tube. The equimolar concentrations were calculated from the following extinction coefficients for the nucleotides at 260 nm (guanine, 12100;

cytosine 7050; thymine 8400; adenine 15200; O^6-methylguanine 5200). The mixtures were placed in a water-bath at 90°C and maintained at that temperature for 10 min after which they were very slowly cooled down to room temperature in an insulated container.

7.2.4 Experiment to determine the level of protein/polynucleotide binding.

Labelling of polynucleotides.

The method for labelling the 5' ends of polynucleotides with [^{32}P] was carried out as described by Maniatis et al. (1982).

In a 1.5 ml Eppendorf tube was placed 20 µg of the polynucleotide (2.0 mg/ml) dissolved in water, 20 units of the T4 polynucleotide kinase, 50 µCi [gamma-^{32}P] ATP (3000 Ci/mmol) and 10 µl of 10x kinase buffer I made up to 50 µl with water. After incubation at 37°C for 30 min, the reaction was stopped by adding 2 µl of 0.5 M EDTA and the mixture was extracted once by adding 50 µl phenol and vortexing for 2 min until an emulsion formed. After centrifuging for 5 min in a microfuge at room temperature, the upper aqueous phase was removed and transferred to a fresh Eppendorf tube and the polynucleotide was precipitated from this aqueous phase by adding 100 µl cold absolute ethanol. After air drying, the polynucleotide was redissolved in 50 µl of TE (pH 7.9). The labelled polynucleotide was

then separated from unincorporated [gamma-^{32}P]ATP using a Nensorb 20 nucleic acid purification cartridge.

Purification of polynucleotides using Nensorb 20 purification cartridges.

a) Priming the Column

The cartridge was clamped to a secure support and the loose packing inside was washed with 2 ml of absolute methanol. A 5 ml disposable plastic syringe was then filled with air and at constant gentle pressure the methanol was pushed through the cartridge (this prewets the sorbent). On the top of the column bed was applied 2 ml of reagent A, and using the 5 ml syringe the liquid was pushed through until the liquid level was just at the top of the bed.

b) Sample loading

To the sample was added 200 µl reagent A which was then gently mixed. The sample was then applied directly to the top of the column bed (1 ng to 20 µg can be applied). With the adaptor and syringe in position, pressure was applied to the syringe until the level of the liquid just reached the top of the resin bed.

c) Sample wash

This procedure removed low molecular weight compounds, unincorporated radioactive nucleotides and excess salt.

On the top of the column bed was added 3 ml of reagent A and using the adaptor and syringe, the liquid was pushed through at a rate of 1-2 drops/s. This was then repeated replacing reagent A with deionised water.

d) Nucleic Acid Elution

On the top of the column was applied 1 ml of 50% methanol, and with the adaptor and syringe in position, the nucleic acid was eluted from the column bed by gentle force whilst 200 μl fractions were collected. The fractions containing nucleic acid were then determined by liquid scintillation counting.

Protein/polynucleotide binding assay

The method for the determination of protein binding to polynucleotide was based on that described by Tisdale (1988).

Each assay contained 5.0 μg labelled [^{32}P] polynucleotide with 10 μl of the DNA(cytosine-5)methyltransferase preparation (approximately 1 μg protein) made up to a total volume of 200 μl with incubation buffer. The polynucleotide had an activity of approximately 100,000 cpm. The binding reaction was performed at 37^{o}C for 20 min in a

shaking water-bath. The complexes were then incubated at 4°C prior to being washed onto a Millipore HA 0.45 μM filter with the following agents of increasing dissociating strength - incubation buffer, 1.0M NaCl, 0.6% sarkosyl + 0.5M NaCl or 0.4% SDS. The filters were then air dried and 8.0 ml of water was added and the radioactivity was determined by counting using a Packard Tri-Carb 2000CA liquid scintillation counter.

7.2.5 Statistical analysis

All results are expressed as mean ± standard error of the mean (SEM) for at least three separate determinations.

7.3 RESULTS

The DNA(cytosine-5)methyltransferase preparation used in these experiments was that extracted from the murine L1210 tumour, described in section three. As noted previously there are a number of limitations associated with this preparation which must be taken into consideration. It has been suggested by Adams et al (1990) that all DNA(cytosine-5)methyltransferases are probably high molecular weight enzymes of molecular weight approximately 190 kDa and therefore, it is likely that the enzyme used in these studies was a degraded form of enzyme as there was no material of this molecular weight 190 kDa present.

It has been observed by Adams et al. (1983) that mild proteolysis can remove or alter some 'enzyme domain' with the result that the de novo activity of the methyltransferase towards double-stranded DNA increases. A model for DNA(cytosine-5)methyltransferase was proposed by Adams et al. (1986) in which the enzyme was envisaged to contain two domains. Domain A binds to the daughter (unmethylated) strand of newly replicated DNA and domain B to the parental (methylated) strand. Domain A is the active site of the enzyme, whilst domain B is an allosteric site, which is either activated when it meets a methyl CG or inhibited when it encounters an unmethylated CG in the parental strand. Domain B is therefore responsible for the maintenance activity of

the methyltransferase. If domain B is lost such as through proteolysis, the enzyme freed from inhibition will methylate DNA de novo. Adams et al. (1983) proposed that DNA(cytosine-5)methyltransferase may switch from the normal maintenance mode to a de novo activity by specific proteolysis in vivo.

However, in the following studies the de novo activity of an enzyme of molecular weight approximately 100 kDa extracted from the murine L1210 tumour was used. It remains a possibility that studies on the de novo activity of this enzyme may reveal properties that are not normally present in vivo, especially since the enzyme was extracted from a tumour. However, studies on the de novo activity of DNA(cytosine-5)methyltransferase do have some importance and are worth investigating since this is the only mechanism by which a new methylation pattern can be introduced. This may be important in gametogenesis and early development when cells show evidence of higher levels of de novo methylation (Jahner et al., 1982).

The inhibition of de novo methylation in vitro by synthetic polynucleotides.

A study of this type has been reported by Bolden et al. (1984) using a partially purified HeLa cell DNA(cytosine-5)methyltransferase. However, this was a study of the maintenance methyltransferase activity

using a hemi-methylated DNA template, whilst the work reported here investigates inhibition of the de novo methyltransferase activity. Bolden et al. (1984) also make no attempt to identify the functional groups in the polynucleotides responsible for the inhibition and the significance in terms of modification by alkylating agents was not discussed. The aim of this section was to identify the functional groups in the polynucleotides which inhibit DNA(cytosine-5)-methyltransferase and to investigate the effect that modification may have on this inhibition.

Various polynucleotides were placed in competition with the M.lysodeikticus DNA, (10 μg being added to the standard 20 μg of M.lysodeikticus DNA), in order to determine whether they had any inhibitory activity. As can be seen from fig 49, calf thymus DNA had no significant inhibitory effect, until >10 μg was added to the standard DNA(cytosine-5)methyltransferase assay. This is presumed to be due to the fact that there are relatively few binding sites for the enzyme in calf thymus DNA compared to M.lysodeikticus DNA. As can be seen from table 20, the pyrimidine polynucleotides, poly[C] and poly[U] had no effect on DNA(cytosine-5)methyltransferase activity, whilst the purine polynucleotides poly[G], poly[I] and poly[X] completely abolished any DNA(cytosine-5)methyltransferase activity. The fact that the purine polynucleotide poly[A] had no effect

was interesting and suggested some form of structure activity relationship. The potency of these purine polynucleotides as inhibitors of DNA(cytosine-5)-methyltransferase is illustrated in fig 50, with an ID_{50} for poly[G] and poly[I] occurring at 4 μg/ml, whilst poly[X] was an even more potent inhibitor with an ID_{50} occurring at 0.4 μg/ml. However, poly[A] had no effect even at a concentration of 100 μg/ml. Fig 51 shows that the inhibition is characteristic of first order kinetics.

By examination of the structure of these polynucleotides a structure activity relationship becomes apparent, see fig 52. The polynucleotides inhibit DNA(cytosine-5)methyltransferase only when they consist of purines and only when a carbonyl group is present at the 6 position. However, the fact that poly[X] produced the most potent inhibition suggests that the carbonyl group at the 2 position also makes some contribution to the binding of DNA(cytosine-5)methyltransferase. The carbonyl at the 2 position is however only available to the DNA(cytosine-5)methyltransferase protein because poly[X] is single-stranded. DNA(cytosine-5)methyl-transferase exerts its action in the major groove of DNA, into which the carbonyl at the 6 position of guanine protrudes. But, if xanthine existed in a DNA duplex the carbonyl at the 2 position would be

unavailable for DNA(cytosine-5)methyltransferase binding, by virtue of it being in the minor groove of DNA.

Similar results were observed for the polydeoxyribonucleotides (table 20), poly[dC] having no effect whilst poly[dG] completely abolished any DNA(cytosine-5)methyltransferase activity, but the complete range was not commercially available. However, it was also interesting to note that the double-stranded copolymers also produced the same result; poly[dA].poly[dT] having no effect, whilst poly[dG].poly[dC] once again abolished any methyltransferase activity. Poly[d(G-C)].poly[d(G-C)] is a substrate for DNA(cytosine-5)methyltransferase and therefore could not be assessed in this assay.

The importance of the carbonyl group at the 6 position of the purine to cause inhibition of DNA(cytosine-5)methyltransferase was investigated further. The following oligonucleotides, poly[d(O^6MG)]$_{20}$ and poly[d(C.O^6MG)]$_{10}$ were synthesised by Dr G Margison and Dr M Mackett of the Paterson Institute, Manchester. Table 20 shows that in competition with M.lysodeikticus DNA poly[d(C.O^6MG)]$_{10}$ causes the methyltransferase activity to drop to 67±3%. There were not sufficient quantities of poly[d(O^6MG)]$_{20}$ to place 10 μg in an assay and therefore the value of 88% activity in table 20 is for 0.9 μg of oligonucleotide. However,

fig 53 compares the inhibitory effect of poly[dG]$_{20}$ with poly[d(O^6MG)]$_{20}$, which shows that 0.2 μg of poly[dG]$_{20}$ is capable of causing a 50% inhibition of the methylation of 20 μg of M.lysodeikticus DNA. In contrast, equivalent amounts of poly [d(O^6MG)]$_{20}$ caused no detectable inhibition of the methylation reaction. Therefore, the inhibition of DNA(cytosine-5)methyltransferase observed with the various polynucleotides and polydeoxyribonucleotides was due to a carbonyl group at the 6 position of a purine and this probably occurs due to binding of the enzyme by hydrogen bonding to this potential recognition site.

As the length of the polynucleotide may have some effect, the number of base pairs in the various polynucleotides and polydeoxyribonucleotides was obtained from the suppliers (table 21). There was no correlation between the length of the nucleic acid and the degree of inhibition. In order to confirm whether the length of the nucleic acid had any effect on the inhibition, poly[G]$_{400}$, poly[dG]$_{600}$ and poly[dG]$_{20}$ were assessed in a competition assay and as can be seen from the dose response curve in fig 54, there was no significant difference between the degree of inhibition produced. The ras antisense oligonucleotide (5'-GGGCCGGGGCCGAGGCCGGC-3'), also had no inhibitory effect when in competition with M.lysodeikticus DNA. Although it contains a high proportion of guanines (13/20), there is only a

maximum of 4 guanines adjacent to each other. Presumably strings of guanines are required for binding to occur. Also as can be seen in table 20, GMP caused only slight inhibition even at 10 mM and therefore a polymer is required for inhibition, but as yet the minimum chain length has not been determined.

Polynucleotide/protein binding assay

The system used to assess the stability of the complex formed between the polynucleotide and the protein in the methyltransferase preparation was that used by Tisdale (1988). The polynucleotides were labelled 5' with [^{32}P]ATP using T4 polynucleotide kinase. The polynucleotide (5 μg) was then incubated for 20 min with 10 μl of the enzyme preparation (approximately 1 μg protein) during which time proteins in the methyltransferase preparation form complexes with the polynucleotide. An assessment of the stability of the polynucleotide/protein complex was then made by washing the incubation mixture onto a Millipore HA 0.45 μm filter with solutions of increasing dissociating strength (incubation buffer, 1.0M NaCl, 0.6% sarkosyl + 0.5M NaCl or 0.5% SDS). The amount of polynucleotide in terms of radioactivity which was then bound to the filter was expressed as a percentage of the total amount of polynucleotide and the results are shown in fig 55.

A control was carried out in which 5 μg of the labelled polynucleotide was washed onto the filter with incubation buffer and in this case all the polynucleotide was washed through the filter. Therefore, the retention on the filters was dependent on the formation of a complex between the polynucleotide and the protein in the methyltransferase preparation.

The most stable complex was formed between poly[G] and the nuclear proteins in the enzyme preparation. The percentage of polynucleotide bound after washing with incubation buffer was 44%. After washing with 1 M NaCl the binding of poly[G] was reduced to 20%, whilst washing with 0.6% sarkosyl + 0.5M NaCl reduced binding to 9%. After washing with 0.5% SDS the binding was reduced to <1%.

The next most stable complex was formed between poly[X] and the nuclear proteins in the methyltransferase preparation. The percentage of polynucleotide bound after washing with incubation buffer was 26%. After washing with 1 M NaCl the binding of poly[X] was reduced to 4%, whilst washing with 0.6% sarkosyl + 0.5 M NaCl reduced binding to 2%. After washing with 0.5% SDS binding was reduced to <1%.

Poly[I], poly[A] and poly[C] formed complexes of similar strength with the nuclear proteins in the methyltransferase preparation. The percentage of polynucleotide bound after washing with incubation buffer was 17%, 13% and 14% respectively. After

washing with 1 M NaCl the binding was reduced to 9%, 6% and 10% respectively, whilst washing with 0.6% sarkosyl + 0.5 M NaCl reduced binding to 8%, 4% and 7% respectively. After washing with 0.5% SDS binding was reduced to 3%, 1% and 3% respectively.

The least stable complex was formed between poly[U] and proteins in the methyltransferase preparation. The percentage of polynucleotide bound after washing with the incubation buffer was 7%. After washing with 1 M NaCl or 0.6% sarkosyl + 0.5 M NaCl the binding was reduced to 5%. After washing with 0.5% SDS the binding was reduced to <1%.

Therefore, poly[G] and poly[X], which were inhibitors of DNA(cytosine-5)methyltransferase, formed more stable complexes with the proteins in the enzyme preparation than poly[A], poly[C] and poly[U], which were not inhibitors of DNA(cytosine-5)methyltransferase. However, poly[I] which inhibits DNA(cytosine-5) methyltransferase with the same degree of potency as poly[G], forms only a weak complex with the proteins in the DNA(cytosine-5)methyltransferase preparation. There is no correlation between the extent of complex formation and the potency with which the polynucleotides inhibit DNA(cytosine-5)methyltransferase, i.e. in terms of the inhibition of DNA(cytosine-5)methyltransferase poly[X]>poly[G] = poly[I]>poly[A] whilst the extent of complex formation between the poly-

nucleotide and the proteins in the methyltransferase preparation is poly[G]>poly[X]>poly[I]>poly[A]. This assay has its limitations in that the methyltransferase preparation is by no means homogenous and other proteins may be responsible for the observed binding. In view of the purity of the methyltransferase preparation no conclusions can be drawn from these results.

The effect of O^6-methylguanine residues on the enzymatic methylation of cytosine in synthetic polydeoxyribonucleotides.

It has been shown that guanine residues and in particular the O^6 position of guanine is an important recognition and binding site for DNA(cytosine-5)methyltransferase. In addition, the carbonyl group at the 6 position of guanine was the only functional group identified as a binding site using polynucleotides as inhibitors and as a consequence, the effect of O^6-methylguanine on the enzymatic methylation of cytosine in synthetic polydeoxyribonucleotides was the only modification investigated.

The effectiveness of guanine residues in binding DNA(cytosine-5)methyltransferase can be demonstrated from the results presented in fig. 53 which show that 0.2 μg of the oligonucleotide poly[dG]$_{20}$ is capable of causing a 50% inhibition of the methylation of 20 μg of M.lysodeikticus DNA. In contrast, equivalent

amounts of poly[dO^{6}MG]$_{20}$ caused no detectable inhibition of the methylation reaction. This suggests that formation of such a lesion in DNA may lead to reduced binding of the DNA(cytosine-5)-methyltransferase with the result that the adjacent cytosines are not methylated.

In order to examine this possibility, pairs of oligonucleotides have been synthesised using the phosphoramidite method. The oligonucleotides were chosen to contain -CG- rich sequences capable of methylation by DNA(cytosine-5)methyltransferase, and in one member of the pair, all guanine residues in -CG- linkage were replaced by an O^6-methylguanine residue.

The alternating co-polymers, poly[d(G-C)]$_{10}$ and poly[d(O^{6}MG-C)]$_{10}$ were synthesised. When this work was initiated, it was not known whether any of these oligonucleotides would behave as substrates for DNA-(cytosine-5)methyltransferase, therefore a 26 mer reported by Bolden et al (1986a) to be an excellent substrate was synthesised: 5'-CCGGCCATTACGGATCCG-TCCTGGGC-3'. Finally, part of the promoter region of the human Harvey ras proto-oncogene (Ishii et al., 1985), a 30 mer from 517-547 5'-CCCGCCCCGCCCCGGCCTCG-GCCCCGGCCC-3' was synthesised.

The oligonucleotides were assessed for their ability to accept [^{3}H]methyl groups from S-adenosyl-L-(methyl-^{3}H)methionine during incubation with

DNA(cytosine-5)methyltransferase, alone or after annealing to shorter complementary strands containing only normal bases. The de novo activity of the DNA(cytosine-5)methyltransferase was assayed as opposed to the maintenance activity, for a number of reasons. Firstly, the inhibitory action of polynucleotides studied earlier in the section was with the de novo activity of the methyltransferase. Therefore, to make any comparisons between the two sets of experiments valid the same activity of the enzyme should be studied. Secondly, in order to study maintenance activity it would have been necessary to synthesise oligonucleotide duplexes which were hemi-methylated in one strand with respect to 5-methylcytosine. The choice of which strand and which cytosines to replace with 5-methylcytosine would complicate the experimental situation and would make the interpretation of the results very difficult.

In the case of both the 30 mer ras sequence and the 26 mer Bolden sequence containing only normal bases, the incorporation of methyl groups from SAM transferred by DNA(cytosine-5)methyltransferase plateaued at approximately 10 μg (fig 56). Consequently, all the following experiments were carried out using an excess of oligonucleotide (20 μg), in

order that any differences observed for methyl incorporation were not due to insufficient oligonucleotide.

The ability of the oligonucleotides to behave as substrates for DNA(cytosine-5)methyltransferase are shown in table 22. The results were compared with M.lysodeikticus DNA which contains a high level of -CG- and is a good substrate for the methyltransferase. Both the 26 mer Bolden sequence (373) and the 30 mer ras sequence (354) contain only normal bases and had high acceptance ability, with the latter incorporating three times as many methyl groups as an equivalent amount of M.lysodeikticus DNA (table 22). They contain three and five potential methylation sites (5'-CG-3') respectively, and no attempt has been made to determine which -CG- pairs were methylated by the methyltransferase.

In contrast, the antisense sequences ras 20 mer (379) and Bolden 16 mer (380) did not act as substrates for de novo methylation. Bolden et al (1986a) observed that the 26 mer strand, antisense to oligonucleotide number 373 used here was a substrate for HeLa DNA(cytosine-5)methyltransferase but possessed only 20% of the capacity of the complementary strand to accept methyl groups. This suggests that the Bolden antisense 16 mer (380) used

in these experiments was too short for enzyme binding or the enzyme can distinguish between two types of -CG- pairs in DNA.

These results were confirmed by denaturing gel electrophoresis and fluorography (fig 56). The 26 mer Bolden sequence (lane 2) and the 30 mer ras sequence (lane 7) were shown to contain radioactivity at the respective positions. There appears to be more radioactivity associated with the ras sequence, although a quantitative assessment of the gel was not made. There was some radioactivity associated with higher molecular weight material for both the 26 mer Bolden sequence (lane 2) and the 30 mer ras sequence (lane 7). This may be due to self annealing of the oligonucleotides, which remains annealed despite the denaturing conditions. There was no radioactivity associated with the 16 mer Bolden antisense strand (380) or the 20 mer ras antisense strand (379) in fig 57 (lane 4 and 9 respectively).

When the Bolden 26 mer (373) was hybridised to the antisense 16 mer (380), the substrate activity towards DNA(cytosine-5)methyltransferase was increased, with greater than five times the number of methyl groups incorporated. In this case radioactivity was incorporated into both the 26 mer (373) and the 16 mer (380) as determined by denaturing gel electrophoresis and fluorography (fig 57). This suggests that the complementary sequence becomes

methylated by maintenance methylation after de novo methylation of the primary sequence. However, when the ras 30mer (354) was hybridised to the antisense 20 mer (379), the substrate activity towards DNA(cytosine-5)methyltransferase was decreased with greater than seventeen times less methyl groups being incorporated.

Radioactivity was incorporated into both the 30 mer (354) and the 20 mer (379) but to a lesser extent, as determined by denaturing gel electrophoresis and fluorography (fig 57). In this gel (lane 10), there was some radioactivity associated with higher molecular weight material, which is likely to be oligo nucleotides which remain annealed despite the denaturing conditions. This suggests that the complementary sequence becomes methylated although to a very low extent, by maintenance methylation after de novo methylation of the primary sequence.

The fact that the annealed ras duplex (354/379) was a poorer substrate than the 30 mer ras sequence (354), was difficult to explain. This occurs despite the complementary sequence (379), which is not a substrate by itself, becoming methylated after annealing to 354. One possible explanation was that the high proportion of guanines (13/20) in the ras antisense strand bind to the methyltransferase and cause the inhibition. However, this was unlikely

since the ras antisense strand (379) was shown not to inhibit the transfer of methyl groups from SAM to M.lysodeikticus DNA by DNA(cytosine-5)methyltransferase (table 20).

In contrast to these results, the replacement of guanine in all 5'-CG-3' positions with O^6-methylguanine in the 30 mer ras sequence (309) completely destroyed the activity of the oligonucleotide as a substrate for DNA(cytosine-5)methyltransferase, even after annealing to the normal complementary strand (379). This result was confirmed by denaturing gel electrophoresis and fluorography in which no radioactivity was associated with these oligonucleotides.

The replacement of guanine in all 5'-CG-3' positions with O^6-methylguanine in the 26 mer Bolden sequence (312) drastically reduced the activity of the oligonucleotide as a substrate for DNA(cytosine-5)methyltransferase. A weak band of radioactivity was detected associated with the 26 mer, when analysed by denaturing gel electrophoresis and fluorography (fig 57, lane 3). The 26 mer Bolden sequence containing O^6-methylguanine (312) did not show any ability to behave as a substrate for DNA(cytosine-5)methyltransferase (table 22) in the methyltransferase assay. Presumably, the level of radioactivity was too close to the background to allow any discrimination.

After annealing the 26 mer Bolden sequence containing $\underline{O}^6$-methylguanine (312) to the normal complementary strand (380), the duplex did not behave as a substrate as determined by the methyltransferase assay (table 23). However, analysis by denaturing gel electrophoresis and fluorography (fig 57, lane 6) revealed a weak band at 16 bases and a relatively strong band at 17 bases. Therefore, radioactivity appears to be associated with the 16mer (Bolden antisense, 380) despite no radioactivity being associated with the 26 mer sense strand containing $\underline{O}^6$-methylguanine (312). One can therefore merely make the observation that the 16mer antisense Bolden sequence (380) is not a substrate by itself, but becomes a substrate when annealed to the Bolden sense-strand containing normal bases (373) or $\underline{O}^6$-methylguanine (312). In the latter case, it was a poor substrate as methylation would not occur through the maintenance methyltransferase activity. The puzzling observation was that a relatively strong band appeared at 17 bases (fig 57, lane 6), despite there being no oligonucleotide of that length in the reaction. This may represent an unknown activity of the DNA(cytosine-5)methyltransferase which warrants further investigation, but as yet no explanation can be made.

Poly[d(C-G)]$_{10}$ and poly[d(C.O^{6}MG)]$_{10}$ were shown not to behave as substrates for DNA(cytosine-5)methyltransferase in the methyltransferase assay (table 22). These oligonucleotides were not investigated any further.

Does O-6 alkylation reduce complementation/self complementation.

The ras and Bolden sequences may have a double-stranded structure to a certain extent, due to a limited degree of self complementation. Therefore, another explanation for the data presented in this section may be that the presence of an O^6-methyl-guanine group reduces the ability of the oligonucleotides to anneal, and it may be that the enzyme can consequently only methylate double-stranded DNA. The ras and Bolden sequences are quite self complementary and with the high concentrations used they may anneal to themselves. It was therefore important to exclude the possibility that O^6 alkylation simply prevents self annealing to occur to produce double-strand regions, consequently preventing it from being a substrate. This would ideally be performed by determining the melting temperatures for the annealed oligonucleotides, but as a temperature controlled spectrophotometer was not available another approach had to be taken.

It is generally thought that $\underline{O}^6$-methylguanine pairs with thymine rather than cytosine (reviewed by Swann, 1990). However, using a set of oligonucleotide duplexes in which $\underline{O}^6$-methylguanine was situated opposite adenine, guanine, cytosine or thymine, Gaffney and Jones (1989) observed that $\underline{O}^6$-methylguanine forms the most stable base pair with cytosine rather than thymine.

Table 24 illustrates the results of an experiment in which the single-stranded oligonucleotides and the annealed oligonucleotides were heated for 10 min at 100°C and then cooled on ice and the change in absorbance at 260 nm was measured.

The ras normal, ras $\underline{O}^6$ sequences and the Bolden normal sequences all increase in absorbance after cooling, which suggests that they all form some double-stranded regions and that inhibition of self complementation does not occur. The change in absorbance for the $\underline{O}^6$ alkylated ras sequence was slightly greater than the ras normal sequence, which suggests that more self complementation occurred for the ras $\underline{O}^6$ sequence and not vice versa, as would be predicted if inhibition of self complementation was the explanation for methyltransferase inhibition. The change in absorbance for ras normal and annealed ras were similar, as were ras $\underline{O}^6$ and ras $\underline{O}^6$ annealed, which would not be the case if methyltransferase

inhibition occurred as a result of an inhibition of annealing. Therefore, a decrease in the level of complementation was not observed with O^6-methylguanine in these experiments.

7.4 DISCUSSION

The binding characteristics of DNA(cytosine-5)methyltransferase have been investigated using synthetic polynucleotides and polydeoxyribonucleotides as competitive inhibitors of the *de novo* methylation of *M.lysodeikticus* DNA. *De novo* methylation of DNA proceeds at a rate only 3 to 4% of that of maintenance methylation (Bolden *et al.*, 1984). The methyltransferase methylates the *C*-5 position of cytosine in the preferred recognition sequence 5'-CG-3', but the rat liver methyltransferase has also been shown to methylate 5'-CA-3', 5'-CT-3' and 5'-CC-3' (Hubrich-Kühner *et al.*, 1989). The results presented suggest that the presence of guanine is an important binding site for DNA(cytosine-5)methyltransferase. This was first suggested by Drahovský and Morris (1972) after they discovered that poly[dG].poly[dC] was a potent inhibitor of *de novo* methylation *in vitro*. Although these workers showed that poly[dI].poly[dC] was as inhibitory as poly[dG].poly[dC] and that the amino group of guanine appears not to be required for binding, they did not make any further attempt to identify the functional group actually responsible for binding.

The carbonyl group at the 6 position of guanine has been implicated as being an important binding site for DNA(cytosine-5)methyltransferase, by the fact that poly[A] had no inhibitory activity whilst

poly[G], poly[I] and poly[X] all destroyed the activity at a concentration of 10 μg/ml. Bolden et al. (1984) made a study of the inhibition of maintenance methyltransferase activity by polynucleotides in vitro and showed that poly[G] and poly[dC].poly[dG] were potent inhibitors of maintenance DNA methyltransferase, whilst poly[A], poly[U] and poly[C] had no significant inhibitory activity. These results are in agreement with the inhibition of de novo methyltransferase activity reported in this thesis.

However, the most striking inhibition observed by Bolden et al. (1984) was observed with poly[dA].poly[dT], despite the fact that poly[dA]. poly[dT] and poly[d(A-T)] by themselves caused no inhibition. Contrary to this observation, poly[dA].poly[dT] was shown in my system not to inhibit the de novo methyltransferase activity. Bolden et al. (1984) were unable to provide an explanation for the potent inhibition of maintenance activity that they observed with poly[dA].poly[dT].

These workers also observed that poly[dC] but not poly[C] caused significant inhibition of maintenance methylation, whilst neither of these caused significant inhibition of de novo activity in my system. Bolden et al. (1984) suggest from this

result that the deoxyribose sugar has some involvement in binding, whilst my results suggest that the effect of the sugar is negligible.

Poly[I] in my system was equally potent as poly[G] and poly [dG] in causing inhibition of de novo methyltransferase activity, whilst Bolden et al. (1984) observed that poly[dI] was not an inhibitor of the maintenance methyltransferase activity.

These differences observed for the two series of experiments on the in vitro inhibition of DNA(cytosine-5)methyltransferase by polynucleotides may occur as a result of the difference in enzyme specificities of the two methyltransferases; Bolden et al (1984) used DNA(cytosine-5)methyltransferase extracted from HeLa cells grown in culture, whilst in my system murine L1210 cells grown in vivo were used.

