DNA METHYLTRANSFERASE FROM *PISUM SATIVUM*

BY

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ABBREVIATIONS

The abbreviations used in this Thesis are in agreement with the recommendations of the editors of the Biochemical Journal, [Biochem. J. (1983) 209 : 1-27], with the exception of the following.

m^5C	5-methylcytosine
m^4C	N^4-methylcytosine
m^6A	N^6-methyladenine
SDS	Sodium dodecylsulphate
PMSF	Phenylmethylsulphonylfluoride
TCA	Trichloroacetic acid
AdoMet	S-adenosyl-L-methionine
mtDNA	Mitochondrial DNA
PEPCK	Phosphoenolpyruvate carboxykinase
Pgk	Phosphoglycerate kinase
Dhfr	Dihydrofolate reductase
Aprt	Adenine phosphoribosyl transferase
Hprt	Hypoxanthine phosphoribosyltransferase
tk	Thymidine kinase
Ac	Activator element
Ds	Dissociator element
Spm	Suppressor-mutator element
rbcS	Ribulose-1,5-bisphosphate carboxylase (small subunit)
PCA	Perchloric Acid

LIST OF CONTENTS PAGE

LIST OF FIGURES

Page

LIST OF TABLES

Summary

The objective of the work reported in this thesis was to isolate and characterise a plant DNA methylase and to compare its properties to those of mouse ascites DNA methylase. This was of particular interest because plant DNA contains methylated cytosine in both CG dinucleotides and CNG trinucleotides, whereas animal DNA contains methylated cytosine in CG dinucleotides only.

High DNA methylase activity was detected in the nuclei from pea shoot tips. Methylation of endogenous DNA was observed, suggesting the presence of undermethylated sites indicative of a delay in complete methylation of DNA following replication.

DNA methylase was purified and characterised from pea seedlings. A 0.2M NaCl extract of pea nuclei was purified using successive chromatography on Heparin Sepharose and Mono Q. Only one peak of enzyme activity was obtained in these and other purification steps. The product of methylation of pea DNA by pea DNA methylase was identified as 5-methylcytosine. The 3' nearest neighbours to the methylated cytosine were determined with pea DNA and found to be G, A, T and C, consistent with the methylation of CG and CNG sequences by the purified pea DNA methylase. Therefore the tentative conclusion is that one species of enzyme is capable of methylating CG and CNG sequences in plant DNA.

The most purified enzyme preparation obtained was enriched 19,000 fold over the tissue homogenate. Molecular exclusion chromatography indicated that pea DNA methylase had a native molecular mass of about 160,000, similar to the high molecular weight, mammalian DNA methylases. An attempt was made to confirm the identity of this high molecular weight band as DNA methylase by assaying for DNA methylase *in situ* in a non-denaturing polyacrylamide gel. However no activity could be detected. Furthermore, in immunochemical experiments using an antiserum to mouse ascites DNA methylase, no cross-reactivity with any electrophoretic band was detected.

Other protein bands of 80,000Da and 40,000Da were obtained with other preparations of pea DNA methylase, and in aged preparations the 160,000Da band was found to be degraded indicating a high susceptibility to proteolysis.

Pea DNA methylase showed a preference for pea DNA over animal and bacterial DNA as substrate. Native pea DNA was a ten-fold better substrate than denatured Micrococcus luteus DNA. This is in contrast to the mammalian enzymes which show little activity with homologous DNA. The enzyme showed a strong preference for hemimethylated double-stranded DNA but it was also able to methylate ϕX174 RF DNA, showing that it is also capable of methylation de novo. Despite its susceptibility to proteolysis, pea DNA methylase had a capacity for continued DNA methylation over many hours in vitro.

Pea DNA methylase exhibited cooperative binding to its DNA substrate, except at limiting DNA concentrations when linear reaction kinetics were observed. These results are consistent with the mechanism proposed in 1971 by Drahovsky and Morris whereby the enzyme methylates DNA by binding irreversibly, and sequentially adding methyl groups to the correct sites by migrating along the DNA strand. The preference of pea DNA methylase for native pea DNA as substrate suggested that the mechanism of action of the pea enzyme might differ from that of the mouse ascites enzyme. A further difference from the ascites DNA methylase, consistent with this possibility, was the observation that in contrast to the ascites DNA methylase, pea DNA methylase showed a similar sensitivity to inhibition by NaCl with denatured and native DNA substrates.