Alternatively, the difference may be due to the different activities of the enzymes that were studied; Bolden et al. (1984) studied inhibition of the maintenance activity using hemimethylated DNA as a substrate, whilst my studies were on the de novo activity of the enzyme. De novo methylation of DNA proceeds at a rate of only 3 to 4% of maintenance methylation (Bolden et al., 1984). The enzyme used by Bolden et al. (1984) had been through more stages of purification than that used in my system and had an activity of 25600 units/mg protein compared with 3000 units/mg protein. However, the number of methyl

groups transferred under the conditions of the assay were similar in both systems, i.e. in my system 0.45 pmol were transferred in the control whilst Bolden et al. (1984) had controls values which varied from 0.24 pmol to 3.3 pmol.

An area of concern in the work of Bolden et al. (1984) was that the specific activity of the SAM that they used was 2400 cpm/pmol. This means that in most of their assays a very low value of 8000 cpm would be incorporated into the DNA. There was also no indication of any statistical treatment of their data and it appears that for the inhibition of DNA(cytosine-5)methyltransferase by synthetic polynucleotides, the results presented are for a single experiment. I therefore view their data with some scepticism and in light of my results consider that the inhibition of DNA(cytosine-5)methyltransferase by poly[dA].poly[dT] that they observed may have been an error, especially since poly[dA], poly[dT] or poly[d(A-T)] did not cause any inhibition.

The importance of the guanine residue for binding has been further supported by the work of Bolden et al. (1986b) who showed that fully methylated poly[dG-5MC].poly[dG-5MC] was a powerful inhibitor of HeLa DNA(cytosine-5)methyltransferase.

The enhanced inhibitory effect of poly[X] over poly[I] and poly[G], suggests that if there is an additional carbonyl group at the 2-position of the

purine moiety, such as with poly[X], this becomes available for hydrogen bonding. This is likely to occur only when present in the single-stranded form, as the 2-position of the purine would be unavailable to DNA(cytosine-5)methyltransferase for binding by being located in the minor groove. This merely exemplifies the importance of the carbonyl group within purines for methyltransferase binding as xanthine is not present in DNA.

In support of the fact that the O^6 position of guanine is an important binding site, Drahovský and Morris (1972) demonstrated that poly[dI].poly[dC] was equally as potent an inhibitor as poly[dG].poly[dC]. Despite this, Bolden et al. (1984) demonstrated that poly[dI] was not an inhibitor. One would expect therefore, that if the carbonyl at the 6 position of the purine is a binding site, then DNA containing other purines bearing a carbonyl at the 6 position would also be substrates for the methyltransferase. This is indeed the case with poly[dI-dC].poly[dI-dC]. Not only does it behave as a substrate for DNA(cytosine-5)methyltransferase, it is also an order of magnitude better than poly[dG-dC].poly[dG-dC] (Pedrali-Noy and Weissbach, 1986). This is presumed to be due to the fact that poly[dI-dC].poly[dI-dC] has a lower melting temperature and may therefore permit better enzyme binding.

The carbonyl group at the 6 position of the purine appears to be a very important binding site for DNA(cytosine-5)methyltransferase. However, it must be emphasised that there must be another binding site within the purine molecule. This is inferred from the fact that although the pyrimidine polymers, poly[C], poly[U] and poly[dC] contain carbonyl groups they do not cause any significant inhibition. Therefore, there must be another site within the guanine molecule which is common to inosine and xanthine, which is involved in the methyltransferase binding. The other site likely to be responsible for hydrogen bonding with DNA(cytosine-5)methyl-transferase is the N-7 position of guanine, as this also protrudes into the major groove of DNA and is common to xanthine and inosine. However, it would appear that this is not as strong a binding site as the carbonyl, as the presence of an N-7 purine alone, such as that which is present in poly[A], is not sufficient to cause binding and inhibition.

The importance of the sugar residues in determining DNA recognition and binding is debatable, but my results on the inhibition of DNA(cytosine-5)methyltransferase by polynucleotides would suggest that they have little importance in 5'-CG-3' recognition. DNA(cytosine-5)methyltransferase cannot methylate RNA, but can presumably bind to RNA. Bolden et al. (1984) identified RNA as a powerful

inhibitor of DNA(cytosine-5)methyltransferase in crude cellular extracts, and my results indicate that the sugar residue plays no part in the binding of the methyltransferase to the polynucleotide, as poly[G] causes the same degree of inhibition as poly[dG]. Bolden et al. (1984) concluded from the observation that poly[C] is without effect whereas poly[dC] shows some inhibition of enzymatic activity, that the methyltransferase recognises only the cytosines attached to a deoxyribose moiety. In conclusion, it would appear that the nature of the sugar residue is less important in methyltransferase binding, but is of vital importance in determining whether the nucleic acid will finally be methylated.

The degree of inhibition observed with the guanine homopolymers, does not vary with the length of the polymer, 400 bp, 600 bp or 20 bp. Presumably there is a cut off point at which inhibition is not observed, GTP (Bolden et al., 1984) and in my system GMP does not cause any significant inhibition. Bolden et al. (1984) showed that poly[dG](12-18) at a concentration of 10 μg/ml, when added to their methyltransferase assay had 34% of the control DNA methyltransferase activity remaining at this concentration. It would therefore be of some interest to determine the minimum length required to cause inhibition, as this would give some indication of the number of hydrogen bonds involved in methyl-

transferase binding and the specificity of the interaction. The poor strength of hydrogen bonds would indicate that a number would be required to give any strength.

The effectiveness of guanine residues in binding DNA(cytosine-5)methyltransferase was demonstrated by the observation that 0.2 μg of poly[dG]$_{20}$ caused 50% inhibition of the methylation of 20 μg of M.lysodeikticus DNA. Equivalent amounts of poly[dO^{6}MG]$_{20}$ caused no detectable inhibition, and so this suggested that the formation of such a lesion in DNA may lead to reduced binding of DNA(cytosine-5)methyltransferase. The O^6 position of guanine protrudes into the major groove of DNA and is therefore available for recognition and hydrogen bonding by those enzymes including DNA(cytosine-5)methyltransferase which are present in the major groove. Therefore, alkylation at the O^6 position of guanine may prevent it being recognised as such by DNA(cytosine-5)methyltransferase and may therefore prevent enzymatic methylation of an adjacent cytosine.

In order to examine this possibility, pairs of oligonucleotides were synthesised using the phosphoramidite method. The oligonucleotides were chosen to contain -CG- rich sequences and in one member of the pair, all guanine residues in the -CG- linkage were replaced by the O^6-methylguanine

residue. As predicted, the replacement of guanine with O^6-methylguanine either completely suppressed enzymatic methylation in the case of the sequence which constitutes part of the promoter of the human Harvey ras proto-oncogene, 517-547 (Ishii et al., 1985) 5'-CCCGCCCCGCCCCGGCCTCGGCCCCGGCCC-3' or severely reduced enzymatic methylation in the case of the 26 mer 5'-CCGGCCATTACGGATCCGTCCTGGGC-3'.

Alkylation of the O^6-position of guanine residues in DNA by chemical alkylating agents, such as environmental N-nitroso compounds is thought to be an important promutagenic lesion in both bacteria (Karran and Marinus, 1982) and mammalian cells (Medcalf and Wade, 1983; Newbold et al., 1980; Margison and O'Connor, 1989). In animal models the formation and persistence of O^6-alkylguanine is correlated with the tissue or species specificity of certain antitumour agents (Babich and Day, 1987; Yarosh, 1985; Saffhill et al., 1985). These effects are normally correlated with the mispairing properties of the modified guanine residues, although few models exist to explain how DNA damage at a specific site may lead to the induction to neoplasia or stop the growth of neoplastic cells.

An alternative explanation for the mutagenic and carcinogenic effect of O^6-guanine alkylation, may be that this modification prevents the recognition of -CG- sequences and therefore reduces the enzymatic

methylation of cytosine in DNA. It would be useful at this stage to make an estimation of the probability that $\underline{O}^6$-methylguanine will occur at a site in DNA to affect enzymatic methylation of an adjacent cytosine, after treatment of cells with a methylating agent.

Consider a cell line with a poor capacity to repair $\underline{O}^6$-methylguanine, such as the human lymphoblastoid cell line GM892. According to the data of Bull and Tisdale (1987), treatment of these cells with the methylating drug temozolomide at a concentration of 0.1 mM would produce approximately 0.4 pmoles of methyl groups bound per μg of DNA, of which approximately 5% would exist as $\underline{O}^6$-methylguanine (Bull, 1988). Using a figure of 3% for the total level of 5-methylcytosine in the cellular genome (Tisdale, 1989) and the probability that guanine in -CG- sequences are twice as likely to be methylated at the $\underline{O}^6$-position (Briscoe and Cotter, 1984), then the probability that an $\underline{O}^6$-methylguanine will be at a site to affect methylation of an adjacent cytosine will be approximately 0.5×10^6 (see APPENDIX 5).

DNA(cytosine-5)methyltransferase is not unique in being inhibited by the replacement of guanine with $\underline{O}^6$-methylguanine in appropriate DNA sequences. DNA cleavage by certain restriction endonucleases has been shown to be inhibited when their recognition

sequence contains O^6-methylguanine in place of guanine. It has been shown by Boehm and Drahovsky (1980), that the ability of several restriction enzymes to digest lambda phage DNA was severely impaired after treatment *in vitro* with MNU. It was then reported by Green *et al*. (1984) that the restriction enzyme PstI will not cleave oligonucleotides containing O^6-methylguanine and this property was used by Wu *et al*. (1987) to devise a sensitive assay for O^{6}AT, based on the inhibition of restriction endonuclease cleavage. A more extensive study has recently been reported by Voigt and Topal (1990) in which oligonucleotides were synthesised containing O^6-methylguanine at unique positions. These were then annealed with their complementary strand and the ability of a range of restriction endonucleases to cleave these oligonucleotides was investigated. DNA cleavage by the restriction endonucleases, HpaII, HinPI, NaeI, NarI, PvuII and XhoI was inhibited when a single guanine was replaced by O^6-methylguanine within the appropriate recognition sequence.

The effect of O^6-alkylation of guanine on the methylation of adjacent cytosine may explain the observed hypomethylation of DNA after the treatment of cells grown in culture with alkylating agents. The antitumour imidazotetrazinone temozolomide has been shown to cause hypomethylation in the human

erythroleukemia cell line K562 three days after treatment (Tisdale, 1986). The imidazotetrazinones, temozolomide and ethazolastone have also been shown to cause hypomethylation in the human lymphoblastoid cell line, GM892 four and five days respectively after treatment (Tisdale, 1989). The methylating carcinogen, MNU has been shown to cause hypomethylation of DNA in Raji cells (Boehm and Drahovský, 1981a). In contrast to the hypomethylation effect of MNU, Krawisz and Lieberman (1984) observed no change in the 5-methylcytosine content of Raji cells as well as a mouse thymic lymphoma cell line S49 and human diploid fibroblasts, even after doses of MNU which resulted in a 95% inhibition of replication in the 24 h after treatment. In both of these reports, labelled deoxycytidine was used to determine the level of 5-methylcytosine and both reports indicate a 5-methylcytosine content of 3.6% in Raji cells in the absence of MNU-treatment.

Holliday (1979) proposed a theory for carcinogenesis, in which damage to DNA which is susceptible to repair mechanisms, could lead to the loss of specific methyl groups from 5-methylcytosine, which in turn could lead to changes in gene expression. The data presented in this section adds further support to this theory and provides a mechanism through alkylation at the O^6 atom of guanine by which alkylating carcinogens may act.

Further support for Holliday's epigenetic theory of carcinogenesis comes from agents which do not react with DNA, but are carcinogenic and reduce the level of DNA(cytosine-5)methylation. One such example is ethionine, which is a liver carcinogen in rats (Farber, 1956). The effect of ethionine administration to rats (300 mg/kg) after partial hepatectomy was studied by Cox and Irving (1977). As a result of competitive inhibition of S-adenosyl-L-methionine synthetase, there was an increase in the hepatic level of SAE to levels 30-40 times greater than SAM, and DNA synthesised after ethionine administration was methyl deficient. Ethionine at a concentration of 4 mM has also been shown to cause hypomethylation of DNA in Friend erythroleukemia cells grown in culture, with a corresponding increase in the expression of globin genes four to five days after treatment (Christman et al., 1977).

Another such agent is 5-azacytidine, an analogue of 5-methylcytosine which can be incorporated into DNA, but cannot be methylated and as a consequence is a strong inhibitor of DNA(cytosine-5)methylation (reviewed by Doerfler, 1983; Jones, 1986; Riggs and Jones, 1983). 5-Azacytidine has been shown to be carcinogenic in mice and rats. In mice an increased incidence of lung tumours has been observed in both sexes and an increased incidence of granulocytic tumours has been observed in females (IARC, 1981). In

the Fischer rat, 5-azacytidine increased the incidence of testicular tumours, had hepatic tumour promoting properties and was able to induce transplacental carcinogenesis (Carr et al., 1988).

However, a number of reports have been published which appear to contradict the theory that an inhibition of DNA(cytosine-5)methylation can be correlated with a carcinogenic effect. Firstly, the potent carcinogen MNU was shown to produce no detectable change in the methylation levels of the human lymphoblastoid cell line Raji, a mouse thymic lymphoma cell line S49 and human diploid fibroblasts. This contradicts a previous report by Boehm and Drahovský (1981a), in which MNU was shown to cause hypomethylation of DNA in Raji cells.

Secondly, injection of the potent carcinogen dimethylnitrosamine into rats after partial hepatectomy, produced no effect on DNA(cytosine-5)methylation in rat liver, which is the target organ for the carcinogen (Craddock and Henderson, 1979). MMS does not induce liver tumours in this system but caused an increase in the level of DNA(cytosine-5)methylation. However, it is a reduction in the level of DNA(cytosine-5)methylation and the resultant expression of aberrant genes which is thought to bring about a carcinogenic effect. There is no reason to believe that all carcinogens modify the level of DNA(cytosine-5)methylation and consequently

the carcinogenic activity of dimethylnitrosamine may be explained by mechanisms other than through an inhibition of DNA(cytosine-5)methylation.

Thirdly, it has been demonstrated that some carcinogens can increase the activity of DNA(cytosine-5)methyltransferase *in vivo*. The activity of *de novo* DNA(cytosine-5)methyltransferase has been studied in various tissues of the rat after administration of a single dose of MNU (Pfohl-Leszkowicz and Dirheimer, 1986). They observed that organs in which DNA(cytosine-5)methyltransferase activity was increased after MNU-treatment, such as the brain, are more susceptible to MNU-induced tumours. They suggested that there was a relationship between the effect on DNA(cytosine-5)methyltransferase activity and carcinogenesis by MNU.

Bravo and Salas (1989) observed a 55% increase in the methyltransferase activity of male rat liver 14 days after treatment with the carcinogen *N*-2-acetylaminofluorene. By contrast, methyltransferase activity in female rats was reduced by 33% after similar treatment. Female Sprague-Dawley rats are relatively resistant to hepatocarcinogenesis by *N*-2-acetylaminofluorene, while in male rats the liver is the target for the carcinogen. These authors suggest that male rats are more prone to hepatocarcinogenesis than female rats, because they predict that greater

changes in the methylation status of male rat livers would occur in response to N-2-acetylaminofluorene treatment. However, from their data I would have predicted female rats to be more prone to hepatocarcinogenesis as the DNA(cytosine-5)methyltransferase activity of the liver appears to drop 14 days after treatment with N-2-acetylaminofluorene.

Despite both of these papers reporting an increase in DNA(cytosine-5)methyltransferase activity after carcinogen administration, no attempt was made to determine whether this has any effect on the methylation status of the DNA. Therefore these changes in methyltransferase activity may have been insignificant.

The results from *in vitro* experiments have shown that many carcinogens can inhibit DNA(cytosine-5)methylation (Chan *et al.*, 1983; Wilson and Jones, 1983), which may occur through reaction with the DNA substrate or reaction with the enzyme. Despite the fact that in living cells in culture (Krawisz and Lieberman, 1984) and *in vivo* (Craddock and Henderson, 1979), the effects on DNA(cytosine-5)methylation have not been obvious, extremely small changes in the level of DNA methylation may have occurred. In theory, the inhibition of one critical methylation would be sufficient to induce a tumour if this led to the expression of an aberrant gene. The data presented in this section provides a mechanism by

which a single O^6-alkylation of guanine may prevent the methylation of an adjacent cytosine, which if this occurred at a critical site could lead to gene expression.

Alkylation at the O^6-position of guanine has been shown to be important in the inhibition of DNA methylation by DNA(cytosine-5)methyltransferase *in vitro*. However, this does not exclude other sites of alkylation as potential inhibitors of DNA methylation. The major base position for alkylation by MNU, ENU, MMS, EMS, temozolomide and ethazolastone is N-7 guanine (Beranek *et al.*, 1980; Bull, 1988; Lawley, 1984) which is also within the major groove of DNA.

Nyce *et al.* (1983) proposed a model by which 7-methylguanine and not O^6-methylguanine was the critical lesion for the inhibition of DNA(cytosine-5)methylation. 7-Alkylguanine is removed from DNA by a base excision process whilst O^6-alkylguanine is repaired by a methyltransferase reaction where the integrity of the base remains intact (reviews, Hanawalt *et al.*, 1979; Sancar and Sancar, 1988; Lindahl and Sedgwick, 1988). Therefore, as 7-methylguanine produces 'gaps' in the DNA during repair, it was proposed to be more likely to inhibit DNA(cytosine-5)methylation than O^6-methylguanine, in which the base integrity does not change.

The ability of DNA and poly[dG-dC].poly[dG-dC] alkylated with MNU or dimethyl sulphate to behave as a substrate for DNA(cytosine-5)methyltransferase was investigated by Pfohl-Leszkowicz et al. (1983a). Treatment with dimethyl sulphate, which caused up to 30% methylation of the N-7 guanines or 10% of N-3 adenines did not affect the ability of the DNA to behave as a methyltransferase substrate. MNU, which in addition to N-7 guanine and N-3 adenine methylation also produces O^6-methylguanine and methylphosphotriesters in DNA. There was a 40% reduction in the ability of MNU-treated DNA to behave as a substrate for DNA(cytosine-5)methyltransferase when 1% of the guanines were converted to O^6-methylguanine. Although a discrimination between methylphosphotriesters and O^6-methylguanine as the inhibitory lesion could not be made by these authors, this experiment does illustrate that 7-methylguanine is not important in the inhibition of DNA(cytosine-5)methyltransferase.

The alkylation of cytosine residues is also likely to affect any subsequent enzymatic methylation of this base. Modification at the O^2 and N-3 position of cytosine has been shown to occur for MMS, EMS, MNU and ENU treatment of DNA (Beranek et al., 1980; Lawley, 1984), but the level is usually less than 1% of the total level of alkylation (ENU is an exception in which O^2 ethylation of cytosine represents 2.9% of

the total alkylated bases). Mutagens such as bisulfite and hydroxylamine can add to the 5,6-double bond of cytosine (Singer and Grunberger, 1983). These agents would presumably also be effective blocks of the enzymatic methylation of cytosine, as they would interfere with the mechanism of methyltransferase action (Wu and Santi, 1987; Santi *et al.*, 1983) which is thought to occur through the 5,6-double bond.

Therefore, alterations in DNA/protein interactions as a result of alkylation at the O^6 position of guanine could provide an epigenetic basis for the action of certain carcinogens. The promoter regions of many genes contain -GC- runs which are the preferred site of damage by many alkylating agents (Mattes *et al.*, 1988), for example the C-Ha-*ras* oncogene which contains seven -GC- runs in the 5'-flanking region (Ishii *et al.*, 1985).

The importance to the cell of maintaining the appropriate 5-methylcytosine status may be one reason why so much energy is expended by many cell types, in the apparently wasteful 'suicide' protein mechanism for removing methyl groups from O^6-methylguanine residues (Olson and Lindahl, 1980; Yarosh *et al.*, 1983).

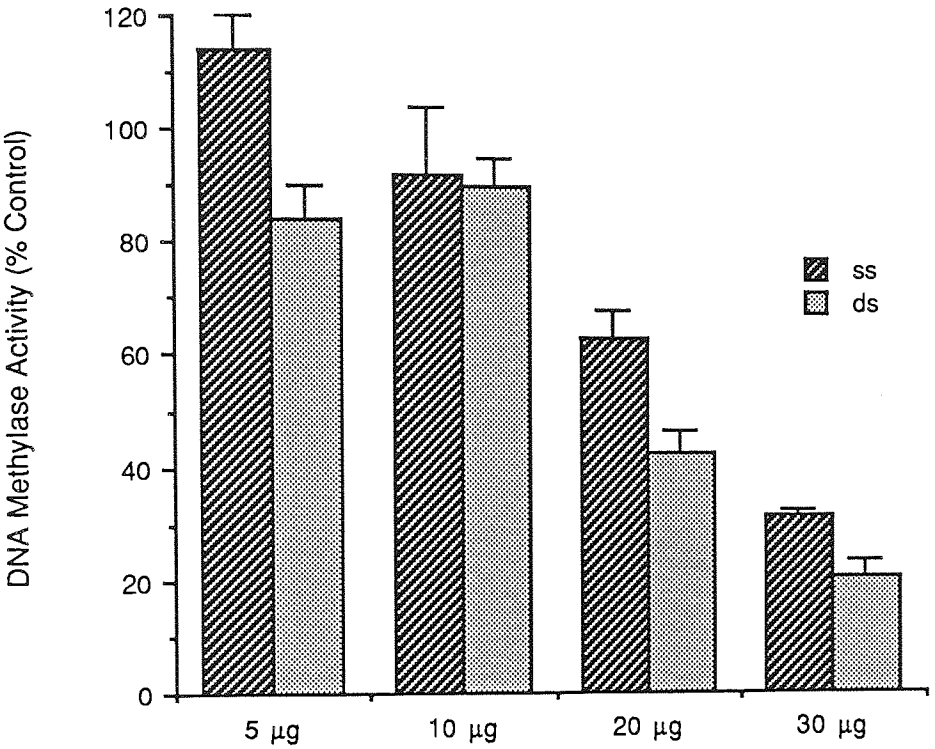


Figure 49: The effect of single and double stranded calf thymus DNA on DNA(cytosine-5)methyltransferase activity.
The two forms of calf thymus DNA (single and double stranded) obtained from Sigma were placed in increasing amounts into the standard DNA methylase assay. The standard assay contained 20 µg M.lysodeikticus DNA, 1 µCi SAM and DNA(cytosine-5)methyltransferase and incubations were carried out at 37°C for 4 h in a total volume of 100 µl.
The results are the mean of three experiments ± SEM.

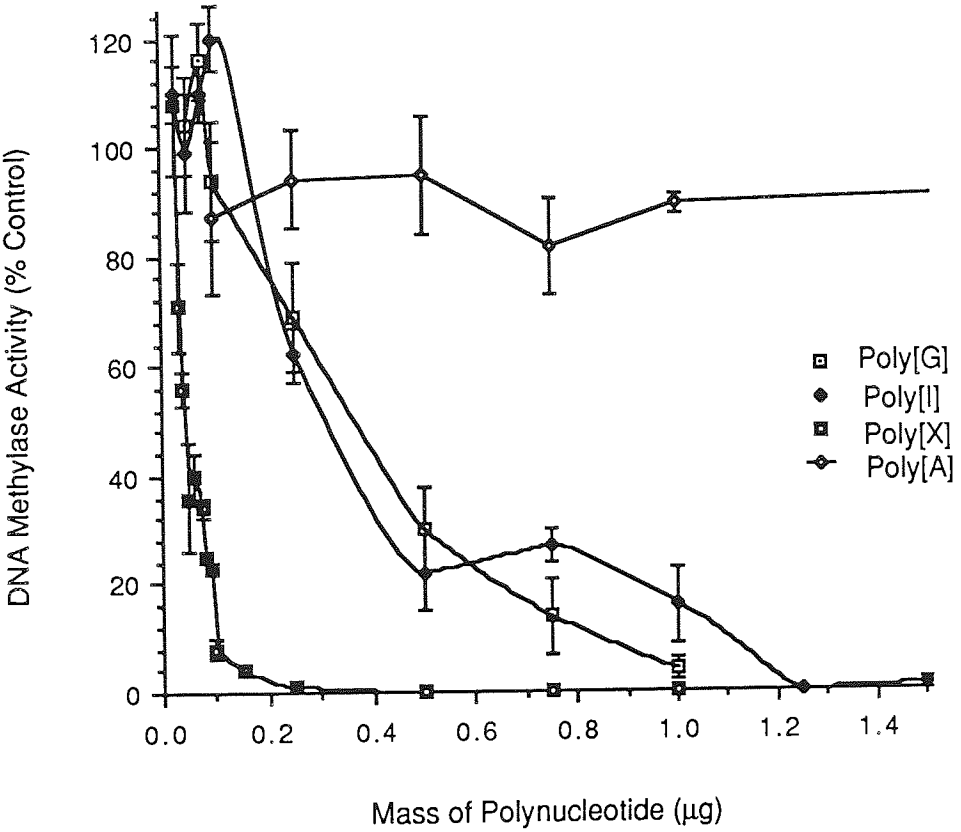


Figure 50: The effect of purine polynucleotides on DNA(cytosine-5)methyltransferase activity - dose response curve. Incubations were carried out at 37°C for 4 h in a total volume of 100 µl containing 20 µg M.lysodeikticus DNA, 1 µCi SAM and DNA(cytosine-5)methyltransferase capable of transferring 0.3-0.5 pmol of methyl groups under the conditions of the assay. The results are the mean of at least three experiments ± SEM.

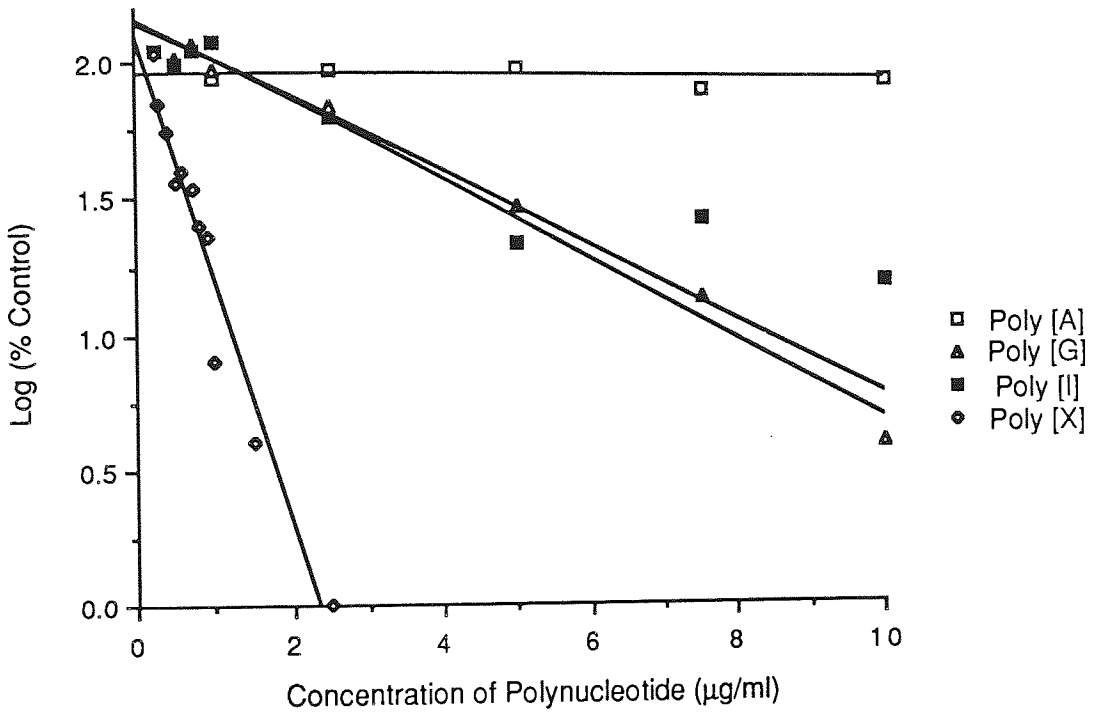


Figure 51: The effect of purine polynucleotides on DNA(cytosine-5)methyltransferase activity - demonstration of first order kinetics.
The data from fig 50 was plotted, log (% control) versus polynucleotide concentration. A straight line indicates that the reaction is first order.

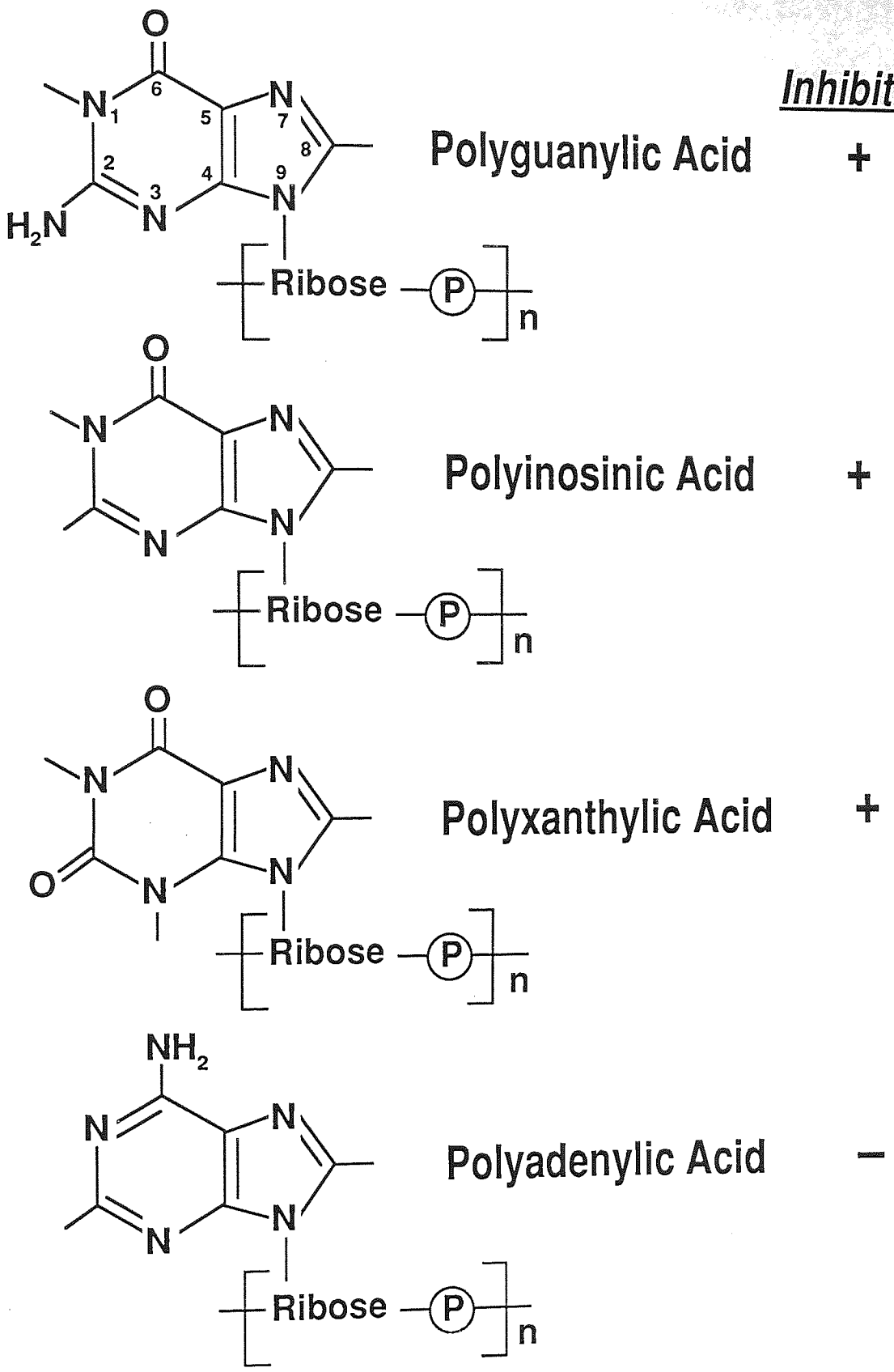


Figure 52: Purine polynucleotides as inhibitors of DNA(cytosine-5)methyltransferase.

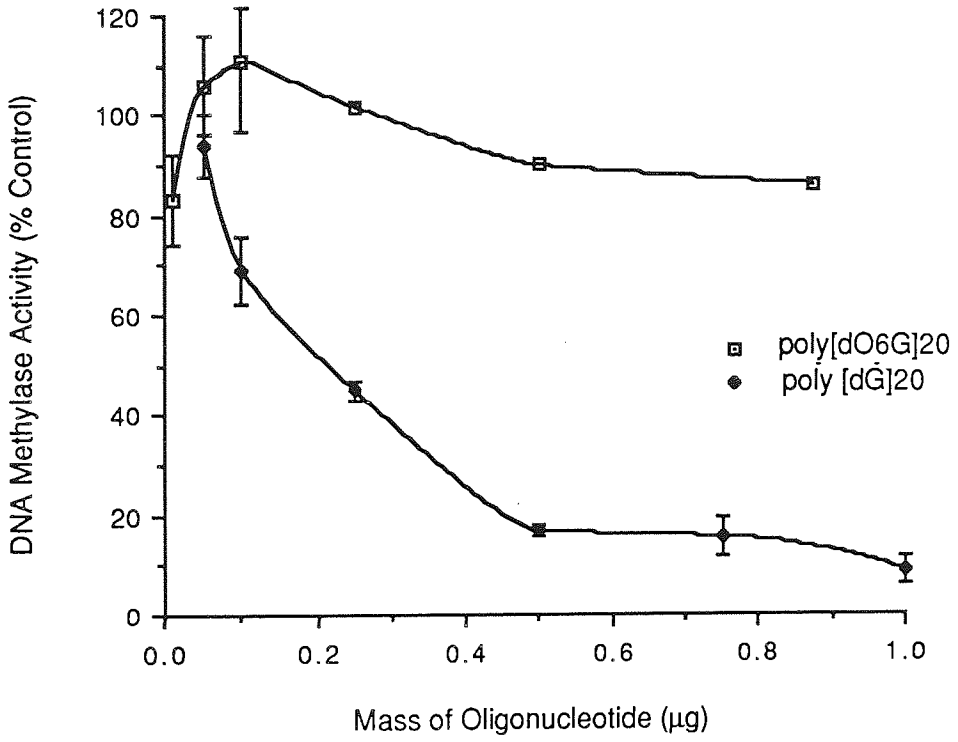


Figure 53: The effect of methylation at the O-6 position of guanine on the ability of poly[dG] to behave as an inhibitor of DNA(cytosine-5)methyltransferase.
Incubations were carried out at 37°C for 4 h in a total volume of 100 µl containing 20 µg M.lysodeikticus DNA, 1 µCi SAM and DNA(cytosine-5)methyltransferase capable of transferring 0.4 pmol of methyl groups under the conditions of the assay. The two oligonucleotides were both 20mers and were homopolymers of either guanine or O^6-methylguanine.
The results are the mean of three experiments ± SEM.

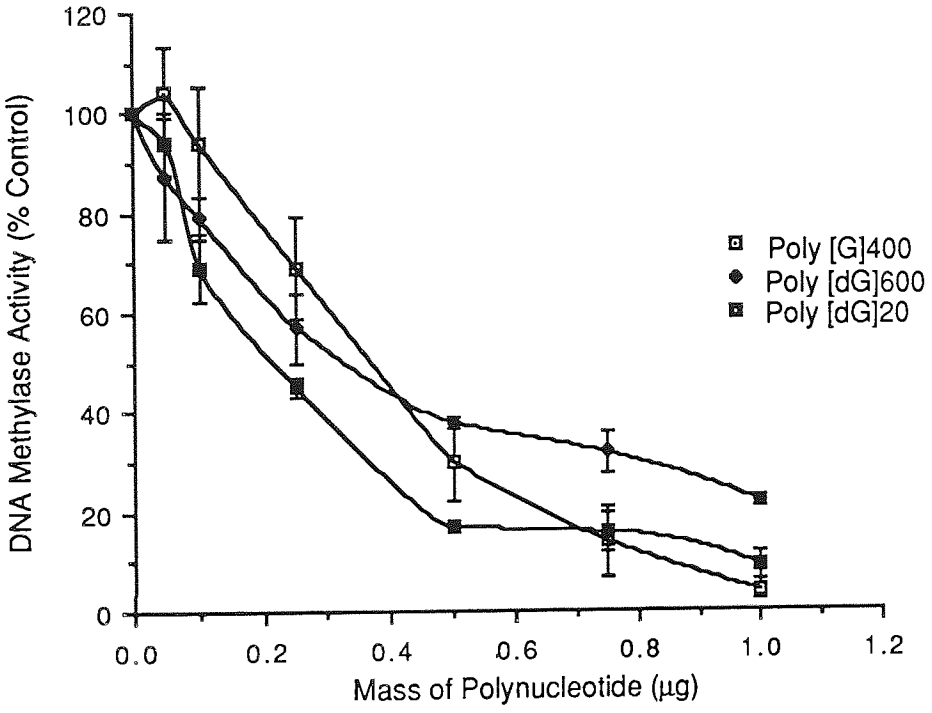
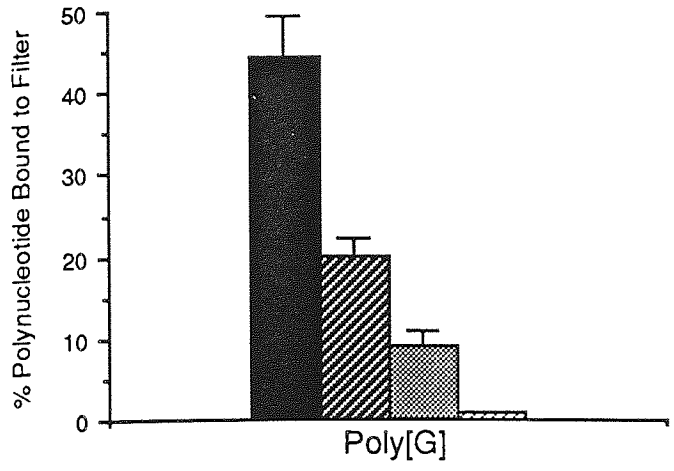
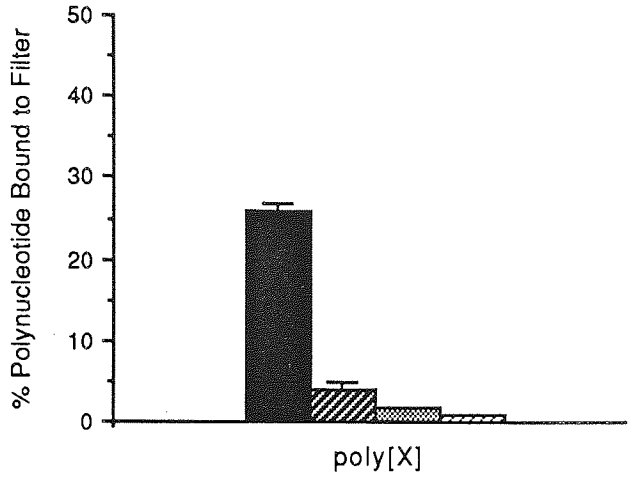


Figure 54: The effect of the base length of guanine polynucleotides and polydeoxyribonucleotides on DNA(cytosine-5)methyltransferase activity.
Incubations were carried out at 37°C for 4 h in a total volume of 100 µl containing 20 µg M.lysodeikticus DNA, 1 µCi SAM and DNA(cytosine-5)methyltransferase capable of transferring 0.4 pmol of methyl groups under the conditions of the assay.
The results are the mean of three experiments ± SEM.


% Polynucleotide Bound to Filter
50
40
30
20
10
0
Poly[G]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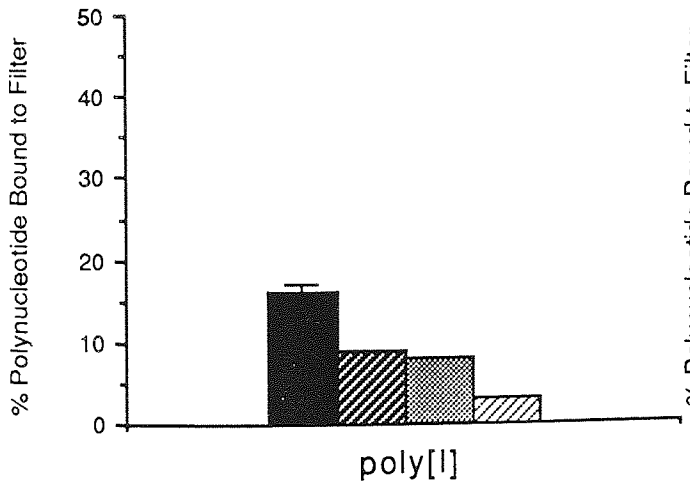
40
30
20
10
0
poly[X]


% Polynucleotide Bound to Filter
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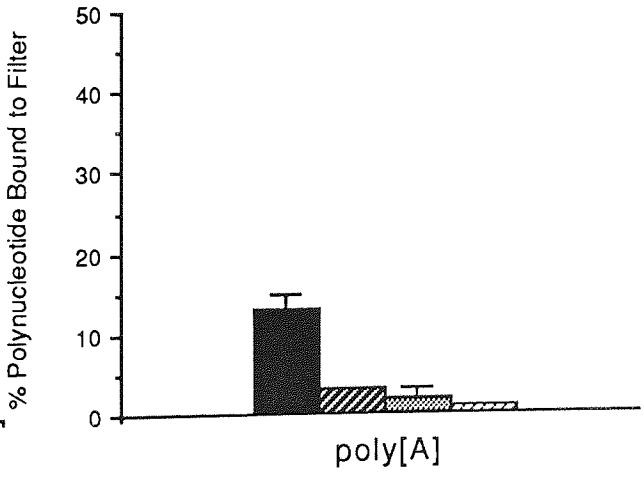
40
30
20
10
0
poly[I]


% Polynucleotide Bound to Filter
50
40
30
20
10
0
poly[A]

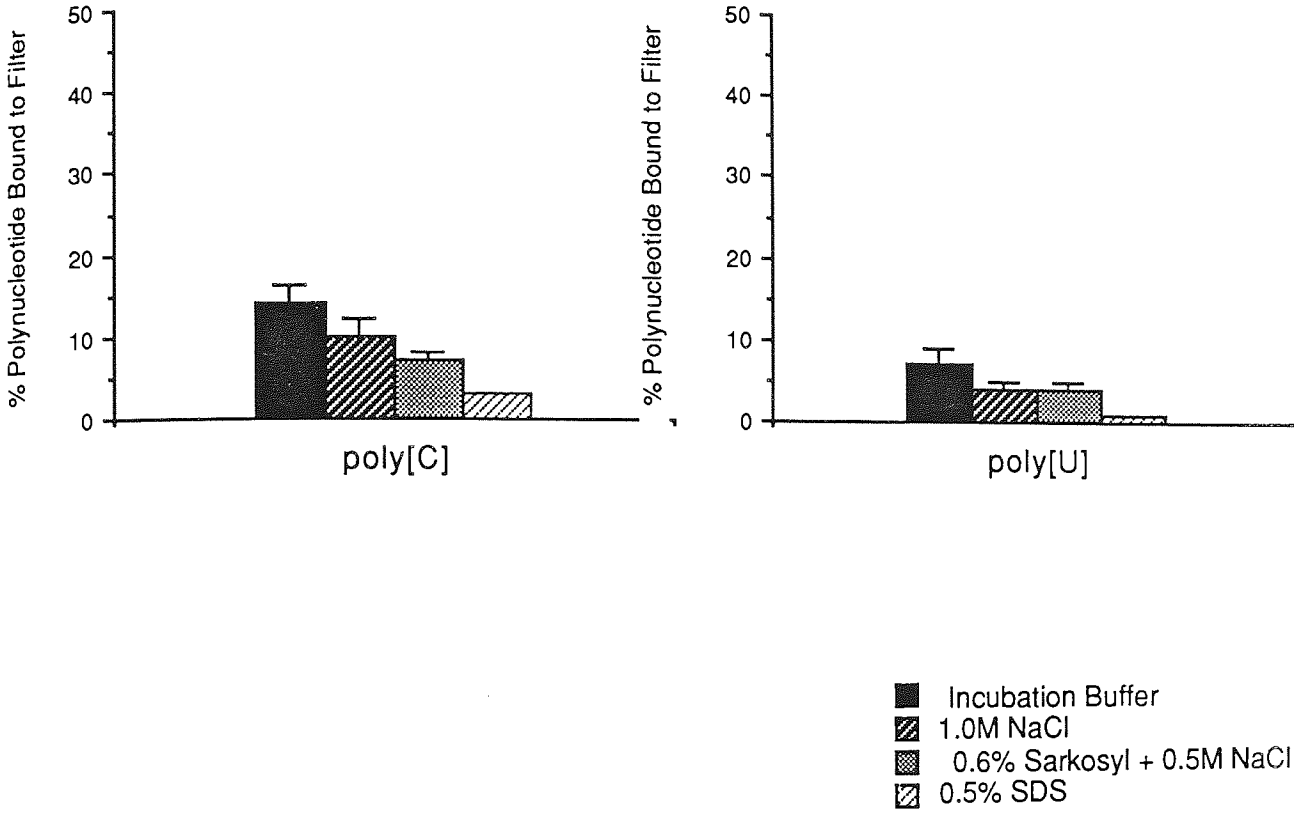


Figure 55: The ability of preformed 5′-[^{32}P] labelled polynucleotides:nuclear protein complexes to treatment with salt and detergents.
The polynucleotides were labelled 5′ with [^{32}P]ATP using T4 polynucleotide kinase. Complexes were then formed during a 20 min incubation at 37°C with 5 µg of the labelled polynucleotide and the DNA(cytosine-5)methyltransferase preparation. The complexes were then incubated for 10 min at 4°C prior to being washed onto a Millipore HA 0.45 µm filter either with the incubation buffer or the dissociating agents indicated.
The results are expressed as the percentage of the polynucleotide bound to the filter and represent the mean ± SEM.

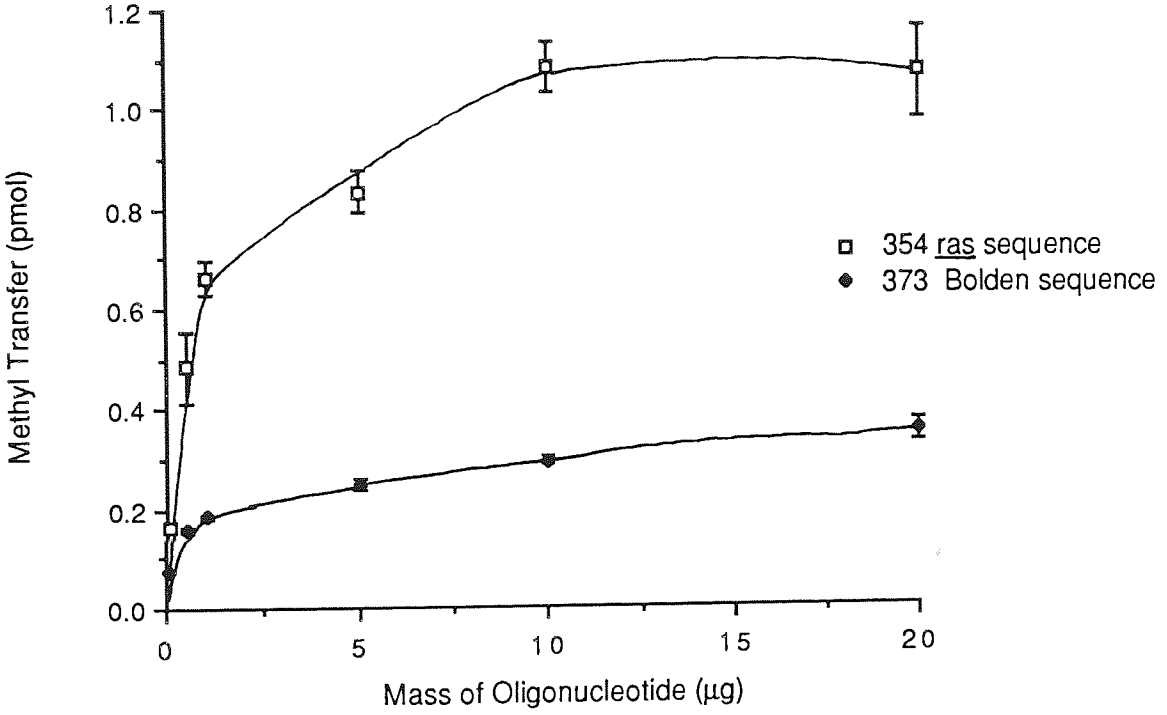


Figure 56: The extent of methylation of the synthetic oligonucleotides 1) 26mer Bolden and 2) 30mer ras sequence by DNA(cytosine-5)methyltransferase.
Incubations were carried out at 37°C for 4 h with 0.5 µCi SAM, a fixed amount of DNA(cytosine-5)methyltransferase and increasing amounts of the oligonucleotides. Assays were carried out in a total volume of 100 µl.
The data represents the mean of three incubations ± SEM.

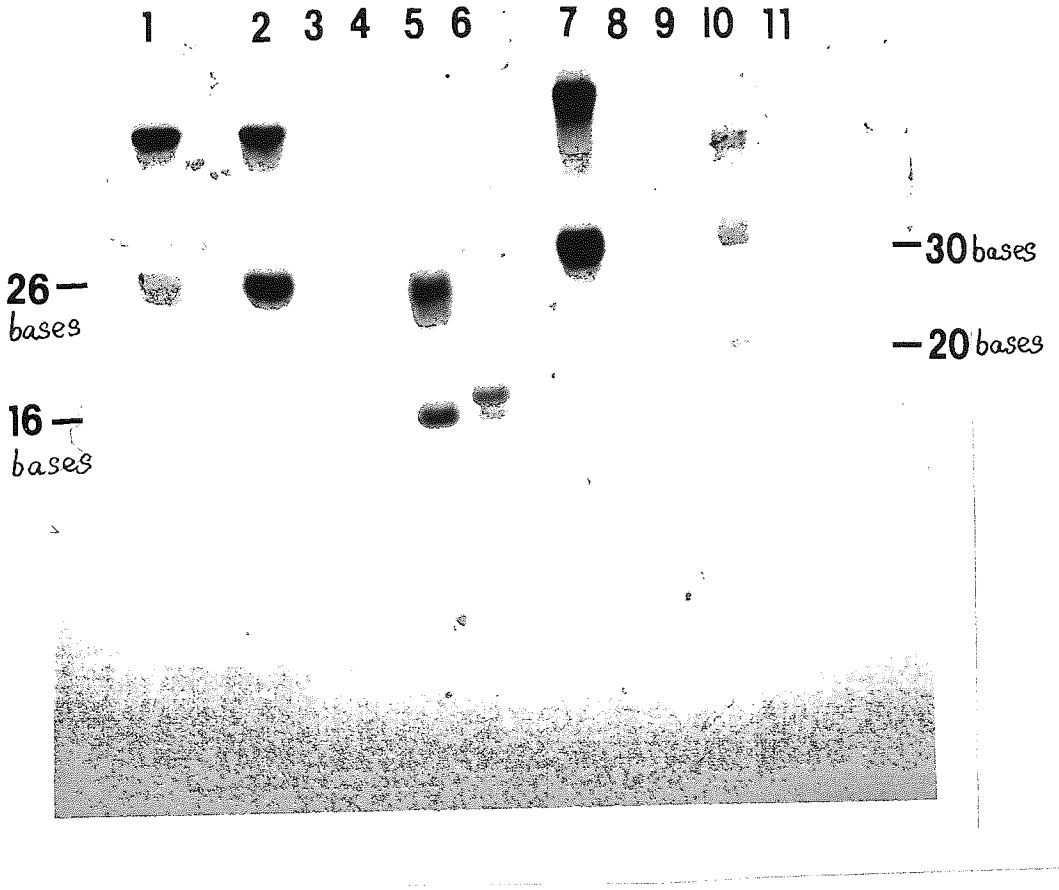


Figure 57: Fluorograph of the Bolden and ras sequences after treatment with DNA(cytosine-5)methyltransferase and [^{3}H] SAM. The samples were run on a 20% urea:acrylamide gel and then fluorographed.
Key:-
1) Bolden sense normal strand (DBU uncoupled) (449),
2) Bolden sense normal strand (ammonia uncoupled) (373),
3) Bolden sense strand containing O^6-methylguanine (312),
4) Bolden antisense strand (380),
5) annealed normal Bolden strands (373/380) (D),
6) annealed Bolden strands, sense strand containing O^6-methylguanine (312/380) (A),
7) ras sense normal strand (354),
8) ras sense strand containing O^6-methylguanine (309),
9) ras antisense strand (379),
10) annealed normal ras strands (354/379) (B),
11) annealed ras strands, sense strand containing O^6-methylguanine (309/379) (C).

	Methylase Activity (% Control)
Polynucleotides (10µg)	
Poly[A]	124±4
Poly[G]	0±0
Poly[I]	0±0
Poly[X]	0±0
Poly[C]	109±10
Poly[U]	106±3
Polydeoxyribonucleotides (10µg)	
Poly d[G]	0±0
Poly d[C.O^{6}G]$_{10}$	67±3
Poly d[O^{6}G]$_{20}$	88 (0.9µg)
Poly d[C]	92±10
Alternating Copolymers (10µg)	
Poly[dA].Poly[dT]	87±10
Poly[dG].Poly[dC]	2±2
GMP 1mM	79±7
10mM	72±10
ras antisense 5'-GGGCCGGGGCCGAGGCCGGG-3'	101±4
Bolden antisense 5'-GATCCGTAATGGCCGG-3'	89±4

Table 20: The effect of synthetic polynucleotides and polydeoxyribonucleotides on DNA(cytosine-5)methyltransferase activity.
The standard assay mixture (100 µl) contained 20 µg M.lysodeikticus DNA, 1 µCi SAM and DNA(cytosine-5)methyltransferase capable of transferring 0.4 pmol of methyl groups under the conditions of the assay. Incubations were carried out at 37°C for 4 h. Assays were carried out in the presence of 10 µg of the polynucleotide or polydeoxyribonucleotide.
The results are the mean of at least three experiments ± SEM.

Polynucleotide/ Polydeoxyribonucleotide	Length (bases)
Poly[U]	238-3195 bases
Poly[A]	312-1145 bases
Poly[C]	approx 400 bases
Poly[G]	approx 400 bases
Poly[I]	not available
Poly[X]	not available
Poly[dC]	550-800 bases
Poly[dA].Poly[dT]	approx 300 base pairs
Poly[dG].Poly[dC]	140-300 base pairs
Poly[dG]	approx 602 bases
$Poly[dG]_{20}$	20 bases
$Poly[d(C.O^6G)]_{10}$	20 bases
$Poly[d(O^6G)]_{20}$	20 bases

Table 21: The length of the polynucleotides and the polydeoxyribonucleotides used in these studies. This information was supplied by the manufacturers.

Oligonucleotide		Methyl Transfer (pmol)	As a % M.lysodeikticus DNA
(372) poly d(C.G)10		0±0	0
(310) poly d(C.O^6G)10		0±0	0
(373) 5'-CCGGCCATTACGGATCCGTCCTGGGC-3'	(Bolden)	0.185±0.042	23±5
(312) 5'-CCXGCCATTACXGATCCXTCCTGGGC-3'	(Bolden)	0±0	0
(380) 5'-GATCCGTAATGGCCGG-3'	(Bolden antisense)	0±0	0
(354) 5'-CCCGCCCCGCCCCGGCCTCGGCCCCGGCCC-3'	(ras)	2.62±0.31	319±38
(309) 5'-CCCXCCCCXCCCCXGCCTCXGCCCCXGCCC-3'	(ras)	0±0	0
(379) 5'-GGGCCGGGGCCGAGGCCGGG-3'	(ras antisense)	0±0	0
DNA (M.lysodeikticus)		0.821±0.055	100

X=O^6-Methylguanine

Table 22: The ability of the polydeoxyribonucleotides to behave as substrates for DNA(cytosine-5)methyltransferase when guanines adjacent to cytosine are replaced by O^6-methylguanine.
Incubations were carried out at 37°C for 4 h in an assay volume of 100 µl containing 20 µg of the appropriate polydeoxyribonucleotide, 1 µCi SAM and DNA(cytosine-5)methyltransferase. The determination of the radioactivity in the nucleic acid was by the Bolden method described in section 7.2.1.
The results are expressed as the mean ± SEM ($n \geq 7$).

Oligonucleotide	Methyl Transfer (pmol)	As a % M.lysodeikticus DNA
Annealed Oligonucleotides		
(373) 5'-CCGGCCATTACGGATCCGTCCTGGGC- 3' (380) 3'-GGCCGGTAATGCCTAG-5'	1.05±0.03	128±4
(312) 5'-CCXGCCATTACXGATCCXTCCTGGGC- 3' (380) 3'-GGCCGGTAATGCCTAG-5'	0±0	0
(354) 5'-CCCGCCCCGCCCCGGCCTCGGCCCCGGCCC-3' (379) 3'-GGGCCGGAGCCGGGGCCGGG-5'	0.15±0.03	18±4
(309) 5'-CCCXCCCCXCCCCXGCCTCXGCCCCXGCCC-3' (379) 3'-GGGCCGGAGCCGGGGCCGGG-5'	0±0	0
DNA (M.lysodeikticus)	0.821±0.055	100

X=O^6-methylguanine

Table 23: The ability of the annealed oligonucleotides to behave as substrates for DNA(cytosine-5)methyltransferase when guanines adjacent to cytosine are replaced by O^6-methylguanine. Incubations were carried out at 37°C for 4 h in an assay volume of 100 µl containing 20 µg of the appropriate annealed polydeoxyribonucleotides, 1 µCi SAM and DNA(cytosine-5)methyl-transferase. The determination of the radioactivity in the nucleic acid was by the Bolden method described in section 7.2.1. The results are expressed as the mean ± SEM (n = 6).

Oligonucleotide	$A_{260nm}(1)$	$A_{260nm}(2)$	$A_{260nm}(2-1)$
(354) ras normal	0.6086	0.6782	0.070
(309) ras O^6	0.6407	0.7352	0.094
(373) Bolden normal	n.d.	n.d.	n.d.
(312) Bolden O^6	0.5820	0.6194	0.037
B(354/379)	0.5961	0.6706	0.075
C(309/379)	0.5962	0.6895	0.093
D(373/380)	0.5863	0.6363	0.050
A(312/380)	0.5982	0.6732	0.075

Table 24: Experiment to determine whether the Bolden and ras sequences exist as double-stranded structures.
Solutions of the oligonucleotides and the annealed duplexes were heated at 100°C for 10 min followed by cooling on ice. The change in absorbance at 260 nm was measured.
The results are the mean of duplicate values.
n.d. = not determined

CHAPTER EIGHT

AN EXAMINATION OF METHODS TO ASSAY DRUGS DESIGNED TO METHYLATE THE C-5 ATOM OF CYTOSINE

8.1 INTRODUCTION

A major project within the Drug Development Group at Aston University was to develop drugs which would specifically methylate the C-5 position of cytosine within DNA. This project was based on the observation that the levels of 5-methylcytosine in DNA are significantly lower in a large series of human tumours and tumour cell lines when compared with DNA from normal cells (Diala et al., 1983; Gama-Sosa et al., 1983). A large amount of experimental evidence has been accumulated that suggests a correlation between decreased levels of 5-methylcytosine within a gene to gene activity (reviewed by Razin and Riggs, 1980; Doerfler, 1983; Riggs and Jones, 1983; Razin and Szyf, 1984). The aberrant expression of particular genes seen in certain tumours may occur as a result of gene hypomethylation, for example the c-Ha-ras oncogene was hypomethylated in six of eight carcinomas (five colonic adenocarcinomas and one small cell lung carcinoma) when compared to adjacent normal tissues (Feinberg and Vogelstein, 1983a).