The specific activity of the most purified DNA methylase was only 1800 units per mg protein, compared with 11,000 for mouse ascites DNA methylase. One possible explanation for this is that additional factors not present in the assay are required for optimal activity. A fraction obtained from the supernatant of nuclei stored in 50% glycerol stimulated bound pea DNA methylase activity ten-fold and solubilised pea DNA methylase activity two- fold. This extract may therefore contain such a factor.

CHAPTER ONE

Introduction

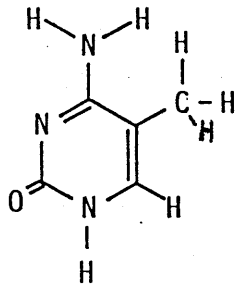
1.1. Occurrence of methylated bases in DNA.

The DNA of many organisms, from bacteria and unicellular eukaryotes to vertebrates and higher plants, contain modified bases other than adenine, guanine, cytosine and thymine. Prominent among these are 5-methylcytosine, N^6-methyladenine, 5-hydroxymethylcytosine and N^4-methylcytosine, found in varying amounts in the DNA from prokaryotic and eukaryotic cells. They are produced post-replicatively by sequence-specific methylases (Shapiro, 1976). This is discussed below in more detail.

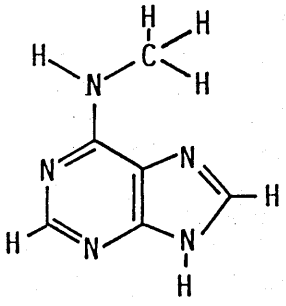
1.1.1. Methylated bases in prokaryotes.

Johnson and Coghill (1925) first reported the presence of 5-methylcytosine in the DNA from Tubercle bacilli. Other workers soon established the presence of other modified bases in the DNA of prokaryotic organisms. These include workers like Dunn and Smith (1955) who reported the presence of the minor base, N^6-methyladenine in the DNA from E.coli 15T⁻. A similar report of the discovery of another minor base, N^4-methylcytosine, was made by Janulaitis et al. (1983) from DNA of Bacillus centrosporus.

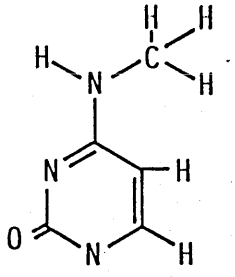
In addition, methylated bases are also present in bacteriophage DNA present in prokaryotic cells. All the cytosine residues in the DNA from the bacteriophage XP12 are present as 5-methylcytosine (Kuo et al., 1968) and bacteriophage T2 contains N^6-methyladenine (Wyatt and Cohen, 1953). 5-hydroxymethylcytosine is present in the DNAs of T-even phages in place of cytosine (Wyatt and Cohen, 1953). The modified bases are shown in Figure 1.



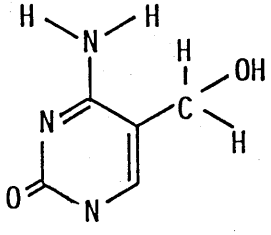
1a. 5-methylcytosine (m^5C)



1b. N^6-methyladenine (m^6A)



1c. N^4-methylcytosine (m^4C)



1d. 5-hydroxymethyl cytosine

Figure 1. Methylated bases in DNA.

1.1.2. Methylated bases in eukaryotes.

The only modified base in the DNA of higher plants and animals is 5-methylcytosine (Figure1). 5-methylcytosine was reported as a component of nucleic acids, from plant and animal cells by some workers over 35 years ago (Hotchkiss, 1948; Beatty and Fischberg, 1950 and Wyatt, 1951). Subsequent reports from experimental results of the base composition analysis of more DNA samples established this modified base as a component of DNA from eukaryotic organisms. These include reports from workers like Thomas and Sheratt (1956) who identified 5-methylcytosine in the DNA from plant leaf and Vanyushin et al. (1970) who analysed DNA from a number of plant and animal cells and confirmed these findings. 5-methylcytosine was present in all DNA examined in varying proportions of the cytosine residues, depending on the species and the particular tissue of the organism.

N^6-methyladenine (Figure1) has also been reported as a component of DNA from unicellular eukaryotes, such as Chlamydomonas reinhardii, (Hattman et al., 1978) and in trace amounts in some eukaryotes such as mosquito, Aedes albopictus (Adams et al., 1979). The major modified base in eukaryotic cell DNA is 5-methylcytosine and all subsequent discussions will be based only on the synthesis and biological significance of this modified base.

1.1.3. Effect of DNA methylation on the physical properties of DNA.

The presence of modified bases in the DNA affects both the buoyant density and the melting temperature of the DNA. The conversion of cytosine to 5-methylcytosine introduces a methyl group into an exposed position in the major groove of the DNA helix.

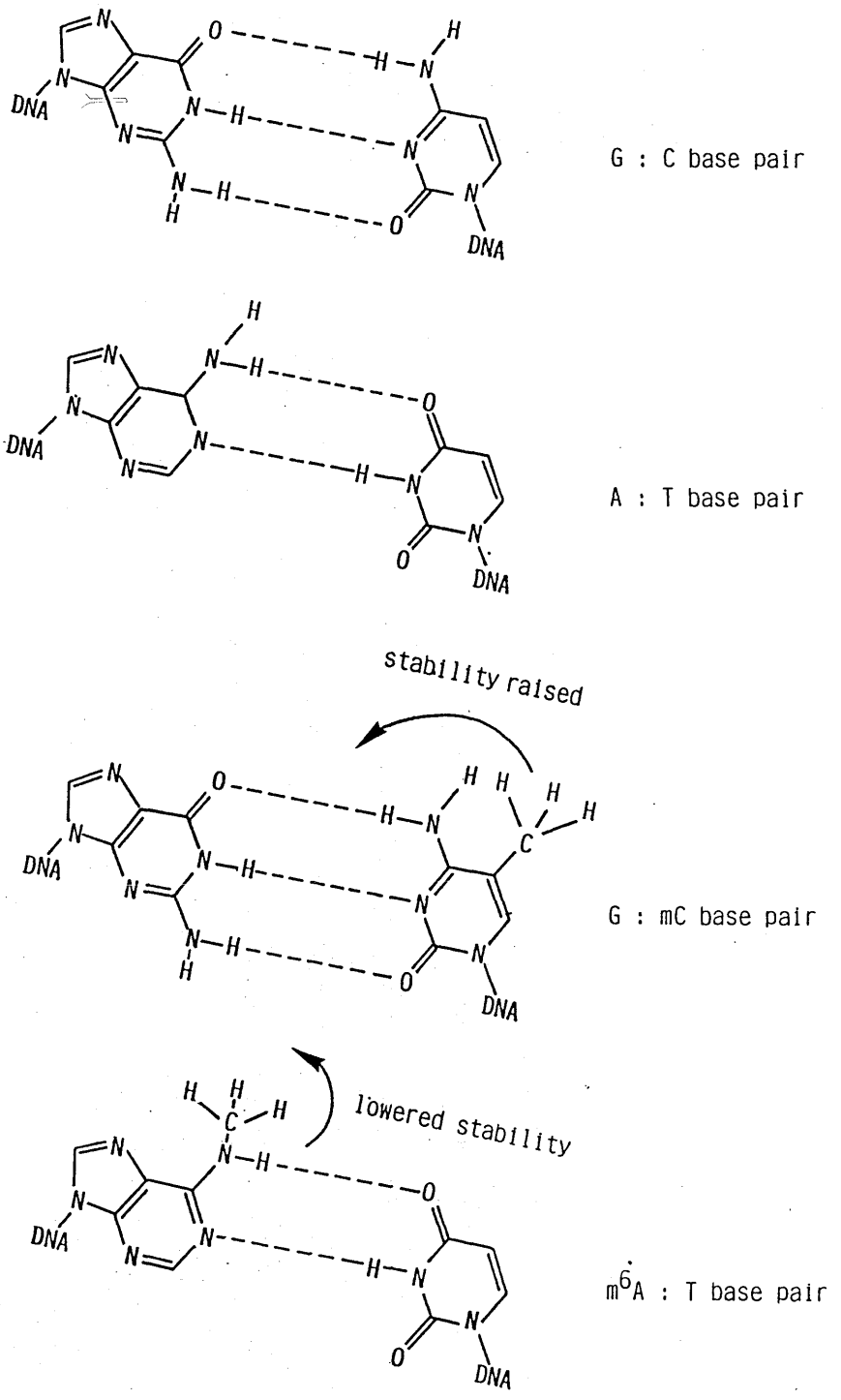


Figure 2. Effect of methylated bases on DNA helical stability. (a) The normal base pairing arrangement found in DNA. (b) Steric effect of methyl groups on the base pairing. (Adams et al., 1986b; Murchie and Lilley, 1989).