A programme of research was then initiated by which potential compounds were synthesised, in the hope that agents capable of methylating the C-5 position of cytosine would appear. The intention for

such drug candidates was that they would 'switch off' over-expressed or aberrant genes in the neoplastic state, such as the ras oncogene.

Two chemical approaches were taken on this project by Mr G M Currie (British Technology Group student) and Professor M F G Stevens (Aston University), for possible means of generating compounds with the ability to methylate the C-5 position of cytosine within DNA.

The initial proposal was to copy the natural process by which DNA(cytosine-5)methyltransferase and S-adenosyl-L-methionine achieve C-5 cytosine methylation. Since the methyl moiety of S-adenosyl-L-methionine exists as a methyl sulphonium, it was decided that the compounds synthesised should have a sulphonium methylating centre of structure $-S^{+}(CH_3)_2$ at one end of the molecule. At the other end of the molecule there would be a nucleophilic thiol (-SH) group, known to be present at the active site of DNA(cytosine-5)methyltransferase in the cysteine of the amino acid sequence Pro-Cys (Bestor et al., 1988). Wu and Santi (1987) have suggested that the rare Pro-Cys dipeptide is involved in catalyzing methyl transfer in pyrimidine methyltransferases. It has been proposed by these authors that the sulfhydryl group of a cysteine residue is the nucleophilic catalyst by which DNA(cytosine-5)methyl-transferases attacks the C-6 of the substrate

cytosine in DNA and activates the C-5 position. Between the sulphonium methylating centre and the thiol activating group, it was proposed to have a linker group which would bind to the major groove of DNA. No suggestion was made of the structure of this 'linker' group. This hypothetical chemical agent would have to specifically recognise cytosine within a DNA sequence and specifically methylate the C-5 position of a cytosine. The source of the methyl group would be that attached to the sulphonium centre of the molecule and consequently such an agent would not require SAM or DNA(cytosine-5)methyltransferase.

The second chemical approach taken for the design of compounds capable of methylating the C-5 position of cytosine, was to synthesise a 'synthetic' enzyme based on the structure of DNA(cytosine-5)methyltransferase. It was proposed that this agent would recognise and bind to an appropriate region of DNA and would use intracellular SAM as the source of the methyl groups. The amino acid sequence of a murine DNA(cytosine-5)methyltransferase has been published (Bestor et al., 1988). It was decided to synthesise a series of peptides based on the active site of this enzyme, in the hope that DNA(cytosine-5)methyltransferase activity may be obtained within a small peptide. It has been suggested by Wu and Santi (1987) that the rare Pro-Cys dipeptide is involved in catalysing methyl transfer in bacterial pyrimidine

methyltransferases. This dipeptide occurs three times within the murine methyltransferase sequence and it has been suggested by Bestor et al. (1988) that the most carboxyl-terminal Pro-Cys dipeptide is most likely to be located at the catalytic centre. It is the sulfhydryl group of the cysteine which is thought to be responsible for the nucleophilic attack on the C-6 atom of cytosine, which leads to activation of the C-5 atom. It was therefore proposed to start with the dipeptide Pro-Cys and synthesise a series of peptides in the hope that DNA(cytosine-5)methyl-transferase activity may be generated.

There are a number of agents which can react with cytosine in DNA. Bisulfite, hydroxylamine, methoxyamine and hydrazine can all add to the 5,6-double bond of cytosine (Singer and Grunberger, 1983), primarily or solely on single-stranded DNA. The monofunctional alkylating agents, such as the alkylsulphonates (MMS, EMS) and the nitrosoureas (MNU, ENU) can alkylate DNA in vitro at the O^2 and N-3 position of cytosine (Beranek et al., 1980; Lawley, 1984) but these reactions generally represent <1% of the total DNA alkylation (except ENU in which O^2-ethylcytosine represents 2.8% of the total DNA alkylated). In homopolynucleotides of cytosine, reaction has also been demonstrated at the N^4 position of cytosine (Sun and Singer, 1974) with the ethylating agents, EMS and ENU. Ionizing radiation

can also damage cytosine within DNA through oxygen derived free radicals, mainly by hydroxyl radical attack at the 5,6 double-bond (reviewed by Breimer, 1988).

Although a number of agents can react with cytosine, there is no report of an alkylating agent that can methylate the C-5 position of cytosine. This section of the thesis describes methods that were used to assay any compounds that were synthesised, in support of this very ambitious medicinal chemistry project.

8.2 METHODS

8.2.1 Development of an assay for agents methylating cytosine at the C-5 position.

Methylation of lambda phage DNA in vitro.

a) In the absence of DNA(cytosine-5)methyltransferase

Lambda phage DNA (5 μg) dissolved in water was treated with the indicated concentration of drug dissolved in water, for 30 min at 37°C in a total volume of 200 μl, unless otherwise indicated. The solution was then saturated with sodium acetate by adding 0.1 volumes of 2.5 M sodium acetate and the DNA was precipitated with 2.5 volumes of cold absolute ethanol. The DNA was redissolved in water and the precipitation process was repeated a further two times and the DNA was washed three times with absolute ethanol. The DNA samples were then air dried ready for restriction enzyme analysis.

b) In the presence of DNA(cytosine-5)methyltransferase.

DNA(cytosine-5)methyltransferase was prepared as described in Section 3.1.1. Lambda phage DNA (5 μg) was treated with the indicated concentration of drug dissolved in the DNA methylase assay buffer and the DNA(cytosine-5)methyltransferase preparation, made up to 100 μl with assay buffer. The mixture was incubated at 37°C for 30 min, after which the reaction was terminated by adding 500 μl sterile 10 mM Tris-HCl pH 7.5, 0.2 M NaCl and 500 μl phenol

reagent. The upper aqueous layer was collected and the DNA was precipitated with 1 ml cold isopropanol and maintained at -20°C for 30 min. The DNA was redissolved in water and the precipitation process was repeated a further two times by adding 0.1 volumes of 2.5 M sodium acetate and 2.5 volumes of cold absolute ethanol. The DNA was then washed three times with absolute ethanol and then air dried ready for restriction enzyme analysis.

Restriction enzyme assay

Lambda phage DNA (5 μg) was treated with drug as described above and was then digested with 10 units of either MspI or HpaII. Digestion was carried out in the appropriate restriction buffer as recommended by the suppliers, in a total volume of 20 μl for 4 h at 37°C. The reaction was stopped by adding 4 μl of 6x concentration loading buffer and then heated in a water-bath at 65°C for 10 min. Samples were then analysed as described in section 4.2.12 using a 1.2% agarose gel, made up in TBE buffer with 0.5 μg/ml ethidium bromide and run at 5 V/cm on an AE-610 submerged agarose EP kit. When the tracking dye was 1-2 cm from the end of the gel, electrophoresis was stopped, and the DNA was revealed using a 302 nm transilluminator and then photographed with a Polaroid MP4 camera and type 665 black and white film.

8.2.2 Experiments to determine whether L-methionine-S-methylsulphonium iodide is a substrate for DNA(cytosine-5)methyltransferase.

DNA(cytosine-5)methyltransferase was prepared as described in section 3.1.1.

a) Competition assay.

To the standard DNA(cytosine-5)methyltransferase assay described in section 3.1.3, was added 10 µl of 0.1 mM, 1.0 mM, 10 mM or 0.1 M L-methionine-S-methylsulphonium iodide dissolved in water, so that the final drug concentrations were 0.01 mM, 0.1 mM, 1.0 mM or 10 mM respectively. Incubations were carried out at 37°C for 4 h with 20 µg M.lysodeikticus DNA and 1 µCi SAM in a total volume of 100 µl.

b) Using L-methionine-S-[^{3}H-methyl] methylsulphonium iodide.

L-Methionine-S-[^{3}H-methyl]methylsulphonium iodide was prepared by Mr G M Currie (British Technology Group student) from [^{3}H-methyl]methionine and iodomethane.

The standard DNA(cytosine-5)methyltransferase assay described in section 3.1.3 was used. Incubations were carried out at 37°C for 4 h with 20 µg M.lysodeikticus DNA, a fixed amount of DNA(cytosine-5)methyltransferase and either 1, 2.5, 5, 7.5 or 10 µCi of S-adenosyl-L-[^{3}H-methyl]methionine or L-methionine-S-[^{3}H-methyl]methylsulphonium iodide (specific activity 72 Ci/mmol and 70 Ci/mmol

respectively). The experiments were run in parallel using the same batch of DNA(cytosine-5)methyl-transferase.

8.2.3 Experiment to determine the ability of peptides to act as specific DNA methylating enzymes.

Each assay contained 20 μg of M.lysodeikticus DNA (2.0 mg/ml in water), poly[dC] (2.0 mg/ml in water) or poly[d(G-C)].poly[d(G-C)] (2.0 mg/ml in water), 1.0 μCi (72 Ci/mmol) S-adenosyl-L-[methyl-^{3}H]methionine and 10 μl of 10 μM or 1 mM peptide (dissolved in water) so that the final concentration was either 1.0 μM or 0.1 mM. Incubations were carried out at 37°C for 4 h in a total volume of 100 μl in a shaking water-bath. The reaction was stopped by adding 40 μg calf thymus DNA (2.0 mg/ml in water) to act as a carrier and 200 μl of 1.0 M NaOH. The mixtures were then heated to 65°C for 20 min in a water-bath, after which they were placed on ice and the DNA was precipitated by adding 80 μl of 5.0 N $HClO_4$. After at least 1 h the DNA was filtered onto a Whatman GF/C glass microfibre filter and washed with cold 5% TCA (w/v) and absolute ethanol. After air drying the filters overnight, the radioactivity was determined by adding 8 ml of toluene/PPO/POPOP scintillant and counting on a Packard Tri-Carb 2000CA liquid scintillation counter.

The following peptides synthesised by Mr G Currie were investigated:

1. Cys-Ala-Pro-Cys-Leu-Pro-Ala-Gly
2. Pro-Cys-Cys-Pro
3. Gly-Phe-Pro-Cys-Pro-His-Phe-Ser
4. Ser-Pro-Cys-Met-Arg-Leu-Ile-His-Leu
5. Pro-Cys-His-Met-Asp
6. Cys-Dimet
7. Dipro-Cys
8. Cys-Gly
9. Cys-Met

8.2.4 Statistical Analysis

All results are expressed as mean ± standard error of the mean (SEM) for at least three separate determinations.

8.3 RESULTS

The assay of sulphonium compounds as potential C-5 cytosine methylating agents.

In anticipation of a range of novel sulphonium compounds to assay, it was proposed to carry out some initial investigations with the use of the isoschizomeric pair of restriction endonucleases, MspI and HpaII in an assay to detect C-5 cytosine methylation.

It was first described by Waalwijk and Flavell (1978) that the cleavage of DNA by the restriction endonuclease HpaII, is prevented by the presence of a C-5 methyl group at the internal cytosine residue of its recognition sequence CCGG. MspI, an isoschizomer of HpaII, cleaves DNA irrespective of the presence of a methyl group at this position. This property of these restriction enzymes has been widely used in molecular biology to study the methylation status of DNA generally or particular genes. It was first shown by McGhee and Ginder (1979), that certain specific methylation sites in the region of the β-globin gene are methylated to different extents in different tissues and Mandel and Chambon (1979), correlated under-methylation and gene activity in the chicken ovalbumin gene using these restriction endonucleases.

Therefore, it appeared feasible that this isoschizomeric pair of restriction endonucleases, could be used in a sensitive *in vitro* assay to

measure potential C-5 cytosine methylations that the sulphonium compounds under investigation may produce. It was decided to use bacteriophage lambda DNA in the assay system, to investigate the capabilities of various sulphonium compounds to act as methylating agents. The recognition sites for the restriction endonucleases MspI and HpaII are illustrated in fig. 58. The nucleotide sequence of bacteriophage lambda DNA has been determined (Sanger et al., 1982) and it consists of 48502 base pairs, which corresponds to 328 MspI/HpaII cleavage sites. This produces a range of clearly visible bands, when separated by agarose gel electrophoresis and any inhibition of cutting by these restriction enzymes by C-5 cytosine methylation would be clearly evident.

A number of commercially available sulphonium compounds (fig. 59 for structures) were investigated in this system. It was hoped to synthesise a molecule with both a methyl sulphonium group and a thiol group within the same molecule, but these commercially available compounds contained only the methyl sulphonium group.

A selection of results from this assay are illustrated in fig. 60 to fig. 63. However, it must be borne in mind that the system was not very reproducible. The first of the sulphonium compounds to be investigated was trimethylsulphonium iodide and a very high concentration of 500 mM had to be used

before any effect could be seen. Lambda phage DNA was incubated with trimethylsulphonium iodide in water at 37°C for various period of time. After extensive washing with water and drying with absolute ethanol, the DNA which was dissolved in the appropriate restriction buffer, was digested with an excess of either MspI or HpaII. The digest was then separated by agarose gel electrophoresis. The results are shown in fig. 60 and it can be seen that as the incubation time is increased, there is an increase in the molecular weight of the restriction fragments after MspI and HpaII treatment, when compared to the control restriction enzyme treated DNA. The control DNA in lanes 1 and 2 had been incubated in water at 37°C for 2 h without the sulphonium compound being present and then processed in exactly the same way as the drug-treated DNA. The DNA in lanes 3, 6 and 9 had been treated with trimethylsulphonium iodide (0.5 M) but was not digested with a restriction enzyme. After 0.5 h incubation with trimethylsulphonium iodide there was an inhibition of cutting by the restriction enzyme HpaII (lane 5), with a reduction in the appearance of low molecular weight bands and the appearance of some higher molecular weight DNA, when compared to the control (Lane 2). After 0.5 h incubation with trimethylsulphonium iodide, there does not appear to be any change in the restriction pattern of the MspI

treated DNA (lane 4) when compared to the control (lane 1). After 1 h incubation with trimethylsulphonium iodide, there was an inhibition of cutting by the restriction enzyme MspI (lane 7) and HpaII (lane 8), with a corresponding reduction in the appearance of low molecular weight bands and the appearance of higher molecular weight DNA when compared to the control (lane 1 and 2 respectively). After 2 h incubation with trimethylsulphonium iodide, there was an inhibition of cutting by the restriction enzyme MspI (lane 10), with a reduction in the appearance of low molecular weight bands and the appearance of higher molecular weight DNA when compared to the control (lane 1). The DNA in lane 11 (2 h incubation with trimethylsulphonium iodide and HpaII digestion) appears to have been completely degraded. There also appears to have been some DNA degradation in lane 6 (1 h incubation with trimethylsulphonium iodide control).

The inhibition of the cutting of the trimethylsulphonium iodide-treated lambda phage DNA by the restriction enzymes appears to be most pronounced for HpaII (lanes 5 and 8). Since HpaII does not cleave the sequence CCGG when either cytosines are methylated, it appeared on first examination that this simple sulphonium compound was methylating C-5 cytosine in a time-dependent fashion. However, it appears that other alkylations at the cytosine

residues within the sequence CCGG can block cutting. In particular, HpaII does not cleave DNA when either cytosine residue is modified to N^4-methylcytosine, whilst cutting by MspI is not affected by this modification (supplier's catalogue).

Cleavage of DNA by the restriction enzyme HpaII is blocked when either of the cytosines within the recognition sequence are 5-methylcytosine or N^4-methylcytosine. Therefore, in figs. 61 to 63 only HpaII was used to assess the methylation status of the drug-treated DNA. Although the rationale for drug design behind the use of sulphonium compounds was not to develop alternative substrates for DNA(cytosine-5)methyltransferase, they were occasionally tested in the presence of this enzyme.

The restriction patterns for dimethyl propargyl sulphonium bromide-treated lambda phage DNA are illustrated in fig. 61 and fig. 62. In both figures, the control DNA in lanes 1 and 2 had been incubated in water at 37°C for 0.5 h in the absence of DNA(cytosine-5)methyltransferase or sulphonium compound and then processed in exactly the same way as the drug-treated DNA. Cleavage of the drug-treated DNA by the restriction enzyme HpaII was inhibited by 75 mM dimethyl propargyl sulphonium bromide (lane 10, fig. 61), where undigested high molecular weight DNA was present. Inhibition of DNA cleavage by HpaII occurred for 0.2 M

dimethyl propargyl sulphonium bromide (lanes 4 and 5, fig. 8) and 0.4 M dimethyl propargyl sulphonium bromide (lane 8 fig. 62) where three new bands of higher molecular weight compared to the control (lanes 1 and 2) were produced. Lane 7, fig. 62 (0.4 M dimethyl propargyl sulphonium bromide) is another example where the DNA has been degraded.

Carboethoxymethyl dimethylsulphonium bromide (dissolved in water) treatment of the lambda phage DNA at high concentrations (0.5 M and 0.6 M) caused the appearance of one or two new high molecular weight bands after HpaII treatment (lanes 10, 11, 13, 14, fig. 62), but they are very faint.

The results suggest that neither dimethyl propargyl sulphonium bromide or carboethoxymethyl dimethylsulphonium bromide are substrates for DNA(cytosine-5)methyltransferase, as there are no significant differences between the restriction pattern for DNA incubated with the sulphonium compound in the presence or absence of DNA(cytosine-5)methyltransferase. This is excepting lane 10 and 11, fig. 61 in which 75 mM dimethyl propargyl sulphonium bromide (lane 10) caused the inhibition of HpaII digestion, whilst in the presence of DNA(cytosine-5)methyltransferase (lane 11) the restriction pattern was similar to the control. This also does not suggest that the sulphonium compound is

a substrate for DNA(cytosine-5)methyltransferase and an explanation for the inhibition of digestion by HpaII (lane 10) is difficult to reach.

The sulphonium compound L-methionine-S-methylsulphonium iodide was then investigated in this system, both in the presence or absence of DNA(cytosine-5)methyltransferase (Fig 63). After HpaII digestion a single band of higher molecular weight compared to the control (lane 2) appeared for concentrations of 50 mM (lane 11) and 75 mM (lane 14) L-methionine-S-methylsulphonium iodide in the presence of DNA(cytosine-5)methyltransferase. Due to the similarity in structure between this compound and the natural methyl donor SAM, it is possible that it may be behaving as a weak substrate for DNA(cytostine-5)methyltransferase.

This series of experiments does not represent a complete experimental investigation of the use of the isoschizomeric pair of restriction enzymes MspI and HpaII in an assay for drug-induced C-5 cytosine methylation. No novel sulphonium compounds were synthesised due to difficulties in the chemistry, and as a result this aspect of the project was dropped. Therefore, a number of important controls were not conducted and should be mentioned at this stage. Firstly, a study of the methylation pattern of lambda phage DNA after treatment with SAM and DNA(cytosine-5)methyltransferase was not investigated. This would

have been a useful positive control in the assessment of the restriction enzyme digests of the lambda phage DNA. Secondly, other alkylations apart from C-5 cytosine within the recognition sequence -CCGG - of the restriction enzymes MspI and HpaII, may inhibit cleavage by these enzymes. An ideal control would have been to methylate lambda phage DNA to various extents with [^{14}C]-methyl labelled methyl methane-sulphonate. Therefore, any inhibition of cutting by MspI or HpaII could be related to the site of methylation.

There were numerous problems associated with the restriction enzyme assay. Firstly, other alkylations within the recognition sequence (-CCGG-) may affect cleavage of the DNA by the restriction enzymes. It is known that N^4-methylcytosine prevents cleavage of DNA by HpaII (information provided by the supplier of the restriction enzymes). Secondly, the DNA(cytosine-5)methyltransferase preparation was impure and may contain other proteins which block recognition by the restriction enzymes. This may include proteins that bind preferentially to 5-methylcytosine rich DNA (Huang et al., 1984; Meehan et al., 1989). Thirdly, the system was not very reproducible. Therefore, after consideration of these problems the assay was unsuitable to make any conclusions from and no further effort was applied to it.

Experiments to determine whether L-methionine-S-methylsulphonium iodide is a substrate for DNA(cytosine-5)methyltransferase.

There was a slight indication from fig. 63 that L-methionine-S-methylsulphonium iodide may behave as a substrate for DNA(cytosine-5)methyltransferase. However, it must be stressed that cleavage of lambda phage DNA treated with L-methionine-S-methylsulphonium iodide and the DNA(cytosine-5)methyltransferase preparation by HpaII, was inhibited only at high concentrations of the sulphonium compound and the reproducibility of these results was poor.

There are strong structural similarities between SAM, the natural substrate for DNA(cytosine-5)methyltransferase and the L-methionine-S-methylsulphonium iodide. Therefore, it was a reasonable proposition that this compound may behave as a weak substrate and consequently warranted some further work. This was investigated by means of a competition assay, in which a fixed amount of labelled S-adenosyl-L-[^{3}H-methyl]methionine (1 μCi) was placed in an assay with increasing concentrations of L-methionine-S-methylsulphonium iodide. One would expect that if the L-methionine-S-methylsulphonium iodide was competing with the S-adenosyl-L-methionine for sites within the DNA(cytosine-5)methyltransferase molecule, there would be a decrease in the amount of radioactivity in terms of methyl groups transferred from [^{3}H-methyl]

SAM to the DNA substrate. However, as can be seen from fig. 64 there was no inhibition of the methylation of M.lysodeikticus DNA by the DNA(cytosine-5)methyltransferase, as the concentration of L-methionine-S-methylsulphonium iodide was increased. In fact there was a slight increase in DNA(cytosine-5)methylation at a concentration of 10 mM L-methionine-S-methylsulphonium iodide which cannot be explained.

In order to clarify whether L-methionine-S-methylsulphonium iodide was a substrate for DNA(cytosine-5)methyltransferase, the S-[^{3}H-methyl]labelled compound was synthesised by Mr G M Currie (British Technology Group student). This was prepared from [^{3}H-methyl]methionine and iodomethane and had a purity of >90%. Experiments were run in parallel using S-adenosyl-L-[^{3}H-methyl]methionine or L-methionine-S-[^{3}H-methyl]methylsulphonium iodide as methyl donors for DNA(cytosine-5)methyltransferase and using M.Lysodeikticus DNA as the methyl acceptor. The results are shown in fig. 65 where the number of methyl groups incorporated into the DNA was compared with the amount of S-adenosyl-L-methionine or L-methionine-S-methylsulphonium iodide (the specific activities were 72 Ci/mmol and 70 Ci/mmol respectively). The amount of radioactivity incorporated into M.lysodeikticus DNA using S-adenosyl-L-[^{3}H-methyl]methionine as the methyl donor was greater

when carried out in the presence of DNA(cytosine-5)methyltransferase. For, L-methionine-S-[^{3}H]methylsulphonium iodide the amount of radioactivity incorporated into the DNA was similar when carried out in the presence or absence of DNA(cytosine-5)methyltransferase.

It cannot be concluded absolutely that no 5-methylcytosine had been produced in the DNA, since very low levels of C-5 cytosine methylation may have occurred. However, considering all the results it seems unlikely that L-methionine-S-methylsulphonium iodide is a substrate for DNA(cytosine-5)methyltransferase.

The effect of peptides to act as synthetic DNA(cytosine-5)methyltransferase enzymes.

Since no novel sulphonium compounds were synthesised, a new approach was taken for the synthesis of potential C-5 cytosine methylating agents. The idea was to develop peptides which would mimic DNA(cytosine-5)methyltransferase and use intracellular SAM as the methyl donor. This approach also provided a very simple assay system to test any potential compounds. The enzyme was replaced with the peptide in the standard DNA(cytosine-5)methyltransferase assay and using S-adenosyl-L-[^{3}H-methyl]methionine as the methyl donor, a transfer of radioactivity in terms of methyl groups could be

easily detected. The peptides synthesised by Mr G Currie were assessed for their ability to catalyse the methylation of various nucleic acid substrates when [^{3}H]SAM was present as the methyl donor.

The results in table 25 show that none of the peptides so far investigated could utilise S-adenosyl-L-[^{3}H-methyl]methionine and methylate DNA. The incubation of DNA or peptide with [^{3}H]SAM produced a background count of 935 ± 67 DPM and 928 ± 48 DPM respectively. The majority of the peptides incubated with DNA and [^{3}H]SAM produced a level of incorporation less than background. However, Pro-Cys-His-Met-Asp at a concentration of 0.1 mM produced an incorporation of 1611 ± 85 DPM, but this was not a significant increase. The positive control in this system was DNA(cytosine-5)methyltransferase in place of the peptide, where generally counts greater than 80,000 DPM were achieved. The nucleic acids, poly[dC] and poly[d(G-C)].poly[d(G-C)] were also investigated for a selection of the peptides, but the level of radioactivity incorporation was less than background.

The fact that the peptides tested could not act as methyl donors was not surprising, since the largest peptide so far investigated was only nine amino acids. A small peptide is hardly likely to

mimic the activity of DNA(cytosine-5)methyl-transferase which consists of 1573 amino acid residues (Bestor et al., 1988).

8.4 DISCUSSION

A number of approaches have been made to develop drugs which have an effect on DNA(cytosine-5)methylation, as chemotherapeutic agents in cancer and viral infection. The development of such drugs has been mainly directed towards agents which inhibit DNA methylation and consequently cause DNA hypomethylation.

The inhibition of S-adenosyl-L-homocysteine hydrolase is one target which has received some attention. This enzyme regulates DNA methylation by controlling the intracellular levels of S-adenosylhomocysteine, which is a potent inhibitor of DNA(cytosine-5)methyltransferase *in vitro* (Adams and Burdon, 1983) and this has been shown to reduce the level of 5-methylcytosine of cells in culture (Kredich and Martin, 1977). This has included the synthesis of carbocyclic analogues of adenosine substituted at the 6'-position (Madhavan *et al.*, 1988), 4',5'-unsaturated 5'-fluoroadenosine nucleosides (McCarthy *et al.*, 1989) and analogues of Neplanocin A (Borcherding *et al.*, 1988). Such an approach appears to be valid as Neplanocin A, one of the most potent inhibitors of SAH hydrolase has been shown to have anti-tumour activity against the murine leukemia L1210 (Yaginuma *et al.*, 1981).

5-Azacytidine is one of the most potent hypomethylating agents and has some use in the clinic, particularly in the treatment of acute myelocytic leukemia (Karon et al., 1973). Interest in this drug has been due to its ability to alter gene activity and this has resulted in the synthesis of many analogues (Carr et al., 1988; Jones and Taylor, 1980) in the hope of finding an analogue which retains the properties of gene activation, without the carcinogenic properties. It seems likely however, that the two activities are the same.

The second approach that has received less attention, is the development of drugs which cause hypermethylation. This would theoretically be more effective in chemotherapy as it is envisaged that the 'switching off' of over-expressed or aberrant genes would be more beneficial than the 'switching on' of genes, which perhaps has carcinogenic consequences. However, the development of agents which inhibit DNA(cytosine-5)methylation is easier to approach through the intervention of the various biochemical pathways which affect the ratio of SAM/SAH. Attempts to cause cellular DNA hypermethylation have involved increasing the concentration of SAM by feeding large amounts of choline and/or methionine in order to push the de novo methyltransferase reaction to the right. Hepatic SAM concentrations are decreased in animals ingesting diets deficient in choline (Zeisel et al.,

1989). It was suggested that this was due to the availability of methionine which is synthesised from choline limiting SAM synthesis. In a study by Mikol et al. (1983), 40% of rats fed a methionine and choline-deficient diet supplemented with DL-homocysteine developed hepatomas and cholangiomas. Supplementing the diet with DL-methionine at 5 g/kg and choline at 2 g/kg body weight afforded some protection for the animals against diethylnitrosamine induced liver carcinogenesis and even when liver cancer occurred there was still some protection against lung metastases.

DNA synthesis inhibitors such as cytosine arabinoside, hydroxyurea, aphidicolin (Nyce et al., 1986) and 1-β-D-arabinofuranosyl cytosine (ara-C), aphidicolin and hydroxyurea (De Haan and Parker, 1988), have been shown to cause DNA hypermethylation. An absolute requirement for such a drug, if there is to be any clinical importance is that the induced hypermethylation is stable in the absence of the drug. Nyce et al. (1986) have shown that this is the case with hydroxyurea, suggesting that the hypermethylated sequences may be heritable. However, hydroxyurea has been shown to cause amplification of the dihydrofolate reductase gene in CHO cells (Mariani and Schimke, 1984). Consequently, the observed hypermethylation may not be of any significance.

Another agent, sodium butyrate (5-20 mM) has been shown to cause hypermethylation as one of its effects. Hypermethylation occurs in the normal WI-38 lung fibroblast cell line as well as the transformed counterparts, SVW-38 and CT-1 (Parker et al., 1986; De Haan and Parker, 1988). An inhibition of DNA synthesis occurs in the normal cells but not in the transformed counterparts and so this is unlikely to be the mechanism by which the hypermethylation occurs.

There have been no reports on the existence of any drugs, or the development of drugs, which can methylate DNA at the C-5 position of cytosine. An initiative of Professor M F G Stevens of the Pharmaceutical Sciences Institute, Aston University, was to develop compounds which would specifically methylate C-5 cytosine within specific gene sequences. The intention was that this would 'switch off' over-expressed or aberrant genes in the neoplastic state, such as the ras oncogene. However, there exists a number of problems with such an approach which were not considered at the onset of the project.