Methylation of cytosine which occurs at the carbon 5 position does not directly affect the hydrogen bonding between guanine and cytosine. The additional methyl group improves stacking interactions between the adjacent base pairs (Figure 2b), leading to an increase in local helical stability, (Kirk, 1967; Ehrlich et al., 1975; Wagner and Capesius, 1981; Adams and Burdon, 1985). This raises the melting temperature of DNA (Gill et al., 1974). This local helical stability effect has been demonstrated by electron microscopic studies (Murchie and Lilley, 1989). They showed that cytosine methylation led to reduced extrusion rates of cruciform DNA by a factor of 1.7 to 2.7, thus revealing the local stabilisation effect of cytosine methylation.

In N^6-methyladenine the nitrogen attached to C^6 cannot participate effectively, in the formation of a hydrogen bond with thymine (Figure 2b), thus causing a local destabilisation of the double helix. This results in a lowering of the melting temperature of the DNA, (Vanyushin et al., 1968; Engel and von Hippel, 1978). Also as a result of the lowered helical stability, the DNA exhibits a faster rate of cruciform extrusion (Murchie and Lilley, 1989).

1.2. Cellular distribution of methylated DNA in eukaryotes.

1.2.1. In organelles of animal cells.

Most of the methylated DNA in animal cell DNA, is found in the nuclear genome. Low levels of 5-methylcytosine have been reported in the DNA from mitochondria in cultured hamster and mouse cells and other animal cell mitochondria DNA (Nass, 1973; Adams and Burdon, 1985 ; Reis and Goldstein, 1983; Pollack et al., 1984) Higher levels of 5-methyl cytosine have been reported in the mitochondrial DNA from beef heart muscle (Vanyushin and Kirons, 1974).

It is almost certain now that 5-methylcytosine is a component of all animal cell mitochondria DNA (Pollack et al., 1984). The 5-methylcytosine residues are present in CpG dinucleotide sequences in mtDNA sequences interspersed with tracts of unmethylated regions. This pattern of methylation is similar to the methylation pattern found in nuclear DNA (Pollack et al., 1984).

1.2.2. In organelles of plant cells.

In higher plants 5-methylcytosine has been reported only in the DNA of the nuclear genome. Plastid DNA is devoid of methylated bases (Baxter and Kirk, 1969; Whitfeld and Spencer, 1968; Hattman et al., 1978; Bohnert, 1982). However, 5-methylcytosine is detectable in low levels in chloroplast DNA of the vegetative cells of the unicellular green algae, Chlamydomonas reinhardii (Bolen et al., 1982; Feng and Chiang, 1984). Higher levels are present in the female gametes, as well as the zygote resulting from the fusion of the male and female gametes, (Sager and Lane, 1972 and 1981; Royer and Sager, 1979; van Grisven and Kool, 1988).

The presence of 5-methylcytosine in non-photosynthetic plastids has recently been reported (Ngernprasirtsiri et al., 1988). It was proposed that this was a general mechanism of transcriptional regulation of non-photosynthetic tissues. However other workers have failed to detect any evidence of methylcytosine, during chromoplast development in the same plant Capsicum annum (van Grisven, 1988).

1.3. Enzymatic methylation of DNA.

1.3.1. Prokaryotic DNA methylases.

In prokaryotes adenine or cytosine can act as the methyl acceptor in enzymatic methylation of DNA.

Most of the DNA methylases in these organisms are associated with endonucleases. The methylases modify specific sites in the DNA and the endonucleases produce double-stranded nicks in DNA. There are three classes of bacterial restriction-modification systems; Types I, II and III. All three systems have specific recognition sites but differ in their modes of cleavage.

Type I restriction-modification enzymes are high molecular weight multisubunit enzymes. They contain 3 nonidentical subunits encoded by separate genes on the E.coli chromosome (Lautenberger and Linn, 1972). They are present in different proportions in the cell. The three subunits are responsible for the nuclease activity, methylation and the sequence specificity of the enzyme. These enzymes require Mg^{2+}, ATP and S-adenosyl-L-methionine for endonuclease and methylase activity. Type I enzymes have complex recognition sites as shown below for EcoK and EcoB restriction-modification enzymes.

EcoK 5'- AACNNNNNNGTGC - 3'
TTGNNNNNNCACG

EcoB 5'- TG*ANNNNNNNN TGCT -3'
AC TNNNNNNNN*ACGA

The recognition sequences consist of a group of three or four bases, (underlined) separated by six (EcoK) or eight (EcoB) unspecified bases, (N), (Adams et al., 1986b). The site of methylation is marked by an asterisk. The cleavage site occurs at least 1000 base pair from the recognition site.