Firstly, it is not known at what stage during the development of a tumour that the gene(s) become(s) hypomethylated with respect to 5-methylcytosine. The project is based on the superficial assumption that a tumour is a direct

result of specific genes losing methyl groups from 5-methylcytosine. However, the hypomethylation observed in many tumours may not occur at the initiation stage but at a much later stage in the process. The stage at which DNA becomes hypomethylated with respect to 5-methylcytosine has been studied in human colon cancer cells (Goelz et al., 1985; Feinberg et al., 1988). Analysis of the 5-methylcytosine content of benign and malignant tumours led to the conclusion that the loss of methyl groups occurs early in the process, since both the benign adenomas and carcinomas were similarly hypomethylated when compared to adjacent normal tissue.

Secondly, the restrictions based on the requirements for chemical reaction of such an agent would have to be enormous. The agent would be required to methylate not only a specific base within DNA, namely cytosine, but also specifically the C-5 position. It would appear impossible to methylate a carbon atom specifically in the presence of competing O- and N-nucleophiles. Although agents such as bisulphite, hydroxylamine, methoxyamine and hydrazine can add to the 5,6-double bond of cytosine (Singer and Grunberger, 1983), none of the monofunctional alkylating agents can do this. A further restriction of such a drug would be that its alkylating activity would have to be specific for a particular gene, identified as being responsible for the tumour. If

one assumes that methylation of the C-5 position of cytosine is capable of 'switching off' a gene, then genomic wide methylation is to be avoided as this would be deleterious to all cells and the specificity of the drug against tumours would be lost. It is also very unlikely that hypomethylation of a single gene determines whether or not it is a cancer cell.

Thirdly, it would be further necessary for this chemical agent to methylate both strands of the DNA. If only one strand was methylated, this could be lost if DNA replication occurred in the absence of maintenance methylation.

Two approaches were taken to generate compounds with the ability to methylate the C-5 position of cytosine within DNA. The initial idea was to synthesise a compound with a sulphonium methylating centre at one end of the molecule, with a nucleophilic thiol (-SH) group at the other end, and an appropriate 'linker' group between them. There was no progress made in the synthesis of compounds bearing this structure and consequently, the chemical approach to the problem was abandoned. There was some preliminary work done on a restriction enzyme assay, to detect drug-mediated C-5 cytosine methylation by sulphonium compounds. There were numerous problems associated with the assay and as no

suitable compounds were synthesised, further work on the development of a restriction enzyme assay was abandoned.

The observed changes in the cutting of lambda phage DNA with restriction enzymes MspI and HpaII after treatment with various sulphonium compounds, may be due to the alkylation of other sites within the recognition sequence, other than C-5 cytosine. This is a reasonable proposition as Green et al. (1984) have shown that the restriction enzyme PstI will not cleave oligonucleotides containing O^6-methylguanine in the restriction site. This concept was used by Wu et al. (1987) who developed an assay for O^6-alkylguanine DNA-alkyltransferase by detecting restriction endonuclease inhibition, due to the presence of O^6-methylguanine in the DNA e.g. HpaII was inhibited by the presence of O^6-methylguanine. Further, the restriction enzyme HpaII does not cleave DNA when either cytosine residue of the recognition sequence (-CCGG-) is modified to N^4-methylcytosine (information supplied by the suppliers of HpaII).

As it appears that some restriction enzymes are inhibited by other alkylations within the DNA, this questions the validity of some papers, which use these restriction enzymes to assess the methylation status of cellular DNA after treatment with alkylating agents. In particular, Boehm and Drahovský (1981) who observed hypomethylation of DNA

in Raji cells after treatment with MNU. The observed effect may have been an under-estimate of the level of hypomethylation if HpaII and MspI used in this study were inhibited by the alkylations produced by MNU.

The second approach for the development of C-5 cytosine methylating agents, was to develop peptides which contain the methyltransferase activity of DNA(cytosine-5)methyltransferase, and use intracellular SAM as the methyl donor. None of the peptides so far investigated showed any ability to utilise SAM and methylate DNA. This was not particularly surprising as the largest peptide so far synthesised was only 9 amino acids long, and this is hardly likely to contain the methyltransferase activity of an enzyme with 1573 amino acid residues (Bestor et al., 1988).

A large proportion of the enzyme would presumably be involved in regulating the activity of the enzyme, and Bestor et al. (1988) suggest that this function lies in the amino terminal portion of the enzyme. This still leaves a polypeptide of 570 amino acids, which is thought to represent the catalytic methyltransferase domain. Apart from utilising the pro-cys sequence which is present at the active site of many DNA methyltransferases, there appears to be no rational design for determining what amino acids should be included in the peptide.

A more logical approach to mimicking the methyltransferase activity of a peptide would be to start off with active enzyme (homogeneous) and by sequential proteolytic digestion, reduce the size of the enzyme until the smallest unit with methyltransferase activity was produced. This would involve the use of a range of proteolytic enzymes under carefully controlled conditions. This peptide could then be sequenced, which would allow production of large quantities for further investigation. By this technique none of the crucial DNA binding/recognition sites are excluded, which would be impossible to predict by progressively building up the peptide from Pro-Cys. Support for this approach comes from Adams et al. (1983) who treated DNA(cytosine-5)-methyltransferase with trypsin and observed an increase in de novo enzyme activity after the enzyme had become more relaxed with regard to DNA structural requirements. The mouse ascites DNA(cytosine-5)methyltransferase of molecular weight 185 kDa has been degraded proteolytically to give polypeptides with methyltransferase activity of 170, 100 and 50 kDa (Adams et al., 1986). The 1000 amino acid N-terminal domain contains a series of 5 to 7 structural units of molecular weight about 20 kDa, which when removed by partial proteolysis leads to the production of a series of catalytically active species. This region is thought to be involved in

DNA binding and recognition, whilst the smaller 570 amino acid and C-terminal domain, because of its similarity to bacterial type II cytosine methyltransferase, is thought to be the methyltransferase domain (Bestor et al., 1988).

RESTRICTION ENDONUCLEASES

MspI cleaves CmCGG and CCGG

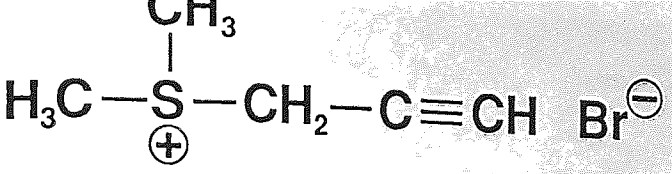
but not mCCGG

HpaII cleaves CCGG

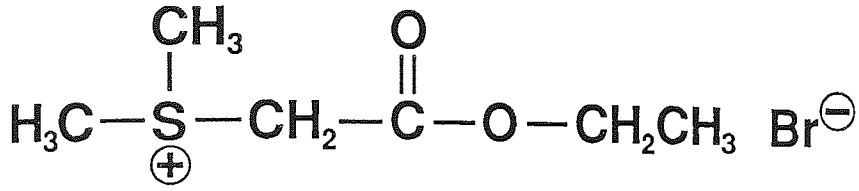
but not mCCGG or CmCGG

mC - could denote either 5-methylcytosine
or N^4-methylcytosine (HpaII).

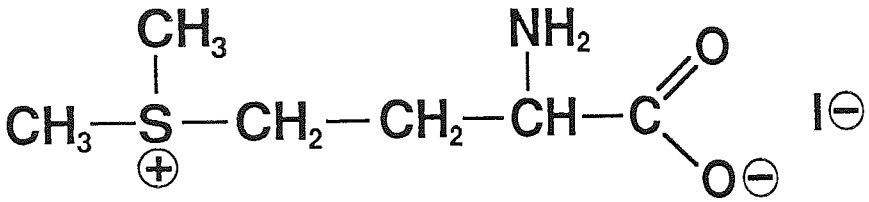
Figure 58: Recognition sites for the restriction endonucleases - HpaII and MspI.



Dimethyl propargyl sulphonium bromide



Carboethoxymethyl dimethylsulphonium bromide



L-Methionine-S-methylsulphonium iodide

S-Adenosyl-L-methionine

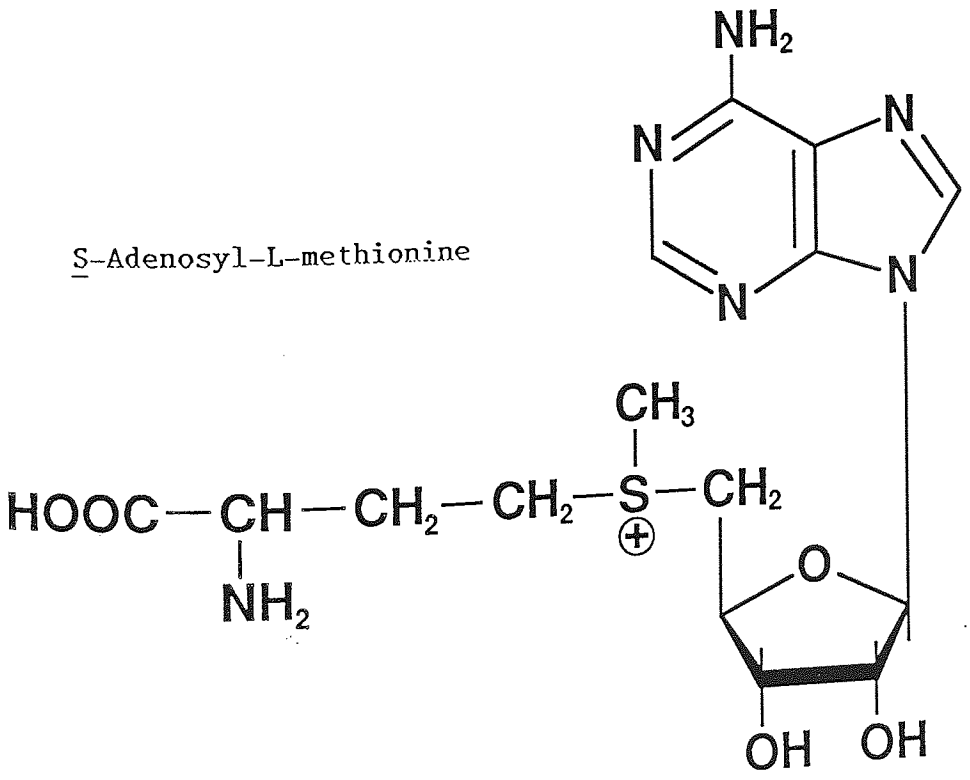


Figure 59: The structure of the commercially available sulphonium compounds used in this study.

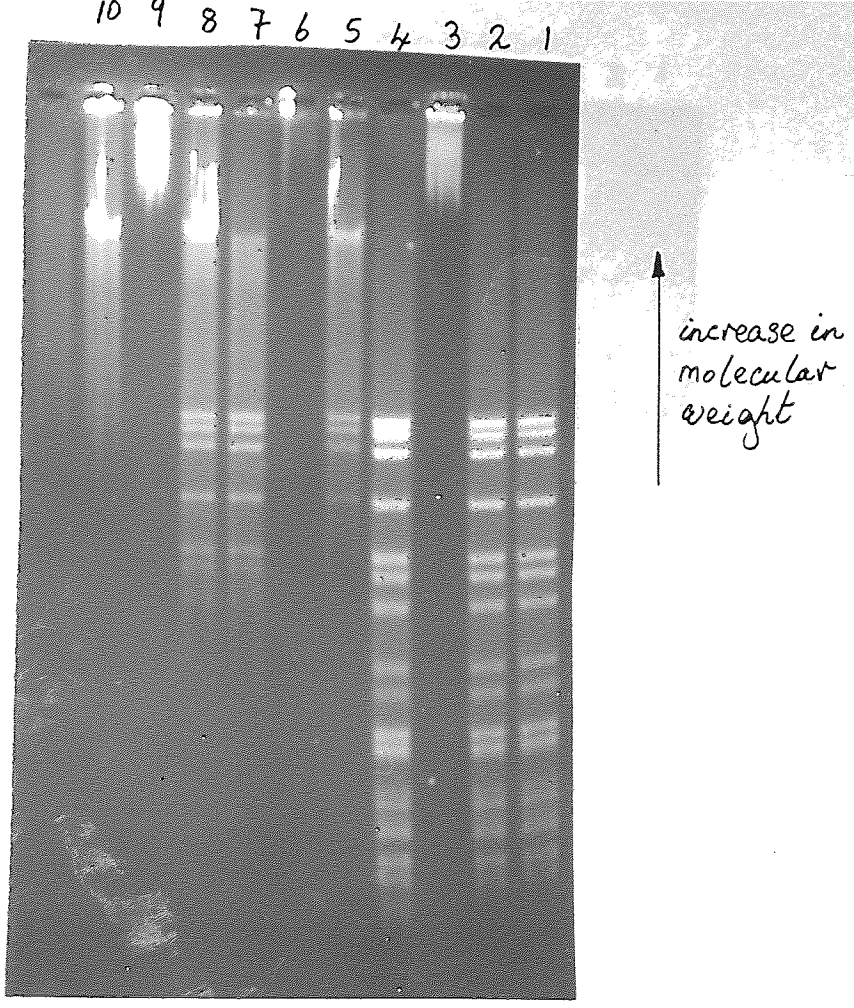


Figure 60: Restriction pattern for the time course of reaction of 500 mM trimethylsulphonium iodide with λ-phage DNA.
Key:- 1) control MspI, 2) control HpaII, 3) 0.5 h control, 4) 0.5 h MspI, 5) 0.5 h HpaII, 6) 1 h control, 7) 1 h MspI, 8) 1 h HpaII, 9) 2 h control, 10) 2 h MspI, 11) 2 h HpaII.

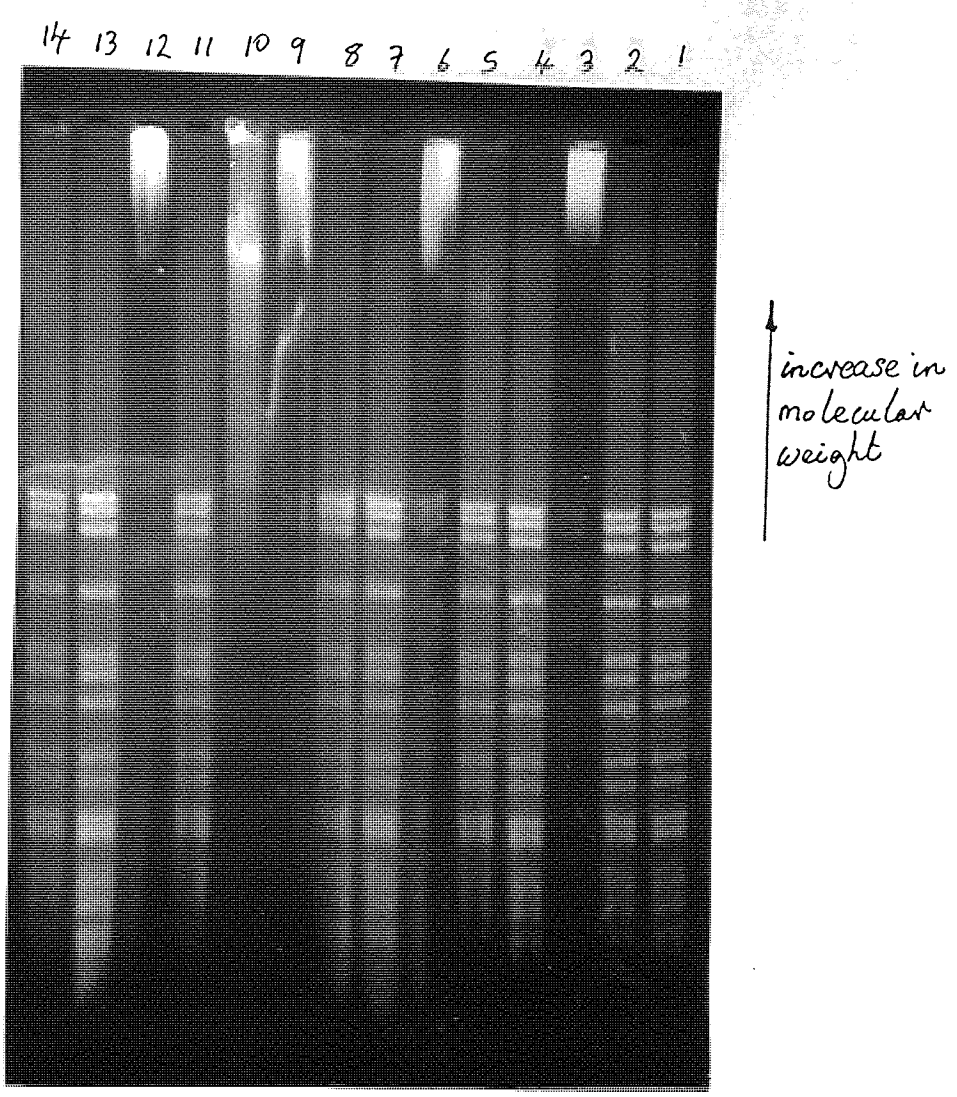


Figure 61: Restriction pattern for dimethyl propargyl sulphonium bromide-treated λ-phage DNA.
Incubation with drug was for 0.5 h.
Key: 1) control MspI, 2) control HpaII, 3) 25 mM DPSB + methyltransferase, 4) 25 mM DPSB, HpaII, 5) 25 mM, + methyltransferase HpaII, 6) 50 mM DPSB + methyltransferase, 7) 50 mM DPSB, HpaII, 8) 50 mM DPSB + methyltransferase, HpaII, 9) 75 mM DPSB + methyltransferase, 10) 75 mM DPSB, HpaII, 11) 75 mM DPSB + methyltransferase, HpaII, 12) 100 mM DPSB + methyltransferase, 13) 100 mM DPSB, HpaII, 14) 100 mM DPSB + methyltransferase, HpaII. The DNA(cytosine-5)methyltransferase was capable of transferring 0.4 pmol of methyl groups under the standard assay conditions.

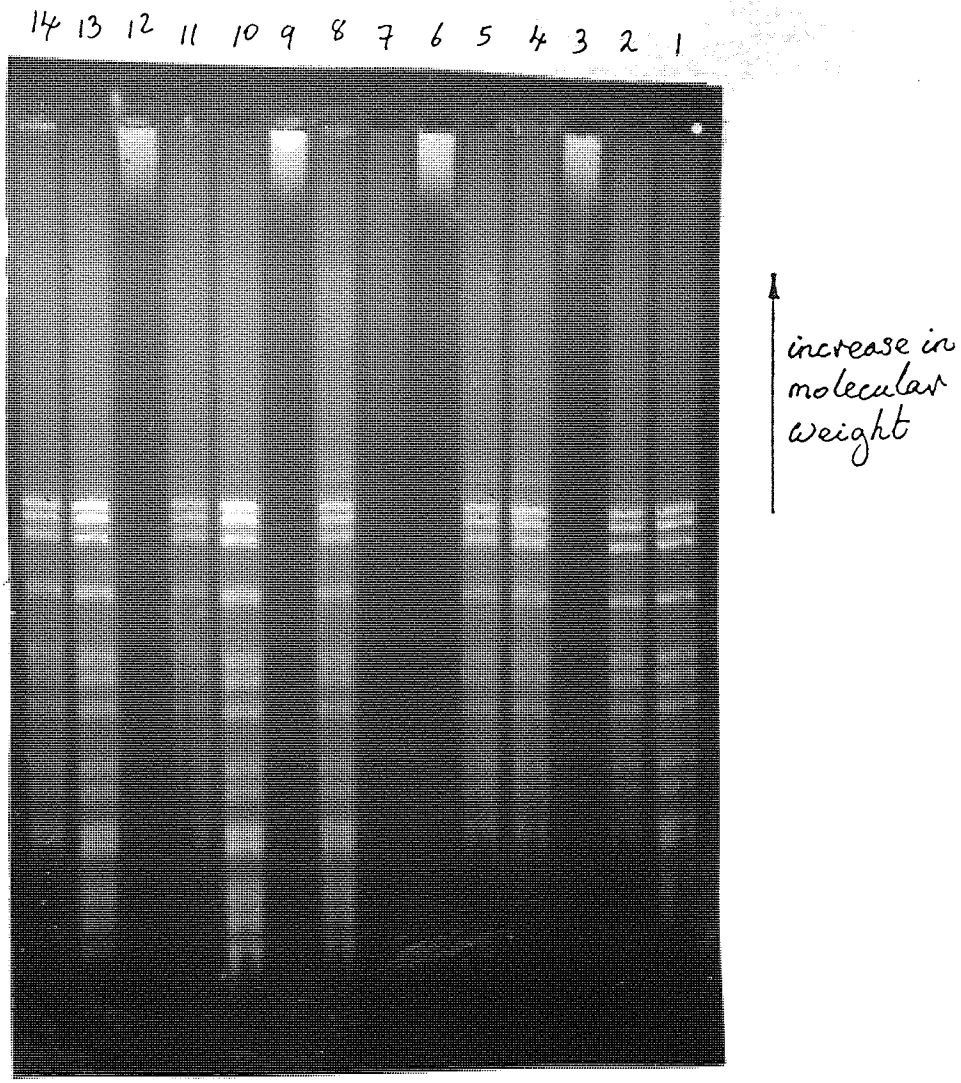


Figure 62: Restriction pattern for dimethyl propargyl sulphonium bromide and carboethoxymethyl dimethylsulphonium bromide-treated λ-phage DNA.
Incubation with drug was for 0.5 h.
Key: 1) control MspI, 2) control HpaII, 3) 0.2 M DPSB + methyltransferase, 4) 0.2 M DPSB, HpaII, 5) 0.2 M DPSB, + methyltransferase, 6) 0.4 M DPSB + methyltransferase, 7) 0.4 M DPSB, HpaII, 8) 0.4 M DPSB + methyltransferase, HpaII, 9) 0.5 M CDSB + methyltransferase, 10) 0.5 M CDSB, HpaII, 11) 0.5 M CDSB + methyltransferase, HpaII, 12) 0.6 M CDSB + methyltransferase, 13) 0.6 M CDSB, HpaII, 14) 0.6 M CDSB + methyltransferase, HpaII. The DNA(cytosine-5)methyltransferase was capable of transferring 0.4 pmol of methyl groups under the standard assay conditions.

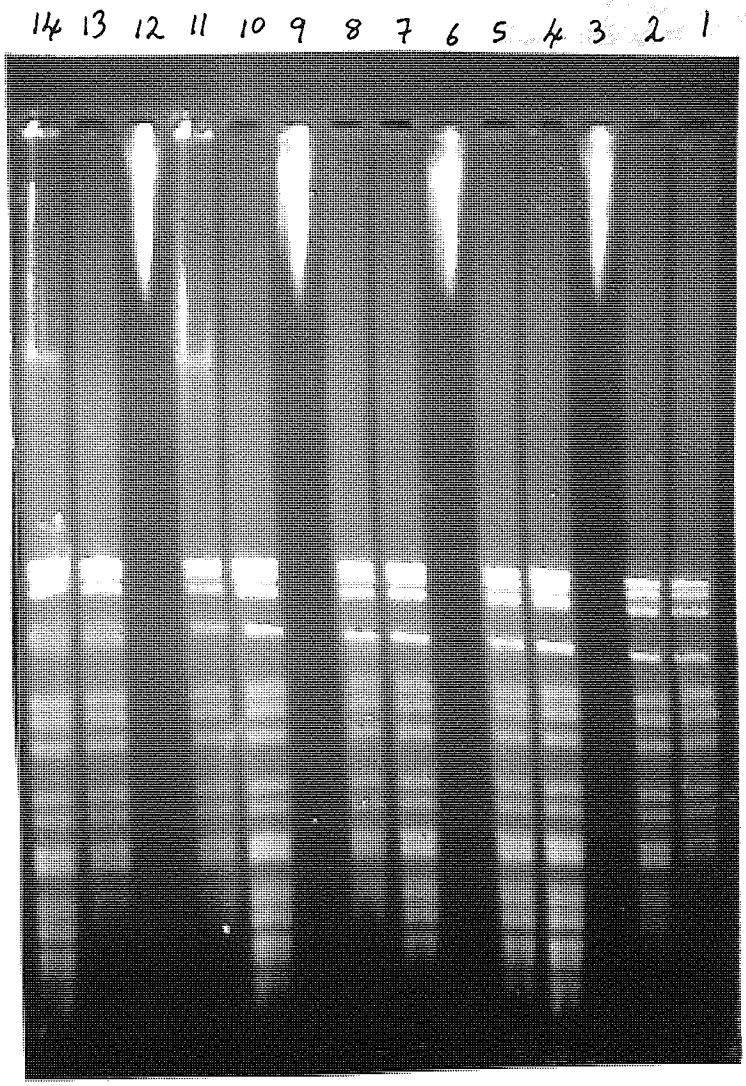


Figure 63: Restriction pattern for L-methionine-S-methylsulphonium iodide-treated λ-phage DNA.
Incubation with drug was for 0.5 h.
Key: 1) control MspI, 2) control HpaII, 3) 12.5 mM + methyltransferase, 4) 12.5 mM. HpaII. 5) 12.5 mM. + methyltransferase, HpaII, 6) 25 mM. + methyltransferase, 7) 25 mM. HpaII, 8) 25 mM, + methyltransferase, HpaII. 9) 50 mM, + methyltransferase, 10) 50 mM. HpaII. 11) 50 mM. + methyltransferase, HpaII, 12) 75 mM, + methyltransferase, 13) 75 mM. HpaII, 14) 75 mM, + methyltransferase, HpaII. The DNA(cytosine-5)methyltransferase was capable of transferring 0.4 pmol of methyl groups under the standard assay conditions.

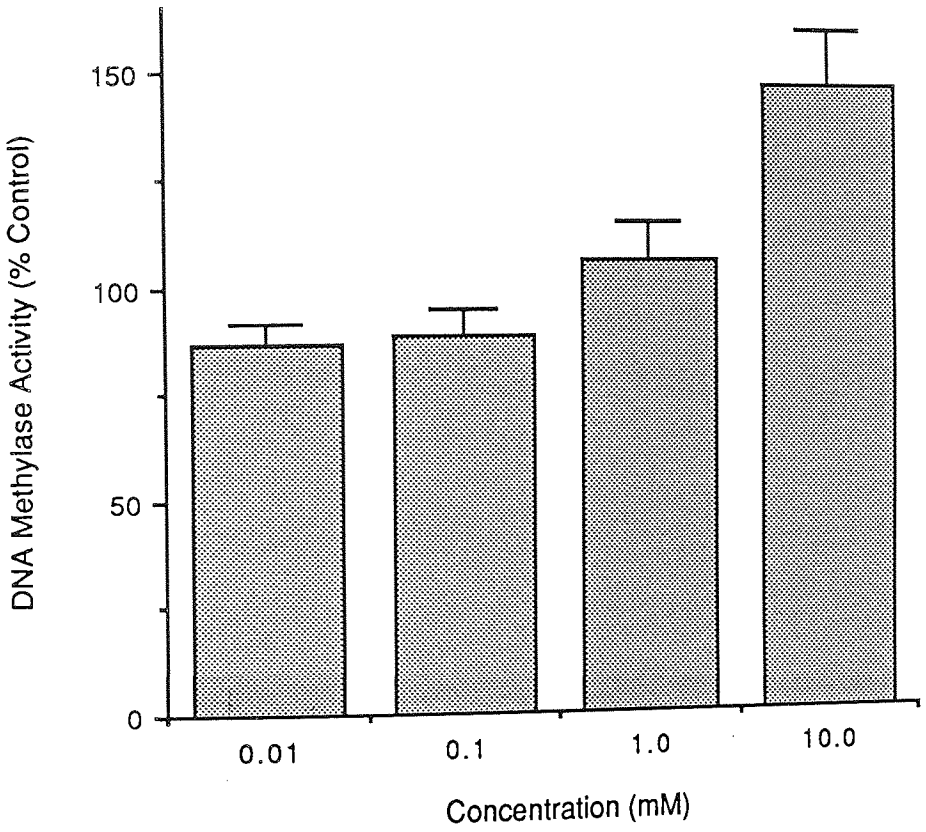
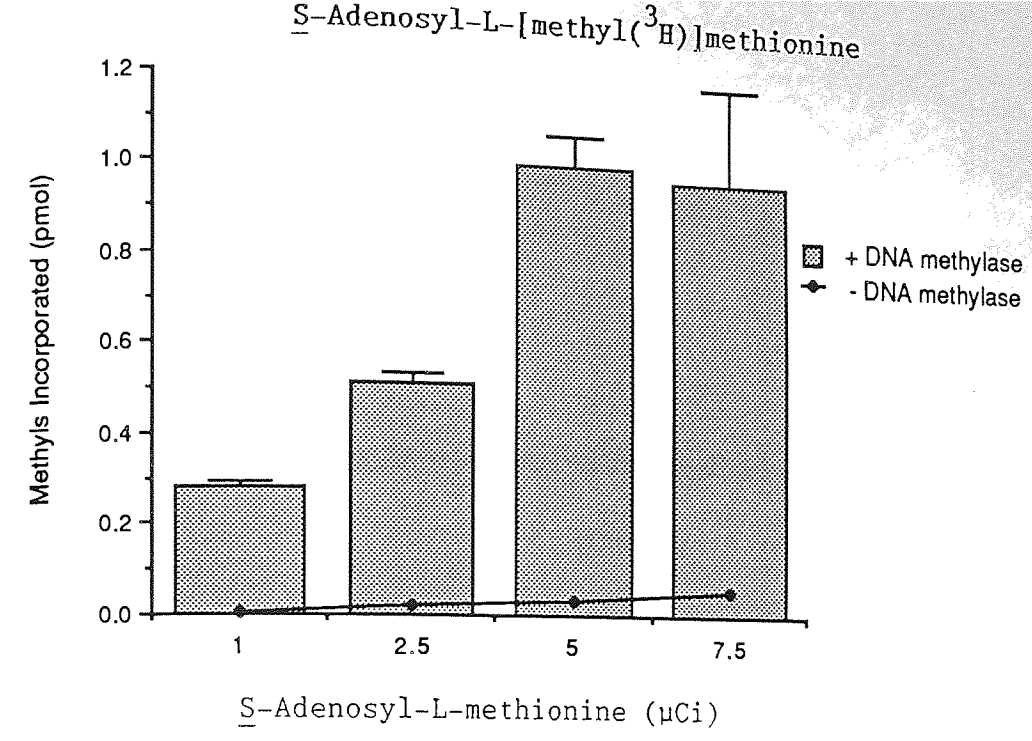


Figure 64: The effect of L-methionine-S-methylsulphonium iodide on the methylation of M.lysodeikticus DNA when in competition with [^{3}H]SAM.
Incubations were carried out at 37°C for 4 h with 20 µg M.lysodeikticus DNA, 1 µCi SAM and a fixed amount of enzyme. L-methionine S-methyl sulphonium iodide was added in increasing concentrations to determine whether it competes with SAM. The results are expressed as the mean ± SEM (n=4).