Type II restriction enzymes are relatively low molecular weight proteins. The endonuclease and methylase are separate enzymes. They each consist of a single polypeptide and act independently of each other.

They require Mg^{2+} for endonuclease activity and S-adenosyl-L-methionine for methylase activity (Roberts 1976 and 1980). The genes for these proteins are encoded on plasmids. Type II methylases transfer methyl groups to either N^6 of adenine, C^5 of cytosine (Roberts, 1984) or N^4 of cytosine (Buryanov et al., 1989). The recognition site for Type II enzymes is four to six base pair palindromic sequences. The recognition sequences for EcoRII and HpaII are shown below. The base methylated is in bold type and the the site cleaved by the nuclease is indicated by an asterisk (Roberts, 1980). The same holds for the same base sequence on the opposite strand, but only one strand cleavage and modification sites are illustrated for the sake of clarity.

EcoRII 5'-*CCA/TGG-3' HpaII 5'-C*CGG-3'
GGT/ACC G GCC

Type III restriction endonucleases have two non identical subunits. They require Mg^{2+} and ATP and are stimulated by AdoMet. The smaller subunit can act alone as a methylase enzyme and requires Mg^{2+} like the Type II methylases (Reiser and Yuan, 1977; Bachi et al., 1979). The restriction sites are non symmetrical and methylation at these sites occur on only one strand of the DNA. The endonuclease cleaves at a site 24 to 26 base pairs from the 3' end of the recognition site (Yuan, 1981). The recognition sites for EcoP1 and EcoP15 are shown below.

EcoP1: 5'- AGACC -3' EcoP15: 5'- CAGCAG -3'

In E.coli there exist also the dam and dcm methylases which do not have associated endonucleases (Barras and Marinus, 1989).

Most of the methylation of cytosine residues in the DNA from E.coli is carried out by the dcm protein which methylates CCA/TGG sequences at the internal cytosine (Razin et al., 1980). The dam methylase methylates adenine in the recognition sequence GATC.

1.3.1.1. Properties of prokaryotic DNA methylases.

A number of DNA methylases from prokaryotic organisms have been purified to homogeneity and the protein structures obtained. A few of them have been cloned and information about the control of their transcription is available (O'Connor and Humphreys, 1982; Adams and Burdon, 1985; Nathan and Brooks, 1989).

Prokaryote methylases have a temperature optimum of about 40^{0}C and a pH optimum between 7 and 8. They are inhibited by NaCl. EDTA is stimulatory and sulphydryl groups are essential. The methyl group donor in all cases is S-adenosyl-L-methionine (Figure 3).

Double stranded DNA substrates are better methyl acceptor for these enzymes than single stranded DNA substrates. The rate of methylation is constant until the DNA substrate is saturated, indicating a monomeric activity (Gunthert and Trautner, 1984). These enzymes are usually present in high concentrations in a cell and newly synthesised DNA is fully methylated shortly after synthesis. The reaction can occur in vitro, with purified enzymes at a similar rate.

The different types of prokaryotic restriction and modification systems and their properties have been summarised in Table 1.

Table 1. Characteristics of restriction-modification systems (Yuan, 1981; Iida et al., 1982).

	Type I	Type II	Type III
1.Resriction and modification activities	Single multi-functional enzyme	Separate endo-nuclease and methylase	Separate enzymes with a subunit in common
2. Protein structure	different subunits	simple	two different subunits
3. Cofactor requirements	ATP, Mg, AdoMet	Mg	ATP, Mg, AdoMet
4. Recognition sequence	EcoB: TGAN8TGCT EcoK:AACN6GTGC	rotational symmetry e.g EcoRI, GAATTC	EcoP1 : AGCC EcoP15 : CAGCAG
5. Cleavage sites	Possibly random at least 1000bp from host specificity site	At or near host host specificity site	24-26 base pair to 3' of host specificity site
6. DNA translocation	Yes	No	No
7. Site of methylation	Host specificity site	Host specificity site	Host specificity site