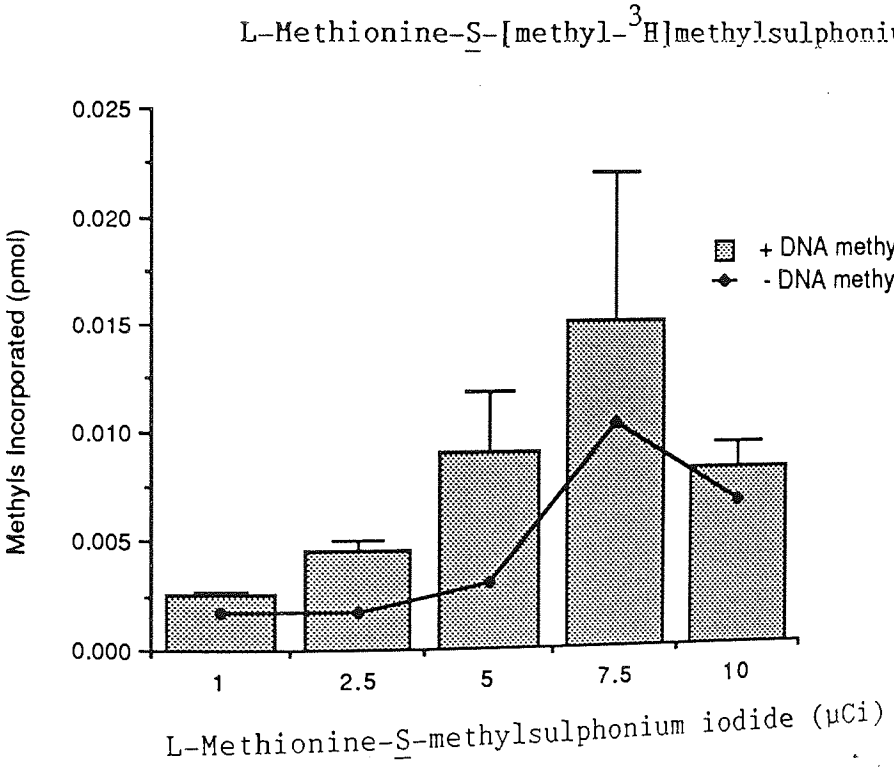


Figure 65: Experiment to determine whether L-methionine-S-methylsulphonium iodide is a substrate for DNA(cytosine-5)-methyltransferase.
a) Incubations were carried out at 37°C for 4 h with 20 µg *M.lysodeikticus* DNA and increasing amounts of S-adenosyl-L-[methyl(^{3}H)]methionine in the presence or absence of DNA(cytosine-5) methyltransferase.
b) Incubations were carried out at 37°C for 4 h with 20 µg *M.lysodeikticus* DNA and increasing amounts of L-methionine-S-[^{3}H]methylsulphonium iodide in the presence or absence of DNA(cytosine-5)methyltransferase.
The experiment was performed three times using the same batch of DNA(cytosine-5)methyltransferase.

CHAPTER NINE

SUMMARY

5-Methylcytosine is the only modified base found naturally in higher eukaryotes and generally 3-6% of DNA cytosine is methylated. The function of DNA methylation is a subject of some discussion, but the majority of work has concentrated on its possible role in controlling gene expression, since a large number of studies have shown that active genes are hypomethylated in expressing cell types and inactive genes are methylated in non-expressing cell types. 5-Methylcytosine in DNA may affect gene expression through its effect on DNA-protein interactions, in particular the binding of transcription factors and its effect on chromatin structure (reviewed by Adams, 1990).

The role of DNA methylation in tumour development has been questioned, since there is a general trend for tumour cells to contain hypomethylated DNA when compared to normal cellular counterparts (Diala et al., 1983; Gama-Sosa et al., 1983). In particular, Vogelstein and colleagues have been carrying out studies on the development of colon cancer in humans. They compared the methylation status of normal colon tissue with benign adenomas and malignant colon carcinomas and provided evidence that the loss of methyl groups occurs as an early event in the development of a tumour (Feinberg and Vogelstein, 1983a, 1983b; Goelz et al., 1985; Feinberg et al., 1988).

A substantial number of experiments have shown that many carcinogens can inhibit DNA(cytosine-5)-methylation *in vitro* through reaction with the methyltransferase or with the DNA substrate (for example Cox, 1980; Chan *et al*., 1983; Pfohl-Leszowicz *et al*., 1983a; Wilson and Jones, 1983). A number of these have also been shown to inhibit DNA methylation in living cells (see table 6). However, there are some notable exceptions, for example MNU which is a potent inhibitor of DNA(cytosine-5)methyltransferase *in vitro* (Pfohl-Leszkowicz *et al*., 1983a, 1983b, 1982; Wilson and Jones, 1983) has been reported not to cause DNA hypomethylation in a range of cell lines, including the human Burkitts lymphoma Raji cell line (Krawisz and Lieberman, 1984). This contradicts the previous report of Boehm and Drahovský (1981a) in which hypomethylation of DNA in Raji cells was observed after treatment with MNU. A major part of this thesis has been to explore the mechanisms by which alkylating agents inhibit DNA(cytosine-5)methyltransferase and cause hypo-methylation of cellular DNA.

A source of DNA(cytosine-5)methyltransferase was required for these studies and for this purpose, enzyme extracted from the murine leukemia L1210 was used. However, there were numerous problems associated with the methyltransferase preparation which should be further acknowledged. Firstly, the

enzyme preparation was impure and may contain proteins which affect the methylation of DNA, for example methylated DNA binding protein (Huang et al., 1984; Meehan et al., 1989) which binds to areas rich in 5-methylcytosine. Secondly, it is possible (but not definite) that the methyltransferase studied was a proteolytically degraded form of enzyme, as there was no material of molecular weight 190 kDa present. It has been suggested that this proteolysis removes a domain responsible for the maintenance methyltransferase activity (Adams et al., 1983; Adams et al., 1986). Therefore, it would appear that such studies on the de novo activity are still valid or perhaps even enhanced, as such treatment has been reported to result in an increase in the de novo activity. It is likely that a large number of studies have been conducted on such proteolytically degraded enzymes (see Adams et al., 1990). Thirdly, the enzyme preparation was unstable over short periods of time and therefore considerable effort was required in these procedures.

An aim of this project was to continue the work of Tisdale and explore the mechanism by which the 3-substituted imidazotetrazinones cause hypomethylation in living cells (Tisdale, 1986; Tisdale, 1989) and cause in vitro inhibition of DNA(cytosine-5)methyltransferase (Tisdale, 1988; Tisdale, 1989). Calf thymus DNA alkylated with temozolomide or

ethazolastone and other alkylating agents, has been shown to be able to inhibit the methylation of M.lysodeikticus DNA by DNA(cytosine-5)methyltransferase when placed in competition with it. No particular site of alkylation arose as being responsible for the inhibition of the methyltransferase and therefore other forms of DNA damage were investigated. It appeared that calf thymus DNA treated with X-rays or DNaseI produced good inhibitors of the methyltransferase, whilst digestion with the restriction endonuclease MspI or sonication did not. This suggested that strand breaks and in particular single-strand breaks were capable of inhibiting the methyltransferase and since the alkylating agents investigated, including temozolomide and ethazolastone produce single-strand breaks in DNA (Zucchetti et al., 1989), this may be one mechanism by which they cause hypomethylation in living cells. It is presumed that the inhibition of methyltransferase activity is due to an increased binding of the enzyme to the alkylated DNA, but attempts to prove this were unsuccessful.

A possible explanation for the inhibition of DNA(cytosine-5)methyltransferase by alkylated DNA, was that the enzyme was inactivated by 'suicide' repair (this is the mechanism by which O6AT removes the

alkyl groups from the O^6 atom of guanine in DNA). Although this was investigated the results generated suggest that this is unlikely.

The treatment of DNA(cytosine-5)methyltransferase with a number of alkylating agents including temozolomide and ethazolastone, was shown to inhibit the methyltransferase. This presumably occurs through reaction with critical sulfhydryl groups. However, as shown for a range of alkylating agents including MNNG, MNU, temozolomide and ethazolastone (Chan et al., 1983; Cox, 1980; Drahovsky and Wacker, 1975; Tisdale, 1989), this only occurs at high concentrations of drug which would be unattainable in the cell. Therefore, although this mechanism may contribute to the hypomethylating effect of temozolomide and ethazolastone in living cells, it is unlikely to be the major cause.

Studies on the inhibition of DNA(cytosine-5)methyltransferase using a range of polynucleotides has identified the O^6-carbonyl group of guanine as an important binding site for the methyltransferase. The O^6-atom of guanine is a reactive site for many alkylating agents including temozolomide and ethazolastone (Bull, 1988), and it appeared feasible that alkylation at this site may destroy the ability of DNA(cytosine-5)methyltransferase to bind to it. Indeed, it was shown that whilst poly[dG]$_{20}$ was a potent inhibitor of DNA(cytosine-5)methyltransferase,

poly[dO^{6}MG]$_{20}$ was not. Oligonucleotides were then synthesised containing O^6-methylguanine within CG sequences and it was shown that O^6-methylguanine severely reduced the ability of certain oligonucleotides to behave as a substrate.

The ability of oligonucleotides to behave as substrates for DNA(cytosine-5)methyltransferase was severely reduced when guanines adjacent to cytosine were replaced by O^6-methylguanine. This observation was highly significant and provided a correlation between those chemical agents which react with oxygen atoms in DNA and their mutagenic/carcinogenic potential via the prevention of DNA(cytosine-5)-methyltransferase recognition. Holliday (1979) suggested an epigenetic mechanism of carcinogenesis through the involvement of 5-methylcytosine and the results presented in this thesis suggest a molecular mechanism by which this may occur. Other sites of alkylation such as N-7 guanine may also prevent methyltranferase recognition, and although this was not investigated, the experiments using polynucleotides as inhibitors of the methyltransferase suggest that this is not a major site.

In conclusion, alkylating agents and in particular the imidazotetrazinones inhibit DNA(cytosine-5)methyltransferase and cause hypomethylation of DNA by a combination of effects. Firstly, reaction with critical sulfhydryl groups

within the enzyme molecule. Secondly, the induction of strand breaks and single-stranded regions may increase the binding of the enzyme to this region and as a consequence slow down the movement of the methyltransferase. Thirdly, alkylation of DNA may interfere with DNA-protein interactions. In particular this may occur through reaction with the $\underline{O}^6$ atom of guanine, with the effect of preventing the methyltransferase from recognising potentially methylatable sites in DNA.

In addition, some effort has been directed towards the development of agents to methylate the $\underline{C}$-5 atom of cytosine, with the potential that this would provide a direct means of blocking gene transcription. However, in order to be able to switch off specific genes, such agents would require enormous degrees of selectivity. There have been difficulties in terms of the chemical approach to this problem, but despite the negative results so far, the pursuit of an agent which will specifically methylate the $\underline{C}$-5 atom of cytosine is justified as the chemotherapeutic potential of such an agent can only be hypothesised without any experimental verification.

A proportion of this thesis has been devoted to certain mechanisms by which alkylating agents cause an inhibition of DNA(cytosine-5)methyltransferase and hence hypomethylation in living cells. However, some

effort has been spent on exploring the mechanisms responsible for the cytotoxicity of the imidazotetrazinones. The cell lines GM892 (Mer-) and Raji (Mer+) were treated with equitoxic concentrations of temozolomide or ethazolastone for various periods of time. The DNA extracted from these cells was then transferred into another cell line (MAC13) and this caused an inhibition of MAC13 cell growth which depended on the length of time the GM892/Raji cells were exposed to the drugs.

The evidence generated pointed towards apurinic sites and single-strand breaks as being responsible for the growth inhibition. However, this was by implication only, as no other forms of damage appeared from the experimental data. These experiments were incomplete at this stage, but open up an area which clearly warrants further investigation.

In conclusion, this thesis has made a significant contribution to the understanding of the mechanisms by which alkylating agents, in particular the imidazotetrazinones, cause hypomethylation of DNA.

APPENDIX

Appendix One:

Specific activity of the DNA(cytosine-5)methyltransferase preparation.

A typical DNA methylase assay may contain 20 µg *M.lysodeikticus* DNA, 1.0 µCi SAM (80 Ci/mmol) and enzyme capable of transferring 100 000 DPM. Incubations were carried out at 37°C for 4 h.

10µl enzyme preparation contains 0.5 µg protein

specific activity of SAM = 80 Ci/mmol
= 80 µCi/nmol
1 µCi SAM = 12.5 pmol

1 µCi SAM/assay $\hat{=}$ 800 000 DPM

In a typical assay 100 000 DPM would be incorporated

$$= \frac{100\ 000 \text{ DPM}}{800\ 000 \text{ DPM/µCi}}$$

$$= 0.125 \text{ µCi}$$

Therefore, 1µCi SAM (12.5 pmol) leads to the incorporation of 0.125 µCi of methyl groups

This is equivalent to 0.125×12.5 pmol

$$= 1.56 \text{ pmol}$$

Amount of protein in 10 µl of enzyme preparation
= 0.5 µg (5.0×10^{-4} mg)

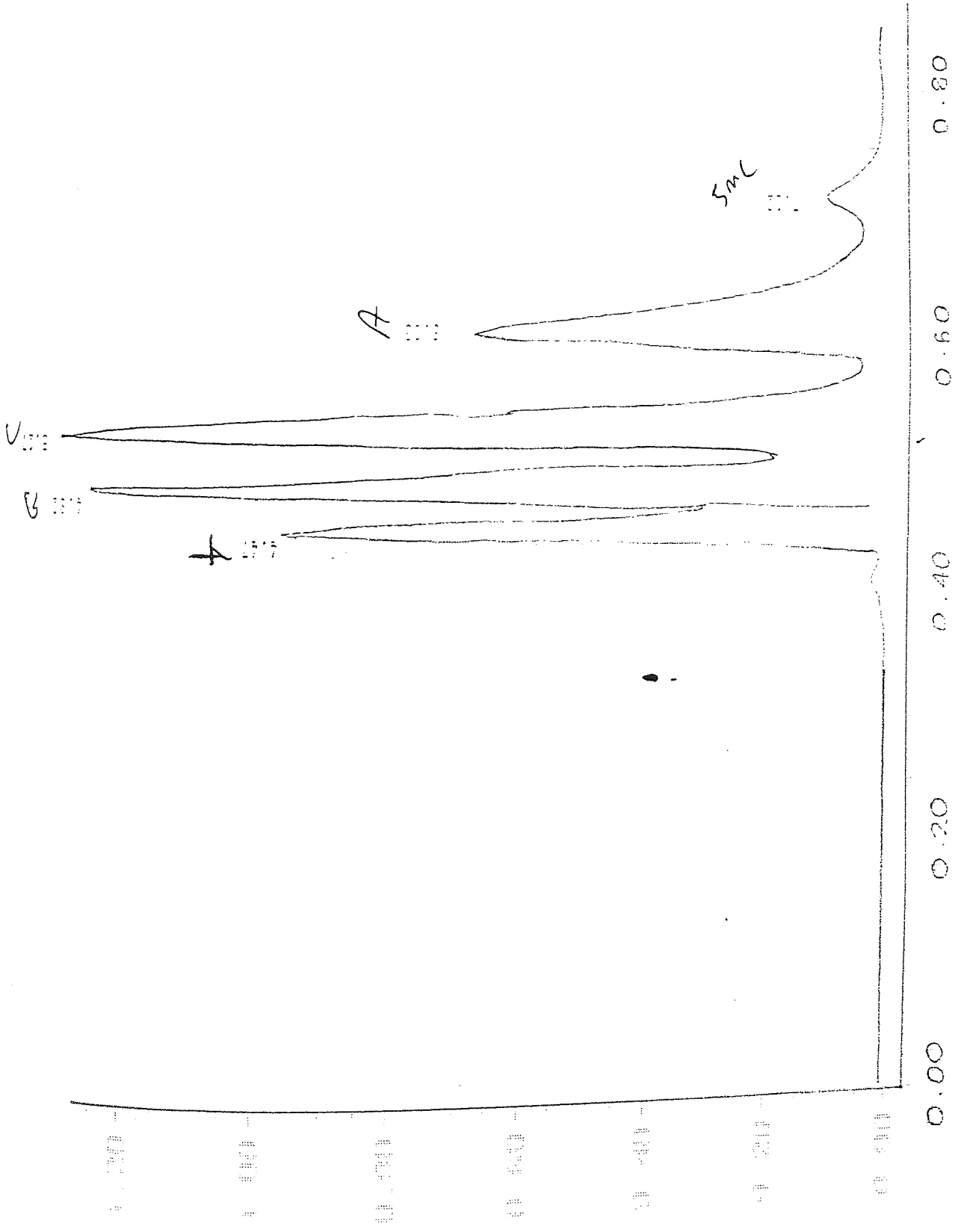
Therefore, specific activity of DNA(cytosine-5)methyltransferase is:

$$= \frac{1.56}{5.0 \times 10^{-4}} \frac{\text{pmol}}{\text{mg}}$$

$$= 3120 \text{ pmol/mg protein}$$

Appendix Two:

Separation of bases by HPLC.



Appendix Three:

Figures for the paper by Zucchetti et al (1989).

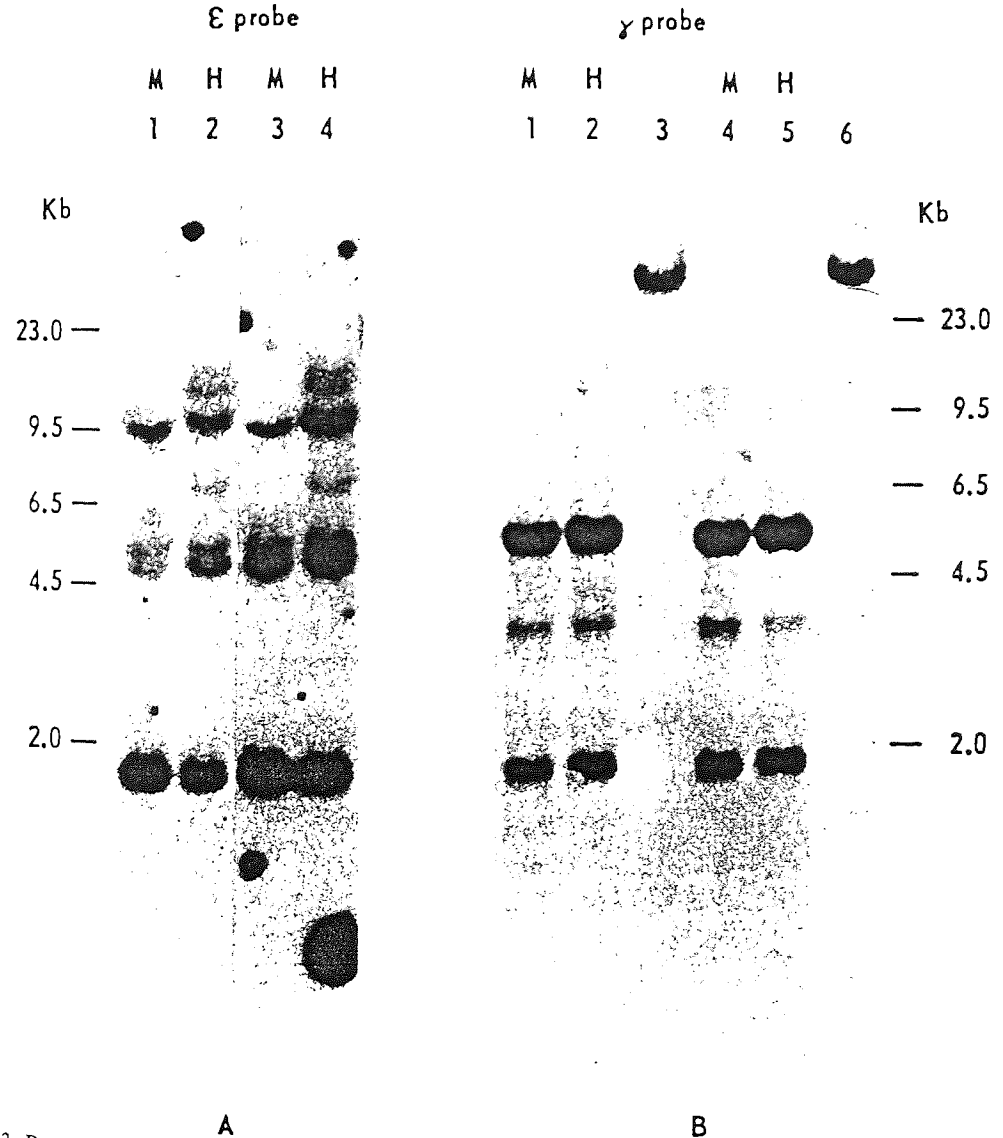


Fig. 3. Pattern of DNA methylation of ε (part A) and γ (part B) globin genes. DNA from untreated (lanes 1, 2) or temozolomide treated (lanes 3 and 4 part A; lanes 4 and 5 part B) K562 cells were digested with MspI (M) or HpaII (H) and hybridized with the 0.7 Kb Bam HI fragment of the ε globin gene (part A) and with the 3.3 Kb HindIII fragment of the Aγ globin gene (part B). Lanes 3 and 6 (part B) correspond to undigested DNA from untreated and from temozolomide treated K562 cells.

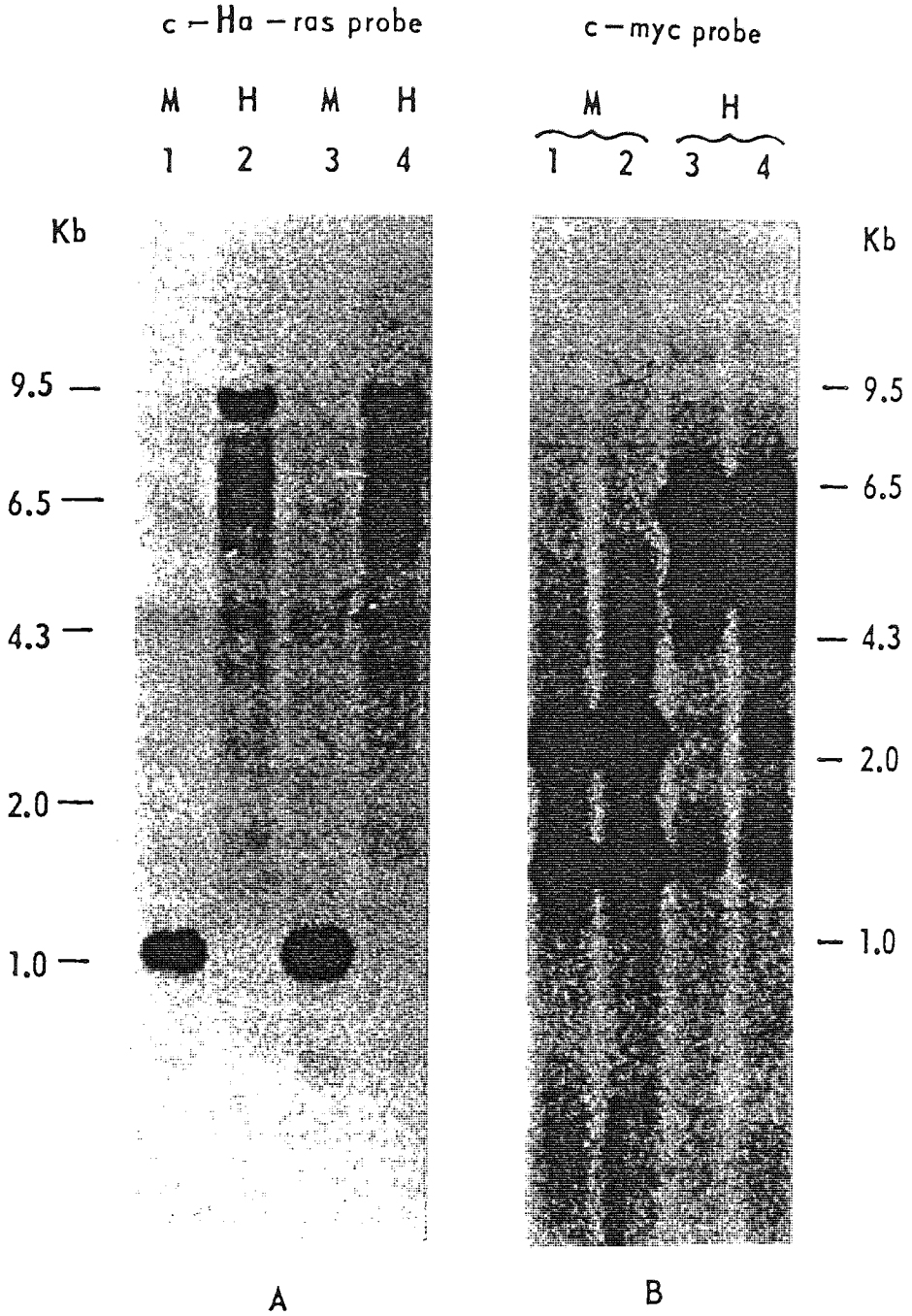


Fig. 4. DNA methylation pattern of c-Ha-ras and c-myc proto-oncogenes. DNAs from untreated (lanes 1 and 2 part A; lanes 1 and 3 part B) and temozolomide treated (lanes 3 and 4 part A; lanes 2 and 4 part B) K562 cells were digested with MspI (M) and HpaII (H) and hybridized with the 6.5 Kb Bam HI fragment of the c-Ha-ras (part A) and with the 1.4 Kb ClaI-EcoRI fragment (third exon) of c-myc oncogenes (part B).

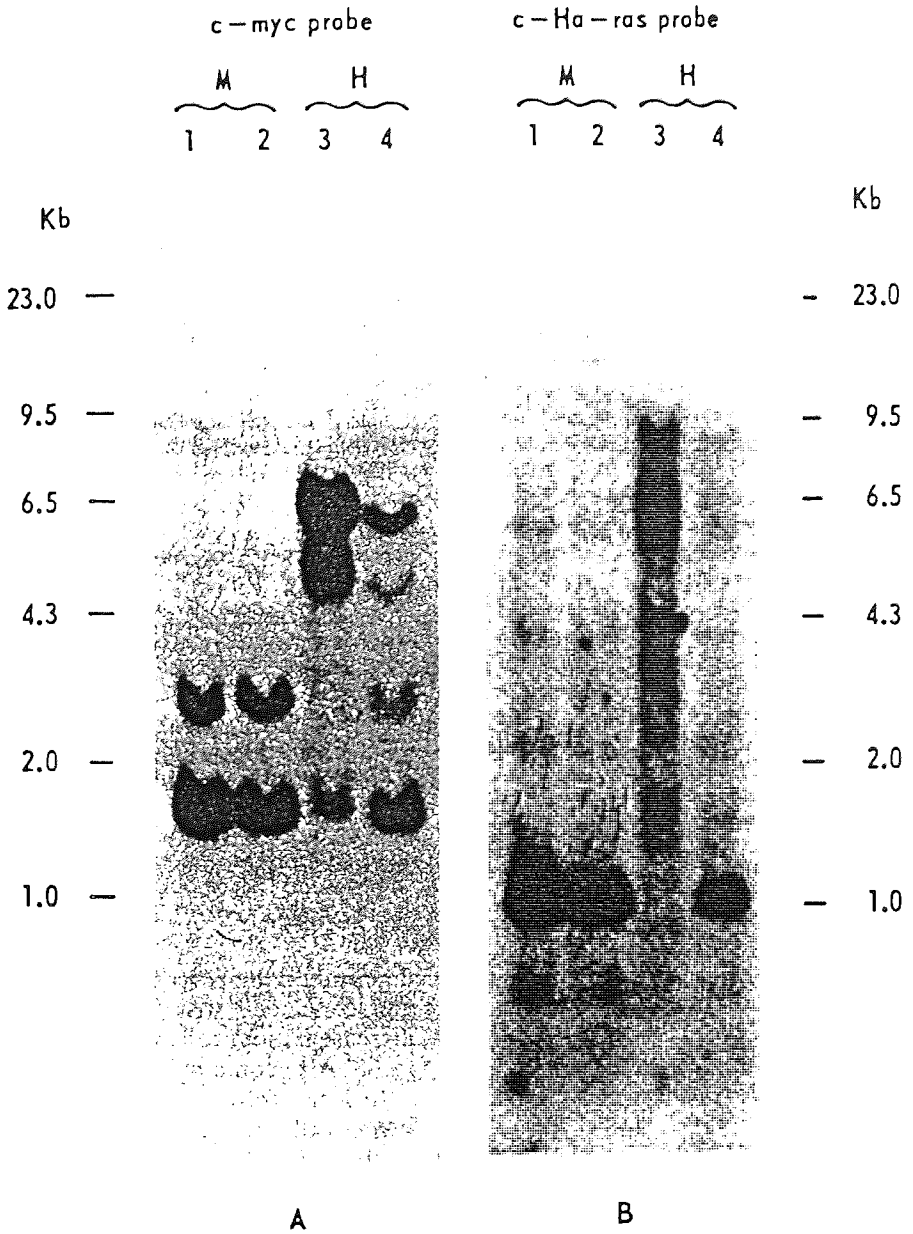


Fig. 5. DNA methylation pattern of c-myc (part A) and c-Ha-ras (part B) proto-oncogenes. DNAs from untreated (lanes 1 and 3) and 5-aza-2'-deoxycytidine treated (lanes 2 and 4) K562 cells were digested with MspI (M) and HpaII (H) and hybridized using the probes described in Fig. 4.

Appendix Four:

Data for chapter six.

MAC13 cells treated with Raji cellular DNA

Sample	Cell No.	Mean ± SEM
No DNA		
Expt 1	106850	118858 ± 6020
Expt 2	124112	
Expt 3	125612	
Raji untreated DNA		
Expt 1	74008	71213 ± 4948
Expt 2	78038	
Expt 3	61594	
Temozolomide 6 h		
Expt 1	26684	19997 ± 5947
Expt 2	8136	
Expt 3	25172	
Temozolomide 12 h		
Expt 1	55878	59972 ± 2768
Expt 2	65246	
Expt 3	58792	
Temozolomide 24 h		
Expt 1	75068	84679 ± 5122
Expt 2	86418	
Expt 3	92552	
Ethazolastone 6 h		
Expt 1	49254	50949 ± 2344
Expt 2	55582	
Expt 3	48010	
Ethazolastone 12 h		
Expt 1	65122	65411 ±1344
Expt 2	67870	
Expt 3	63240	
Ethazolastone 24h		
Expt 1	88050	82478 ± 3446
Expt 2	83204	
Expt 3	76180	

MAC13 cells treated with GM892 cellular DNA

Sample	Cell No.	Mean ± SEM
No DNA		
Expt 1	106264	127749 ± 11008
Expt 2	134332	
Expt 3	142652	
GM892 untreated DNA		
Expt 1	74476	81251 ± 3409
Expt 2	85304	
Expt 3	83972	
Temozolomide 6 h		
Expt 1	31592	19456 ± 6300
Expt 2	16320	
Expt 3	10456	
Temozolomide 12 h		
Expt 1	42188	42809 ± 4371
Expt 2	50672	
Expt 3	35568	
Temozolomide 24 h		
Expt 1	62552	67756 ± 2888
Expt 2	72528	
Expt 3	68188	
Ethazolastone 6 h		
Expt 1	41908	36619 ± 2723
Expt 2	35100	
Expt 3	32848	
Ethazolastone 12 h		
Expt 1	61272	57060 ± 3324
Expt 2	50500	
Expt 3	59408	
Ethazolastone 24h		
Expt 1	62032	66317 ± 3434
Expt 2	63812	
Expt 3	73108	

MAC13 cells treated with X-irradiated calf thymus DNA

Sample	Cell No.	Mean ± SEM
No DNA		
Expt 1	88352	93903 ± 6074
Expt 2	106036	
Expt 3	87322	
Untreated calf thymus DNA		
Expt 1	64464	62577 ± 2755
Expt 2	66116	
Expt 3	57150	
72 rads		
Expt 1	73136	73568 ± 913
Expt 2	72248	
Expt 3	75320	
100 rads		
Expt 1	65250	66405 ± 1786
Expt 2	64056	
Expt 3	69910	
200 rads		
Expt 1	57228	56807 ± 545
Expt 2	57468	
Expt 3	55726	
400 rads		
Expt 1	58454	66079 ± 3864
Expt 2	70976	
Expt 3	68808	

The methylation status of MAC13 cells after treatment with calcium phosphate and DNA extracted from cells treated with imidazotetrazinones.

Sample	C	5MC	$\frac{5MC}{5MC + C} \times 100$
Untreated	2163942	52446	2.4
	2216987	54645	2.4
	2239155	54118	2.4
No DNA	425214	9966	2.3
$(Ca_3(PO_4)_2$	413409	10313	2.4
	417682	10224	2.4
	503613	7432	1.5
	500613	10876	2.1
	502085	10872	2.1
Untreated	385428	9478	2.4
GM892 DNA	416540	9239	2.2
	262894	5944	4.03
GM892 treated with temozolomide			
6 h	9614	1804	15.8
	9448	1968	17
	9526	1709	15.2
12 h	20847	1247	5.6
	25441	2028	7.4
	25585	1950	7.1
24 h	15369	2760	
	18354	1597	8.0
	17917	1691	8.6
GM892 treated with ethazolastone			
6 h	31754	2428	7.1
	30903	1520	4.7
	30356	1532	4.8
12 h	45525	2373	5.0
	44265	2135	4.6
	44931	2100	4.5
24 h	54285	3183	5.5
	52096	2476	4.5
	53979	2449	4.3
Untreated	362758	12716	3.39
Raji DNA	377874	12242	3.14
	197889	5818	2.86

Raji treated with temozolomide			
6 h	177597	7739	4.18
	252086	6130	2.37
	136824	5583	3.92
12 h	156576	5247	3.24
	309024	8451	2.66
	169928	4604	2.64
24 h	491172	15133	2.99
	588217	19156	3.15
	243703	10622	4.18
Raji treated with ethazolastone			
6 h	173188	6533	3.64
	299937	9869	3.19
	152551	5697	3.60
24 h	260613	8336	3.10
	661696	17899	2.63
	331931	10213	2.99

Appendix Five:

Probability of O^6-methylguanine occurring in DNA.

0.1 mM Temozolomide

= 0.4 pmol/µg DNA (Bull and Tisdale, 1987).

5% alkylations exist as O^6-methylguanine

= 0.02pmol/µg DNA

1×10^6 cells contain 10 µg DNA

Therefore, 2×10^{-14} mol 06MG/10^5 cells

= 2×10^{-19} mol 06MG/cell

Approximately 3% bases in DNA exist as 5-methylcytosine

Therefore, 2×10^{-19} mol 06MG/cell x 0.03

= 6×10^{21} mol 06MG adjacent to C

Human genome contains 3×10^9 nucleotides

approximately 25% are G = $0.25 \times 3 \times 10^9$

= 7.50×10^8 G

6.02×10^{23} molecules/mol

$\frac{7.50 \times 10^8}{6.02 \times 10^{23}}$ = 1.25×10^{-15} mol G/cell

Therefore, the probability of O^6-methylguanine being next to a C

$$= \frac{6 \times 10^{-21} \text{ mol 06MG}}{1.25 \times 10^{-15} \text{ mol G}}$$

$$= \underline{4.80 \times 10^{-6}}$$

Appendix 6:

Publications

Hepburn, P.A. and Tisdale, M.J. (1991).
Antitumour Imidazotetrazines - XXV. Growth suppression by DNA from Cells Treated with Imidazotetrazinones. Biochem. Pharmacol. 41 339-3443

Hepburn, P.A. and Tisdale, M.J. (1991).
The Importance of the O^6 position of Guanine Residues in the Binding of DNA Methylase to DNA. Biochim. Biophys. Acta In Press

Hepburn, P.A. and Tisdale, M.J. (1991).
Antitumour Imidazotetrazines - XXVI. Studies on the Mechanism of DNA Hypomethylation by 3-Substituted Imidazotetrazinones. Life Sciences In Press

Hepburn, P.A., Margison, G.P. and Tisdale, M.J. (1991).
Enzymatic Methylation of Cytosine in DNA is Prevented by Adjacent O^6-Methylguanine Residues. J. Biol. Chem. In Press

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ANTITUMOUR IMIDAZOTETRAZINES—XXIV

GROWTH SUPPRESSION BY DNA FROM CELLS TREATED WITH IMIDAZOTETRAZINONES

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Abstract—Transfection of a murine colon adenocarcinoma cell line (MAC13) with DNA extracted from GM892 or Raji cells previously treated with either the methyl- (temozolomide) or ethyl- (CCRG82019) imidazotetrazinones caused a dose-related suppression of cell growth. The effect was proportional to the concentration of DNA transfected and the time of incubation of the donor cell lines with the drugs. It was not shown with X-irradiated DNA suggesting that the effect did not arise from non-specific damage to the DNA. Transfection of MAC13 cells with DNA extracted from GM892 cells was more effective in inhibiting growth than DNA from Raji cells, and temozolomide treated cellular DNA was a more potent growth inhibitor than that from CCRG 82019 treated cells. For both agents the growth inhibitory effect was most marked with DNA extracted 6 hr after drug addition and thereafter the effect decreased up to 24 hr after drug addition. This suggests that the growth inhibitory effect is due to a repairable lesion, and that the terminal mechanism of action of these agents involves targets after DNA.

In the series of 3-alkyl substituted imidazotetrazinones, strict structural requirements are required for the alkyl group to exert effective antitumour activity. Thus, while 3-(2-chloroethyl)- (mitozolomide) and 3-methyl- (temozolomide) imidazotetrazinones are effective antitumour agents, the presence of a 3-ethyl-(CCRG 82019), or higher alkyl substituents, leads to a loss of cytotoxic potency *in vitro* [1] and antitumour activity *in vivo* [2]. A similar structure–activity relationship is shown in the series of antitumour triazenes and nitrosoureas [3]. The antitumour activity of drugs of these types has been linked to alkylation of the O-6 position of guanine in DNA. Thus, cells deficient in the capacity to repair lesions at the O-6 position of guanine (Mer^-) show an increased sensitivity to both a monomethyl or monochloroethyltriazene, compared with a repair proficient cell line (Mer^+) [4]. However, no difference in toxicity between a Mer^- and Mer^+ cell line was observed with an ethyltriazene, suggesting that O^6-alkylation of guanine may not be important for the cytotoxicity of the ethyl derivative. In the case of the imidazotetrazinones, depletion of the repair capacity of a Mer^+ cell line with the free base O^6-methylguanine resulted in an increased sensitivity towards both mitozolomide and temozolomide, but not the ethyl derivative CCRG 82019.

Alkylation of guanine with a chloroethyl group leads to interstrand cross-linking arising from a secondary reaction of the chloroethyl group with the N-3 position of a cytosine residue in the opposite DNA strand after migration to the N-1 position of guanine [6]. However, it is difficult to see how a methyl group at the O-6 position of guanine could lead to cytotoxicity, since cross-linking is chemically impossible. While mutation induction correlates well with the production of O^6-methylguanine in DNA, there is a less clear correlation between cytotoxicity and O^6-methylguanine production. Thus while some authors have shown a correlation between unrepaired O^6-methylguanine and cytotoxicity [7], others have failed to find such a correlation [8]. Some workers have suggested that adducts at the O-6 atom of guanine in DNA are not potentially cytotoxic lesions and that defects other than lack of methyltransferase is responsible for the sensitivity of Mer^- cells to killing by alkylating agents [9]. In order to investigate the potential mechanisms responsible for cytotoxicity we have determined the ability of alkylated DNA to directly influence the growth of a cell line after direct transfection.

MATERIALS AND METHODS

Tissue culture medium and foetal calf serum were purchased from Gibco Ltd (Paisley, U.K.). Temozolomide and CCRG 82019 were synthesized by May and Baker Ltd (Dagenham, U.K.) and all other chemicals were purchased from the Sigma Chemical Co. (Poole, U.K.).

Cell culture. Cell lines Raji (human Burkitts lymphoma, GM892 (human lymphoblastoma) and MAC13 (murine colon adenocarcinoma) were maintained in RPMI 1640 medium containing 10% foetal calf serum under an atmosphere of 5% CO_2 in air. Drugs were dissolved in dimethylsulphoxide (DMSO), such that the final concentration of DMSO in the culture medium did not exceed 0.4%. Both Raji and GM892 cells were exposed to equitoxic concentrations of the drugs at the ID_{50} values (Table 1) for either 6, 12 or 24 hr and the DNA was extracted by the method of Warren [10]. Cells were isolated from culture medium by centrifugation, washed with 0.9% NaCl, treated with 6% 4-aminosalicylic acid and lysed with 10% SDS. After

Table 1. Sensitivity of cell lines to imidazotetrazinones and O^6-alkylguanine-DNA alkyltransferase activity (O^6MeGMT)

Cell line	ID_{50} (μM)*		O^6MeGMT (fmol/mg protein)
	Temozolomide	CCRG 82019	
GM892	10 ± 7	229 ± 20	10 ± 5
Raji	206 ± 20	360 ± 30	634 ± 80
MAC13	77 ± 10	>1000	44 ± 4

Results are expressed as means ± SE from the average of at least three experiments.

* Concentration required to produce 50% inhibition of cell growth.

extraction with phenol reagent the DNA was precipitated with 2-ethoxyethanol, washed extensively with 70% ethanol followed by absolute ethanol, and allowed to dry overnight.

Transfection of DNA into eukaryotic cells. The day before transfection, MAC13 cells were split into individual flasks at a concentration of 5×10^4cells per flask containing 9 mL of new RPMI 1640 medium, plus 10% foetal calf serum and allowed to adhere overnight. The DNA for transfection was sterilized by precipitation with two volumes of absolute ethanol and air dried in a sterile hood. MAC13 cells were treated with 30 μg of DNA co-precipitated with calcium phosphate, which was distributed evenly over the surface of the cells and gently agitated to mix the precipitate and the medium. The cells were then incubated for 5 hr, after which time the medium was removed and the cells were washed twice with 5 mL of 1 ×phosphate buffered saline. The cells were then fed with 10 mL of complete medium, and incubated under an atmosphere of 5% CO_2 in air for 3 to 4 days, when the cells were removed by trypsin treatment, and the cell number was enumerated with a Coulter electronic particle counter, model D.

For the experiments with X-irradiated DNA, cells were transfected with 30 μg of calf thymus DNA which had previously been irradiated with various doses of radiation from 72 to 400 rads.

Statistical analysis. All results are expressed as mean ± SE for at least three separate determinations. Differences have been analysed statistically using Student's *t*-test.

RESULTS

The concentrations of temozolomide and CCRG 82019 required to produce a 50% inhibition of growth of the three cell lines is shown in Table 1. The level of response appears to be related to the concentration of the repair enzyme O^6-methylguanine methyltransferase (O^6MeGMT) in a particular cell line. Thus, GM892 cells which are highly sensitive to the cytotoxic effect of temozolomide, have low levels of the repair protein, while Raji cells which are insensitive, have high levels of the repair protein, and an intermediate level is found in the MAC13 cell line.

In order to try to understand the cellular reactions responsible for cytotoxicity after treatment with the imidazotetrazinones, cellular DNA extracted from GM892 or Raji cells has been transfected into MAC13 cells using the calcium phosphate precipitation technique. Transfection of MAC13 cells with DNA from GM892 or Raji cells, which have only been exposed to the solvent, does cause a reduction in cellular proliferation compared with untreated controls (Fig. 1), and therefore results with DNA obtained from drug treated cells have been compared with those obtained with solvent control. For both Raji or GM892 cells treated with either temozolomide or CCRG 82019 at equitoxic concentrations, the DNA when transfected into MAC13 cells, causes an inhibition of proliferation. Incubation of MAC13 cells with DNA from either cell line in the absence of calcium phosphate had no effect on the subsequent proliferation. The results in Fig. 1 show that the effect is dependent on the length of time the donor cells have been incubated with drug prior to DNA extraction. Thus, for DNA extracted from both GM892 cells (Fig. 1A) or Raji cells (Fig. 1B), the effect is most pronounced with DNA extracted 6 hr after drug addition, and becomes progressively less so 12 and 24 hr after drug addition. Transfection of MAC13 cells with DNA from GM892 cells treated with either temozolomide or CCRG82019 has a more profound effect on the subsequent growth than that extracted from Raji cells, despite the higher concentrations of drugs to which these cells were exposed (Table 1). For temozolomide-treated cells at 6 hr both DNA from GM892 and Raji cells produces a highly significant ($P < 0.001$ and $P < 0.005$, respectively) inhibition of the growth of MAC13 cells. However, at 12 and 24 hr only, GM892 DNA still significantly ($P < 0.005$ and $P < 0.05$, respectively) inhibits the growth of MAC13 cells. Similarly for CCRG 82019-treated cells at 6 hr, both DNA from GM892 and Raji cells produces a significant ($P < 0.001$ and $P < 0.05$, respectively) inhibition of growth of MAC13 cells, while at 12 and 24 hr only GM892 DNA still significantly inhibits growth ($P < 0.005$ and $P < 0.05$, respectively).

DNA extracted from cells treated with CCRG 82019 appears to be a less potent inhibitor of the growth of MAC13 cells than that extracted from cells treated with temozolomide. Thus at 6 hr, DNA from both GM892 and Raji cells exposed to temozolomide is a more potent ($P < 0.05$ and

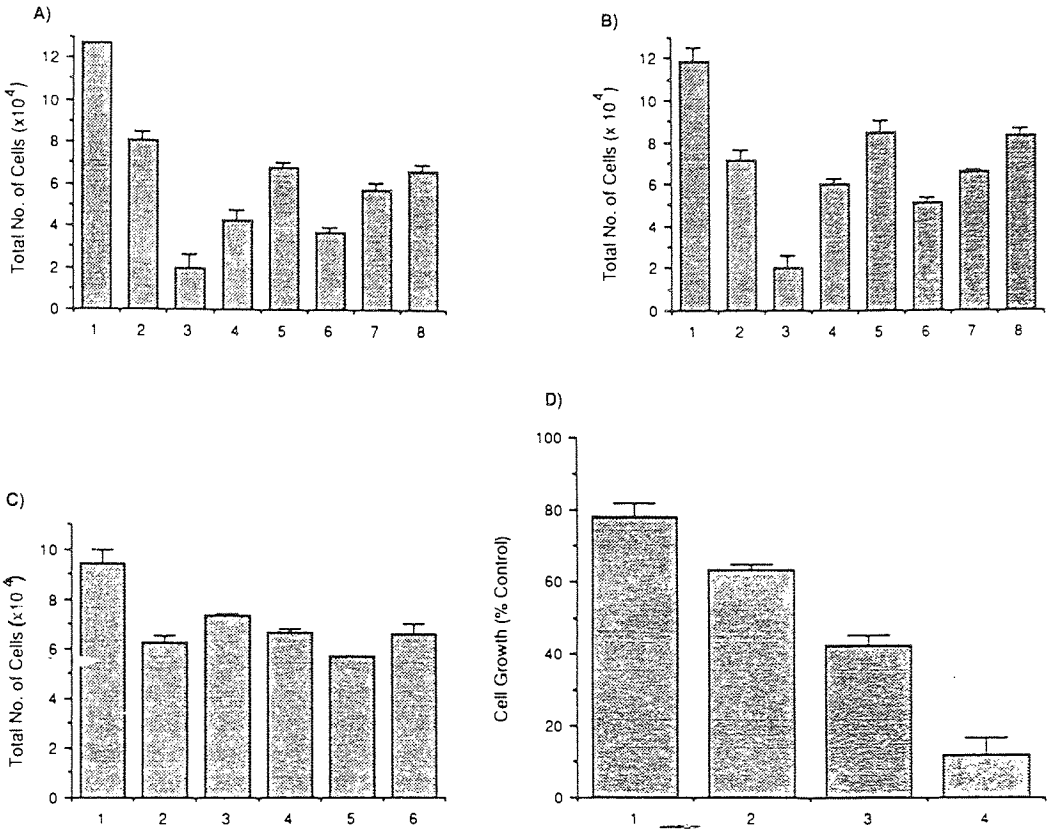

Fig. 1. Effect on growth of MAC13 cells of transfection with DNA isolated from (A) GM892 and (B) Raji cells or (C) calf thymus DNA previously exposed to various doses of X-irradiation and (D) calf thymus DNA treated with 10 mM temozolomide *in vitro*. In (A) and (B) cells were transfected with calcium phosphate co-precipitated DNA (30 μg) isolated from solvent controls cells (2) or cells which had been exposed to temozolomide (3–5) or CCRG 82019 (6–8) at the ID_{50} concentrations for 6 (3, 6), 12 (4, 7) and 24 hr (5, 8) and the effect on subsequent cellular proliferation was determined as compared with non-transfected cells (1). In (C) cell growth in the absence of DNA (1) was compared with that in cells transfected with calf thymus DNA, which was either untreated (2) or subjected to 72 (3), 100 (4), 200 (5) or 400 (6) rads of X-irradiation, while in D cells were transfected with 25 (1), 50 (2), 100 (3) or 150 (4) μg of temozolomide treated calf thymus DNA and cell growth was compared with the equivalent amounts of non-treated calf thymus DNA.

$P < 0.005$, respectively) inhibitor of the growth of MAC13 cells than DNA from cells exposed to CCRG 82019.

For cells exposed to temozolomide for 6 hr, the growth inhibitory effect is proportional to the concentration of drug up to 500 μM (Fig. 2) and is proportional to the concentration of DNA used in the transfection assay (Fig. 3A and B). The inhibition of cell growth is not due to a non-specific effect from damaged DNA, since transfection of MAC13 cells with calf thymus DNA irradiated with up to 400 rads of X-irradiation has no effect on the subsequent growth (Fig. 1C), while transfection with calf thymus DNA previously treated with 10 mM temozolomide causes a progressive decrease in cell growth with increasing concentrations of DNA (Fig. 1D).

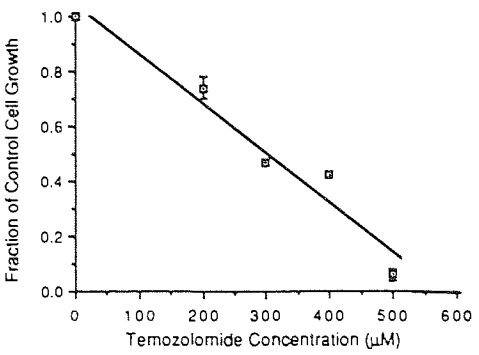

Fig. 2. The effect on growth of MAC13 cells of transfection with DNA (30 μg) isolated from Raji cells exposed to various concentrations of temozolomide for 6 hr. Results are expressed as mean ± SE relative to the effect of DNA from solvent treated control cells.

DISCUSSION

These results suggest that alkylation of DNA by the imidazotetrazinones is not the ultimate reaction, but that the alkylated DNA is capable of exerting other actions in the cell, which are ultimately

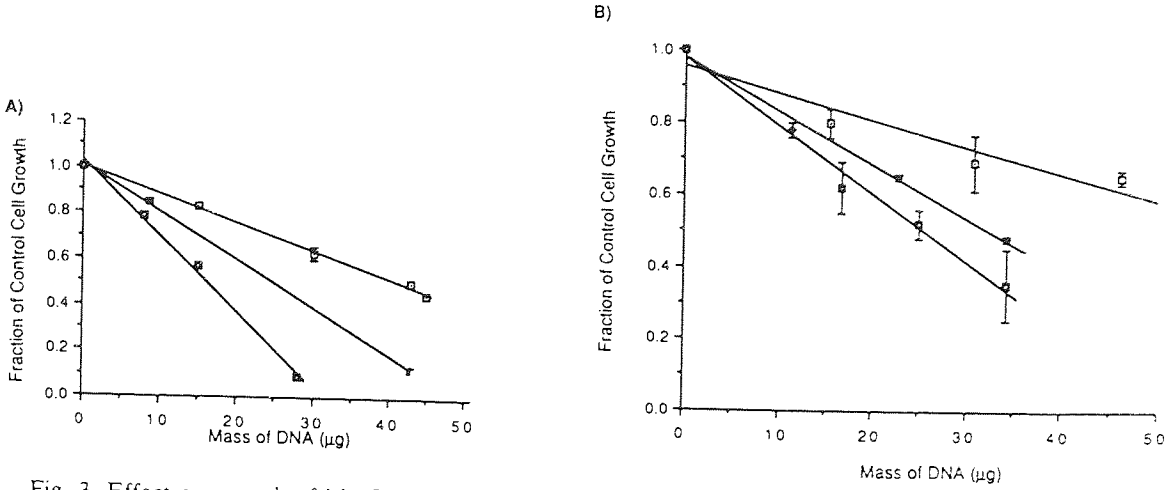

Fig. 3. Effect on growth of MAC13 cells of transfection with various concentrations of DNA isolated from Raji cells 6 hr after treatment with 200 (□), 400 (■) and 500 (□) μM temozolomide (A) or 192 (□), 385 (■) and 480 μM (□) CCRG 82019 (B).

responsible for the inhibition of cellular proliferation. The effect appears not to be due to a non-specific inhibition of proliferation by damaged DNA, e.g. by single-strand breaks since X-irradiated calf thymus DNA does not produce a similar action. However, calf thymus DNA, previously treated *in vitro* with temozolomide, when transfected into MAC13 cells produces growth inhibition in a manner similar to that from DNA extracted from cells exposed to this agent. The time course for the inhibition of growth by transfected DNA suggests a repairable lesion. The greater potency of temozolomide over CCRG 82019, and the greater effectiveness of DNA from GM892 over Raji cells correlates with the direct effect of these agents on the two cell lines. This has previously been attributed to O^6-alkylation of guanine residues, which is related to the repair capacity for this particular lesion. However, at the LD_{50} levels of drugs utilized in the present study, the repair proteins are effectively titrated out, so that there is little removal of O^6-methyguanine over a 24 hr time period [11]. This suggests that the O^6-alkylguanine lesion is not responsible for the growth inhibitory effect of the DNA.

However, two lesions found in both cell lines, which are repairable and which may contribute to the cytotoxicity of alkylated DNA are the formation of 7-alkylguanine and 3-alkyladenine. In addition, the yield of both these alkylated bases in cells exposed to temozolomide (70 and 9.2% of the total products, respectively), is much higher than in cells exposed to CCRG 82019 (24 and 4.9% of the total products, respectively) with the latter forming mainly phosphotriesters [11]. Medcalf and Lawley [12] have studied the rate of removal of alkylated bases from the DNA of *N*-methyl-*N*-nitrosourea (NMU) treated human fibroblasts and have found that removal of both of these bases occurs, but that 3-methyladenine is removed much more rapidly with a time course approximating to that of the alkylated DNA used in the transfection studies reported here. This suggests that the most likely lesion responsible for the growth inhibitory effect of the alkylated DNA is the formation of 3-alkyladenine. 3-Methyladenine has been implicated in the lethal effects of alkylating agents to bacteria [13], but its importance in eukaryotic cells has not been demonstrated.

The cellular reactions responsible for the ultimate toxicity of the transfected DNA are not known. However, it is interesting to note that DNA extracted from GM892 cells previously treated with temozolomide was capable of inhibiting the methylation of *M. lysodeiktious* DNA by purified DNA–cytosine 5-methyltransferase [14], with a time course similar to that with the transfected DNA observed in the present study. This suggests that the repairable lesion was the same in both cases. Modification of DNA by chemical carcinogens interferes with the enzymatic methylation of DNA both *in vitro* [15] and *in vivo* [16]. Although the relationship to the antiproliferative effects of these agents is not known, it is interesting to note that the antileukaemic action of 5-aza-2′-deoxycytidine has been correlated with its effects on DNA methylation [17].

Transfection of MAC13 cells with [^{3}H]thymidine labelled DNA results in an incorporation of 0.3% of the radioactivity into the cell, when 17 μg of DNA is used in the transfection experiment. It is not known whether the transfected DNA is incorporated into the murine genome, or whether the alkylated DNA is exerting its growth inhibitory effect independent of incorporation. Further studies are now being conducted to investigate this novel event.

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Enzymatic Methylation of Cytosine in DNA Is Prevented by Adjacent O^6-Methylguanine Residues*

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The effect of O^6-alkylation of guanine residues on the enzymic methylation of cytosine has been studied using synthetic oligonucleotides in which all guanines in cytosine-guanine sequences at potentially methylatable sites are replaced by O^6-methylguanine. In contrast with the unmodified forms, which showed high acceptance activity for *methyl*-^{3}H-labeled groups from *S*-adenosyl-L-[*methyl*-^{3}H]methionine in the presence of DNA methylase, the modified oligonucleotides were not substrates for the enzyme either in the single-stranded or annealed forms. In view of the importance of cytosine methylation in the down-regulation of certain genes, the potential to affect gene expression by this mechanism may be a contributory factor in the toxic and carcinogenic effects of chemical methylating agents.

Alkylation of the O^6-position of guanine residues in DNA by chemical methylating agents such as environmental *N*-nitroso compounds is thought to be an important promutagenic lesion in both bacteria (Karran and Marcaus, 1989) and mammalian cells (Medcalf and Wade, 1983; Newbold *et al.*, 1980, Margison and O'Connor, 1989). In animal models the formation and persistence of O^6-alkylguanine is correlated with the tissue or species specificity of certain antitumor agents (Babich and Day, 1987; Yarosh, 1985, Saffhil *et al.* 1985). These effects are normally correlated with the mispairing properties of the modified guanine residues, although few models exist to explain how DNA damage at a specific site may lead to the induction to neoplasia or stop the growth of neoplastic cells.

The nature of the neighboring base influences the extent of alkylation of Gua residues (Briscoe and Cotter, 1984) and has led to the proposal (Mathes *et al.*, 1988) that GuaCyt-rich regions in genes could be the preferred sites of damage by these agents. While GuaCyt-rich sites are relatively rare in the whole genome they are associated with the promoter locus typically in the 5′-region of the gene (Bird, 1984). Enzymatic methylation of the Cyt residue at the 5-position by DNA methylase at such GuaCyt-rich sites has often been associated with suppression of gene expression (Bird, 1984). If such sites are also the targets for chemical methylating agents then alterations in Cyt methylation and consequently in gene expression could occur. Using synthetic oligonucleotides containing readily methylatable sites, we have investigated the effect of replacement of Gua by O^6-methylguanine (O^6-MeGua) on the enzymatic methylation of the adjacent Cyt.

EXPERIMENTAL PROCEDURES

Materials—*S*-Adenosyl-L-[*methyl*-^{3}H]methionine (specific activity, 83 Ci mmol^{-1}) and [γ-^{32}P]ATP (specific activity, 3000 Ci mmol^{-1}) were purchased from Amersham International, Bucks, United Kingdom. *Micrococcus lysodeikticus* DNA was purchased from Sigma Chemical Co., Poole, Dorset, U. K. and polynucleotide kinase from Boehringer Corp., Ltd., Lewes, Sussex, U. K. The oligonucleotide $(dG)_{20}$ was purchased from Pharmacia LKB Biotechnology, Milton Keynes, Bucks, U. K., while all other oligonucleotides were prepared by Dr. M. Mackett of the Paterson Institute, Manchester, U. K. by solid state methodology using β-cyanoethyl-*N*,*N*-diisopropylphosphoramidite intermediates obtained from Cruachem Chemical Co. Ltd., Livingston, Scotland and American Biometics Inc., Hayward, CA as described (Atkinson and Smith, 1985; Borowy-Borowski and Chambers, 1987). The oligonucleotides were synthesized on a 1 -μmol scale using a Du Pont Coder 3000 synthesizer. Deprotection was carried out with 10% 1,8-diazobicyclo[5.4.0]undec-7-ene] in methanol at room temperature for 5 days. The purity of the oligonucleotides was determined by end-labeling with ^{32}P from [γ-^{32}P]ATP using polynucleotide kinase followed by electrophoresis on a 20% urea:acrylamide gel and autoradiography.

Enzyme Purification—DNA methylase was purified from L1210 leukemia cells essentially according to the method of Turnbull and Adams (1976). DNA was removed from the nuclear extract by DE52 cellulose, and proteins were consecutively precipitated by the addition of ammonium sulfate to 30–60% saturation. The precipitate from the 60% ammonium sulfate saturation was fractionated on a column of Ultrogel AcA-34, and the active fractions were pooled and dialyzed against 50 mM Tris, pH 7.8, containing 1 mM EDTA, 1 mM dithiothreitol, 50% glycerol, and 50 mg liter^{-1} phenylmethylsulfonyl fluoride. The dialysate was stored at −20 °C and used as the enzyme preparation in this study.

DNA Methylase Assay—This was based upon the method described by Bolden *et al.* (1985). The standard assay contained 20 μmol of Tris-HCl, pH 7.5, 0.5 μmol of dithiothreitol, 0.1 μmol of EDTA, 0.01 μmol of EGTA[1], 1 μCi of *S*-adenosyl-L-[*methyl*-^{3}H]methionine, 20 μg of oligonucleotide, and enzyme in a total volume of 100 μl. Incubation was at 37 °C for 4 h, and, after addition of unlabeled *S*-adenosyl-L-methionine to 1.5 mM, the reaction mix was transferred to Whatman DE81 discs and washed 5 times in 20 mM Na_2HPO_4, pH 8.4, twice in water, and once in ethanol:ether (95:5, v/v) and the radioactivity determined. Control assays were also performed without any added substrate oligonucleotide to distinguish oligonucleotide methylation from other types of methylation, and these values were subtracted from the total methylation obtained. In all cases these represent less than 10% of the total radioactivity incorporated.

RESULTS AND DISCUSSION

In order to examine whether O^6MeGua residues would affect enzymic methylation of 5′-adjacent cytosine residues, we have synthesized pairs of oligonucleotides, using the phosphoramidite method (Table 1). The oligonucleotides were chosen to contain CytGua-rich sequences capable of methylation, and

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[1] The abbreviation used is: EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

TABLE I

Methylation of complementary single-stranded oligonucleotides and annealed duplex forms

Oligonucleotides as substrates for L1210 DNA methylase are shown: X = O^6-methylguanine. The assay was carried out as described under "Experimental Procedures" using 20 μg of the oligonucleotide, and the values represent the mean ± S.E. of between 7 and 10 determinations for the single-stranded and 4 determinations for the duplex forms. For annealing equimolar concentrations of the appropriate oligonucleotides were incubated at a temperature of 70°C or above calculated from the appropriate extinction values of the individual bases at 260 nm (G, 12,100; C, 7050; T, 8400; A, 15,200; O^6MeG, 5200) for 10 min and slowly cooled to room temperature in an insulated container. The results are expressed as means ± S.E.

	Substrate	*methyl*-^{3}H incorporated
		pmol
Oligonucleotides		
a	5′-C C G G C C A T T A C G G A T C C G T C C T G G G C-3′	0.19 ± 0.04
b	5′-C C *X* G C C A T T A C *X* G A T C C *X* T C C T G G G C-3′	0.00
c	5′-G A T C C G T A A T G G C C G G-3′	0.00
d	5′-C C C G C C C C G C C C C G G C C T C G G C C C C G G C C C-3′	2.62 ± 0.31
e	5′-C C C *X* C C C C *X* C C C C *X* G C C T C *X* G C C C C *X* G C C C-3′	0.00
f	5′-G G G C C G G G G C C G A G G C C G G G-3′	0.00
Annealed oligonucleotides		
a	5′-C C G G C C A T T A C G G A T C C G T C C T G G G C-3′	1.05 ± 0.03
c	3′-G G C C G G T A A T G C C T A G-5′	
b	5′-C C *X* G C C A T T A C *X* G A T C C *X* T C C T G G G C-3′	0.00
c	3′-G G C C G G T A A T G C C T A G-5′	
d	5′-C C C G C C C C G C C C C G G C C T C G G C C C C G G C C C-3′	0.15 ± 0.03
f	3′-G G G C C G G A G C C G G G G C C G G G-5′	
e	5′-C C C *X* C C C C *X* C C C C *X* G C C T C *X* G C C C C *X* G C C C-3′	0.00
f	3′-G G G C C G G A G C C G G G G C C G G G-5′	
M. lysodeikticus DNA		0.82 ± 0.06

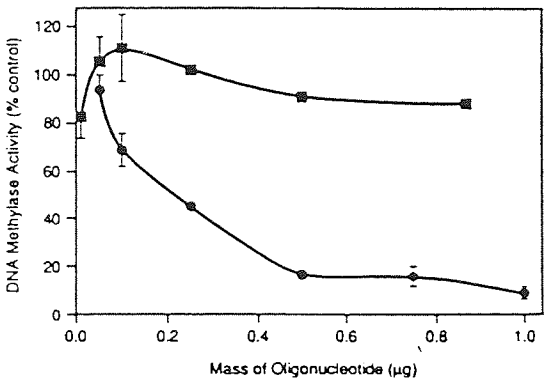

FIG. 1. **Effect of increasing concentrations of $(dG)_{20}$ (●) and $(dO^6MeG)_{20}$ (■) on the methylation of *M. lysodeikticus* DNA by DNA methylase, purified from L1210 leukemia cells.** The results are the mean of three experiments and are expressed as mean ± S.E.

in one member of the pair, all Gua residues in CytGua linkage have been replaced by an O^6MeGua residue. The 26-mer (a) has previously been investigated by Bolden *et al.* (1986), who found it to be an excellent substrate for eukaryotic DNA methylase, while the 30-mer (d) is a CytGua-rich part of the promoter locus (514–547) of the H-*ras* proto-oncogene (Ishi *et al.*, 1985). Chromatographic analysis showed the oligonucleotides to be free of 6-aminopurine, and O^6MeGua was the only modified base present. The oligonucleotides were assessed for their ability to accept *methyl*-^{3}H-labeled groups from *S*-adenosyl-L-[*methyl*-^{3}H]methionine during incubation with DNA methylase, which was partially purified from L1210 leukemia cells (Turnbull and Adams, 1976), either alone or after annealing to shorter complementary strands containing only normal bases. In comparison with *M. lysodeikticus* DNA, chosen because of the higher level (85%) of CytGua, both the 26-mer (a) and the 30-mer (d) had high acceptance ability, with the latter incorporating 3 times as many methyl groups as an equivalent amount of *M. lysodeikticus* DNA (Table I). In contrast the antisense sequences (c and f) did not act as substrates for *de novo* methylation, as previously observed for C (Bolden *et al.*, 1986), suggesting either that the fragments are too short for enzyme binding or that the enzyme distinguishes two types of CytGua pairs in DNA. However, when a and c were hybridized, substrate activity was high and radioactivity was incorporated into both oligonucleotides as determined by denaturing gel electrophoresis and fluorography (data not shown). This suggests that the complementary sequence becomes methylated by maintenance methylation after *de novo* methylation of the primary sequence. Although radioactivity was also incorporated into the antisense strand f in the annealed oligonucleotide formed from d and f, the total level of incorporation was low in both strands, with d incorporating less methyl groups than in the single-stranded form. The reason for this difference is not known.

In contrast to these results, replacement of Gua in all CytGua positions with O^6MeGua in the 26- and 30-mer completely destroyed the activity of the oligonucleotides as substrates for the DNA methylase even after annealing to the normal complementary strands (Table I).

The mechanism of this inhibition may be related to the lack of binding of the DNA methylase to regions of DNA containing O^6MeGua residues. It has been suggested (Bolden *et al.*, 1984) that guanine residues are important in recognition and binding of DNA methylase to dCytpdGua, the site of cytosine methylation. This effectiveness of guanine residues in binding DNA methvlase can be demonstrated from the results presented in Fig. 1, which show that 0.2 μg of the oligonucleotide $(dG)_{20}$ is capable of causing a 50% inhibition of the methylation of 20 μg of *M. lysodeikticus* DNA. In contrast, equivalent amounts of $(dO^6MeGua)_{20}$ caused no detectable inhibition of the methylation reaction. This suggests that formation of such a lesion in DNA may lead to reduced binding of the DNA methylase with the result that adjacent cytosine residues become hypomethylated.

If we consider a cell line lacking O^6MeGua repair capacity with an extracellular concentration of a drug (temozolomide)

of 0.1 mM then 0.4 pmol of alkylated products is found bound per μg of DNA of which 5% are O^6MeGua (Bull and Tisdale, 1987). Using a figure of 3% for the total 5MeCyt in the cellular genome and the possibility that Gua in GuaCyt sequences is twice as likely to be methylated at the O^6-position (Briscoe and Cotter, 1984), then the probability that an O^6MeGua will be at a site to affect methylation of an adjacent cytosine will be between 1 in 10^5 and 1 in 10^6.

DNA cleavage by restriction enzymes that recognize a unique DNA sequence has also been shown to be inhibited by the replacement of Gua by O^6MeGua in the appropriate sequence (Voigt and Topal, 1990), a suggestion that such modification in the major groove of DNA generally inhibits the ability of proteins to recognize a particular DNA sequence. The effect of O^6-alkylation of Gua on the methylation of adjacent Cyt would explain the observed hypomethylation of DNA after treatment with methylating carcinogens (Boehm and Drahovsky, 1981) and could provide an epigenetic basis for the action of carcinogens (Holliday, 1979). In addition it would explain the enhanced expression of oncogenes such as Ha-*ras* which have been shown to be hypomethylated in primary human carcinomas (Feinberg and Vogelstein, 1983). In addition, hypomethylation could cause widescale disruption of gene expression which may be responsible for, or related to, the cytotoxic properties of the alkylating agents. The importance to the cell of maintaining the appropriate cytosine methylation status may explain the apparently wasteful suicide protein mechanism for removing O^6MeGua residues (Yarosh *et al.*, 1983).

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Importance of the O^6 position of guanine residues in the binding of DNA methylase to DNA

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Key words: DNA methylase; Guanine O^6; DNA binding; DNA recognition

Methylation of *Micrococcus lysodeikticus* DNA by purified DNA methylase isolated from L1210 leukaemia cells is potently and specifically inhibited by both hetero and homoribo and deoxyribopolynucleotides containing guanine residues. The inhibitory effect is unaffected by chain length, but is abolished when the O^6 residue of guanine is substituted as in poly[d(O^6MeG)]$_{20}$. Potent inhibition is also shown by polyinosinic and polyxanthylic acids, but not by polyadenylic acid or by heteropolymers containing adenine and thymine. These results suggest that the 6-position of the purine nucleus is important in binding of the DNA methylase to a particular region of the DNA duplex and that the hydrogen bonding properties of this group are important in enzyme recognition.

Introduction

Methylation of cytosine residues in DNA is thought to play an important role in the regulation of gene expression. Thus, inactive genes are frequently hypermethylated in the promoter region, while active genes are hypomethylated [1]. Although not all eukaryotic genes are regulated by methylation, it appears to be important in the regulation of housekeeping genes [2].

In animals the site of methylation is the 5-position of cytosine contained in the sequence CpG. Non-methylated CpG is clustered in GC-rich islands [3], and most vertebrate genes are associated with such islands, usually in the 5′-region of the gene. Both the primary DNA sequence and the spacing between the CG groups appear to be important in the direction of the DNA methylase [4], and for maintenance methylation the presence of 5-methylcytosine in the parental DNA is of fundamental importance.

Factors influencing the binding of the DNA methylase to particular CpG sites are not known, although guanine residues, as well as adjacent adenine and thymine residues, have been suggested as being important [5]. The possible importance of guanine residues is also suggested by the inhibition of the methylation of DNA and poly(dG-dC) · poly(dG-dC) when the guanine residues are methylated in the O^6-position by *N*-methyl-*N*-nitrosourea (NMU) [6].

In order to investigate the recognition features of the DNA duplex responsible for the methylation of specific cytosines in CpG sequences, we have looked at the effect of oligonucleotides, which are not substrates for the DNA methylase, on the methylation of *M. lysodeikticus* DNA, which is a substrate, because of the high GC content [7].

Materials and Methods

S-Adenosyl-L-[*methyl*-^{3}H]methionine (specific activity 83 Ci mmol^{-1}) and [γ-^{32}P]ATP (specific activity 3000 Ci mmol^{-1}) were purchased from Amersham International, Bucks. *M. lysodeikticus* DNA and all other chemicals were obtained from Sigma, Poole, Dorset. Polynucleotides were obtained from Sigma except poly(G) and poly(C) which were purchased from Fluka Chemicals, Glossop, Derbyshire and poly(dG) which was purchased from Pharmacia LKB Biotechnology, Milton Keynes, Bucks. The oligonucleotides, poly[d(C · O^6MeG)]$_{10}$ and poly[d(O^6MeG)]$_{20}$ were prepared by Dr. G. Margison and Dr. M. Mackett of the Paterson Institute, Manchester by solid state methodology using β-cyanoethyl-*N*,*N*-diisopropyl phosphoramidite intermediates obtained from Cruachem Chemical, Livingstone, U.K. and American Biometics, Hayward, CA,

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U.S.A. as described by Borowy-Borowski and Chambers [8]. They were synthesised on a 1 μmol scale using a Dupont Coder 3000. Deprotection was carried out with 10% DBU (1,8-diazabicyclo[5.4.0]undec-7-ene) in methanol at room temperature for 5 days. The purity of the oligonucleotides was determined by end-labelling with ^{32}P from [γ-^{32}P]ATP using polynucleotide kinase (Boehringer, Lewes, Sussex) followed by electrophoresis on a 20% urea : acrylamide gel and autoradiography.

Preparation of DNA methylase

DNA methylase was prepared from L1210 murine leukaemia cells passaged either in vitro or in vivo in BDF_1 mice. Cells (at least $1 \cdot 10^9$) were washed at 4 °C in 0.9% NaCl and pelleted by centrifugation at 1500 rpm for 5 min in a Heraeus bench top centrifuge. The cells were then suspended in 10 ml of 10 mM Tris-HCl (pH 7.5), containing 10 mM EDTA and 0.5% Nonidet P40, and homogenised using a Dounce teflon homogeniser for 1 min. The nuclei were pelleted by centrifugation at 4000 rpm for 20 min, washed in 0.25 M sucrose and suspended in 50 mM Tris (pH 7.8), containing 1 mM EDTA, 1 mM dithithreitol, 10% glycerol and 60 mg l^{-1} phenylmethylsulphonyl fluoride (buffer M) containing 0.4 M NaCl and the DNA methylase was purified essentially according to the method of Turnbull and Adams [9] with modifications. DNA was removed from the nuclear extract by DE52 cellulose and proteins were consecutively precipitated by addition of ammonium sulphate to 30 and 60% saturation. The precipitate from the 60% ammonium sulphate saturation was dissolved in buffer M containing 0.4 M NaCl, applied to a column of Ultrogel AcA-34 and eluted with the same buffer. Activity was eluted at 1.25 to 1.75 × void volume, and active fractions were pooled and dialysed against buffer M containing 50% glycerol for 6 h. The dialysate was stored at −20 °C and used as the enzyme preparation in this study.

DNA methylase assay was based upon the method described by Bolden et al. [10]. The standard assay contained 20 mmol Tris-HCl (pH 7.5), 5 mmol dithiothreitol, 1 mmol EGTA, 1 μCi *S*-adenosyl-L-[*methyl*-^{3}H]methionine, 20 μg *M. lysodeikticus* DNA and enzyme preparation in a total volume of 100 μl. After incubation for 4 h at 37 °C the samples were cooled to 0 °C and unlabelled *S*-adenosyl-L-methionine was added to a final concentration of 1.5 mM. The entire reaction mixture was then spotted onto 2.1 cm discs of Whatman DE81, allowed to air dry, and the discs were washed five times in 20 mM Na_2HPO_4, (pH 8.4), twice in water and once in ethanol/ether (95 : 5, v/v). All washes were carried out at room temperature for 5 min. After air drying the filters overnight, the radioactivity was determined by adding 8 ml of toluene, 2,5-diphenyloxazole (PPO) mixture and counting on a Packard Tri-Carb 2000CA liquid scintillation counter. Control

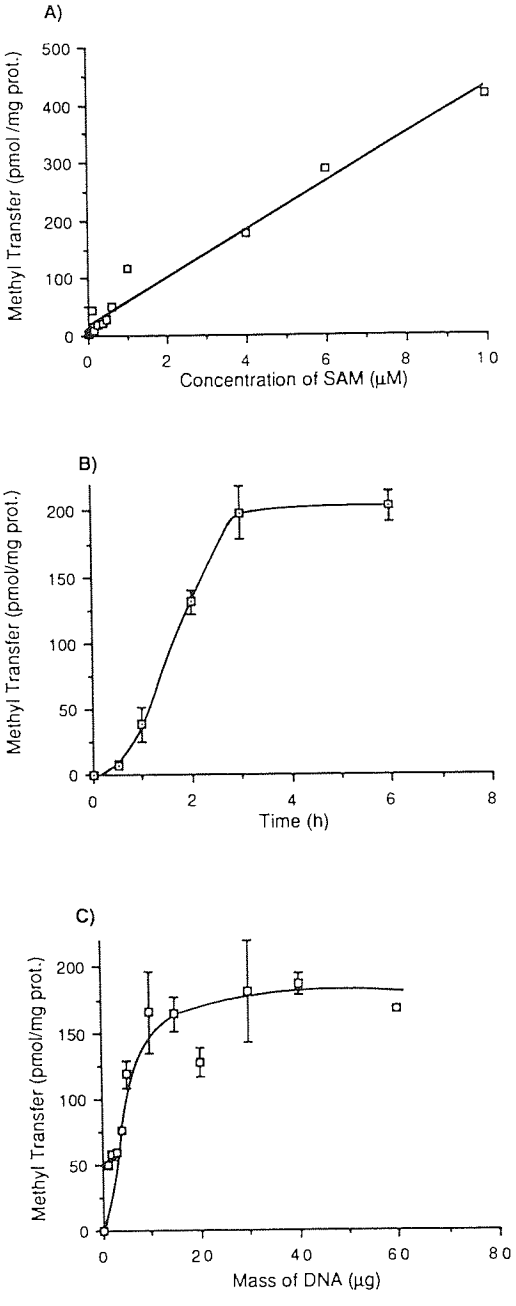

Fig. 1. Kinetics of methylation of *M. lysodeikticus* DNA by DNA methylase purified from L1210 leukaemia cells. (A) Extent of incorporation of methyl groups with variations in the concentration of *S*-adenosyl-L-methionine. (B) Effect of incubation time on the extent of incorporation of methyl groups. (C) Variation in the amount of methyl groups transferred with the mass of DNA in the assay. For A and C the standard incubation time was 4 h.

assays were also performed without any added substrate DNA to distinguish DNA methylation from other types of methylation, and these values were subtracted from the total methylation obtained. In all cases these represented less than 10% of the total radioactivity incorporated. All values represent the mean ± S.E. of at least three separate determinations.

Results

The characteristics of the DNA methylase isolated from L1210 murine leukaemia cells are indicated in Fig. 1. The reaction rate with *M. lysodeikticus* DNA is proportional to the concentration of *S*-adenosyl-L-methionine in the incubation mixture up to 10 μM (Fig. 1A), to the incubation time up to 3 h of incubation (Fig. 1B) and to the mass of the DNA in the assay mixture up to 20 μg (Fig. 1C). The molecular mass of the methylase as determined from the Ultrogel AcA-34 column was 95 kDa and fractions were collected in the molecular mass range 60 to 120 kDa. The size of the enzyme is somewhat smaller than values previously reported for other DNA methylases [11]. There was no detectable deoxyribonuclease or ribonuclease activity.

The results presented in Fig. 2 show the effect of various polynucleotides and polydeoxynucleotides on the methylation of *M. lysodeikticus* DNA by purified DNA methylase. For both the polyribo and deoxyribonucleotides tested, only those containing a guanine residue were potent inhibitors of the methylation process. Thus, while poly(G), poly(dG) and poly(dG) · poly(dC) were all effective inhibitors, poly(C), poly(dC), poly(U), poly(A) and poly(dA) · poly(dT) had no effect on the methylation of *M. lysodeikticus* DNA. Both ribo- and deoxyribopolynucleotides containing G residues were equally effective as inhibitors (Fig. 3), and the inhibitory potency was unaffected by chain length, since even small oligomers such as $poly(dG)_{20}$ could inhibit the DNA methylase, although GMP itself caused only slight inhibition (28 ± 10%) at 10 mM. The O^6-position of the purine moiety appears to be important in the inhibition process since poly(I) and poly(X) were also effective inhibitors (Fig. 4), while when the O^6-position was substituted as in $poly[d(C \cdot O^6MeG)]_{10}$ (Fig. 2) or in $poly[d(O^6MeG)]_{20}$ (Fig. 3) the inhibitory effect was destroyed.

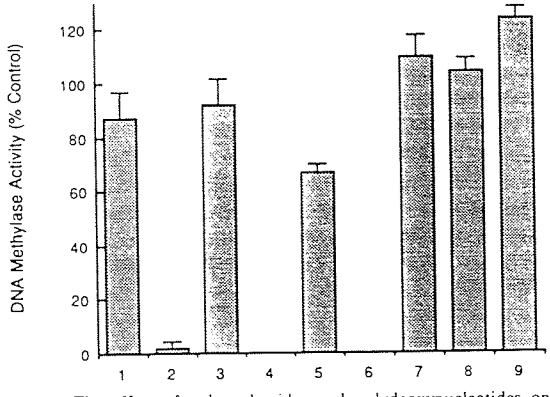

Fig. 2. The effect of polynucleotides and polydeoxynucleotides on DNA methylase activity using *M. lysideikticus* DNA as acceptor. To the standard methylase assay using 20 μg *M. lysodeikticus* DNA was added 10 μg of poly(dA)·poly(dT) (1); poly(dG)·poly(dC) (2); poly(dC) (3); poly(dG) (4); $poly[d(C \cdot O^6MeG)]_{10}$ (5); poly(G) (6); poly(C) (7); poly(U) (8) and poly(A) (9), and the incorporation of [*methyl*-^{3}H] from *S*-adenosyl-L-[*methyl*-^{3}H]methionine was determined as described in Materials and Methods.

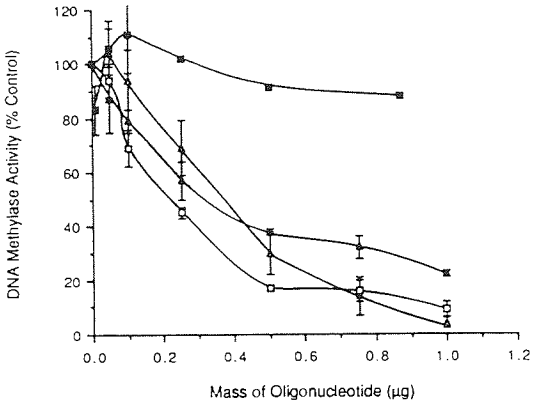

Fig. 3. Effect of increasing concentration of $poly(G)_{400}$ (△), $poly(dG)_{600}$ (▲), $poly(dG)_{20}$ (□) and $poly[d(O^6MeG)]_{20}$ (■) on the methylation of *M. lysodeikticus* DNA by purified L1210 DNA methylase.

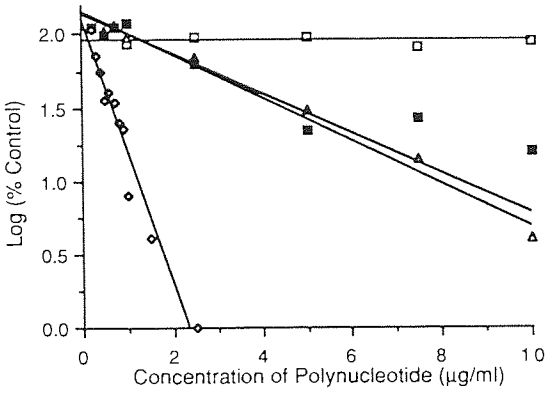

Fig. 4. Effect of increasing concentrations of poly(A) (□), poly(G) (△), poly(I) (■) and poly(X) (◇) on the methylation of *M. lysodeikticus* DNA by purified DNA methylase. The results have been normalised to a first-order plot and are expressed relative to a control incubation containing no oligonucleotide.

Discussion

De novo methylation of DNA proceeds at a rate only 3 to 4% of that of maintenance methylation [5], although the same enzyme is probably responsible for both functions [4,5,7]. Our results are consistent with the hypothesis that the substituent at the 6-position of the purine nucleus is important in binding of the enzyme to an appropriate section of DNA. Thus, the presence of a carbonyl group at the 6-position greatly facilitates binding of the enzyme, since poly(G), poly(I) and poly(X) are all strong inhibitors of the methylation of *M. lysodeikticus* DNA, while a 6-amino function as in poly(A) is without effect. Previous studies [11,12] have shown that the rate of methylation of the synthetic duplex poly(dI-dC) · poly(dI-dC) is 10- to 100-times higher than poly(dG-dC) · poly(dG-dC) or other non methylated DNAs [12,13]. The unmethylated polymer shows substrate inhibition [12] and homopolymers poly(dI) · poly(dC) and poly(dG) · poly(dC) which do not serve as methyl accepting polymers complete with the methyl accepting polymer poly(dG-dC) · poly(dG-dC) in binding to DNA methyltransferase [13]. We have also shown that the non methyl accepting polymer poly(dG) · poly(dC) is able to effectively compete with *M. lysodeikticus* DNA for the methylase.

It is likely that the carbonyl group in the 6-position of the purine nucleus is involved in hydrogen bonding of the DNA duplex to the enzyme since formation of the O-methyl derivative as in poly[d(O^6MeG)]$_{20}$, which destroys the hydrogen bonding properties, also abolishes the inhibitory effect of the polynucleotide. In addition, synthetic oligonucleotides containing O^6MeG in place of G in CG linkage are not substrates for the DNA methylase (unpublished data). Within double-stranded DNA the O^6-position of the purine protrudes into the major groove of DNA, and is therefore available for recognition and hydrogen bonding by those enzymes which are present in the major groove, such as DNA methylase. The enhanced inhibitory effect of poly(X) over poly(I) and poly(G) suggests that if there is an additional carbonyl group at the 2-position of the purine moiety, such as with poly(X), this also becomes available for hydrogen bonding if the DNA is single-stranded.

We have been unable to confirm the results of Bolden et al. [5] that adjacent adenine and thymine residues are important in influencing the choice of sites of methylation by the enzyme. In agreement with the results of the above authors, calf thymus DNA has no inhibitory effect on the methylation of *M. lysodeikticus* DNA, but in our hands neither poly(dA) · poly(dT) nor poly(A) were able to influence the methylation reaction. It is difficult to explain the differences between these two sets of results, which can only arise from the differences in specificity of the methylases used in the two studies.

The increased binding of purified DNA methylase to poly(G) over poly(C), poly(U) and poly(A) would suggest that the enzyme binds loosely and reversibly to G units in the DNA duplex and that the nature of the sugar unit is unimportant. The O^6-position of guanine is a reactive site for a number of carcinogenic and mutagenic alkylating agents and if O^6-alkylation prevents the recognition of guanine as such by DNA methylase, this has important implications in terms of carcinogenesis. Consequently, the inhibition of DNA methylase recognition by the presence of O^6-alkylguanine in DNA may account for the promutagenic and procarcinogenic activity of O^6-alkylguanine as suggested by Loveless [14].

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