1.3.2. Eukaryotic DNA methylases.

1.3.2.1. General.

5-methylcytosine formation is accomplished by a methyl transfer from S-adenosyl-L-methionine (AdoMet) to the 5 position on the cytosine ring in DNA from eukaryotic cells. The formation of an enzyme-DNA complex is probably a first step in the enzymatic transfer of methyl groups (Drahovsky and Morris, 1971; Cantoni, 1975; Deutsch et al., 1976). The reaction is catalysed by the enzyme DNA (cytosine-5-) methyltransferase, (DNA methylase, E.C 2.1.1.37), and the product is DNA containing 5-methylcytosine (Figure 3). The enzyme is found associated with cell nuclei except in calf thymus cells, where most of the enzyme activity is present in the cytoplasm (Sano et al., 1983). The enzymatic reaction of DNA methylation is a post replicative process. It is generally accepted that, over a single cell cycle, most of the methyl groups are added within minutes of the incorporation of deoxycytidine into DNA, (Burdon and Adams, 1969; Keppler, 1970; Adams and Burdon, 1983). However a small fraction of methylation may be delayed for several hours after DNA replication (Kiryanov et al., 1980; Adams et al., 1983).

DNA methylase shows specificity not only to the base methylated but also to the bases adjacent to the site of methylation (Bird, 1980). Two types of DNA-methyltransferase activity have been postulated, maintenance methylation and de novo methylation. The two types of eukaryotic DNA methylation are discussed in more detail below.

1.3.2.2. Maintenance methylation.

DNA replicates semiconservatively, and the immediate product is the hemimethylated daughter strand.

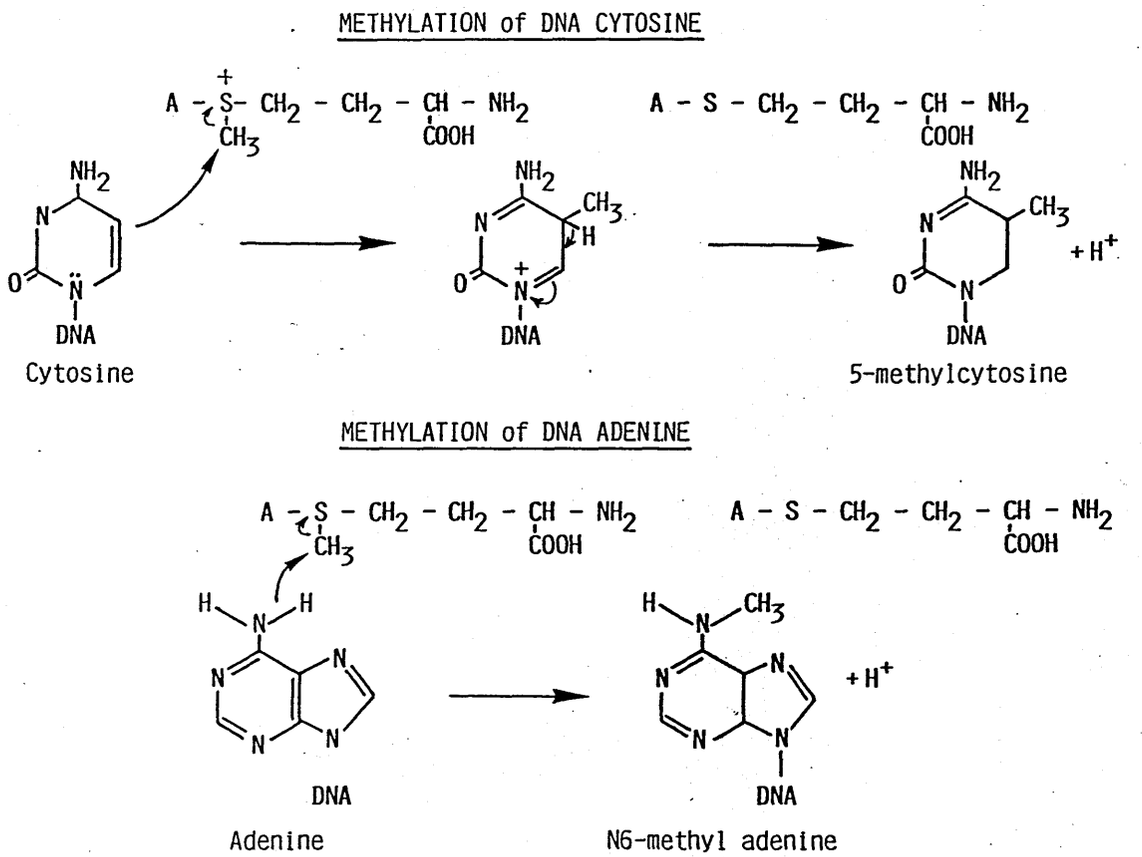


Figure 3. Mechanism of methylation of DNA cytosine and adenine by S-adenosyl-L-methionine. (Adams and Burdon, 1983).

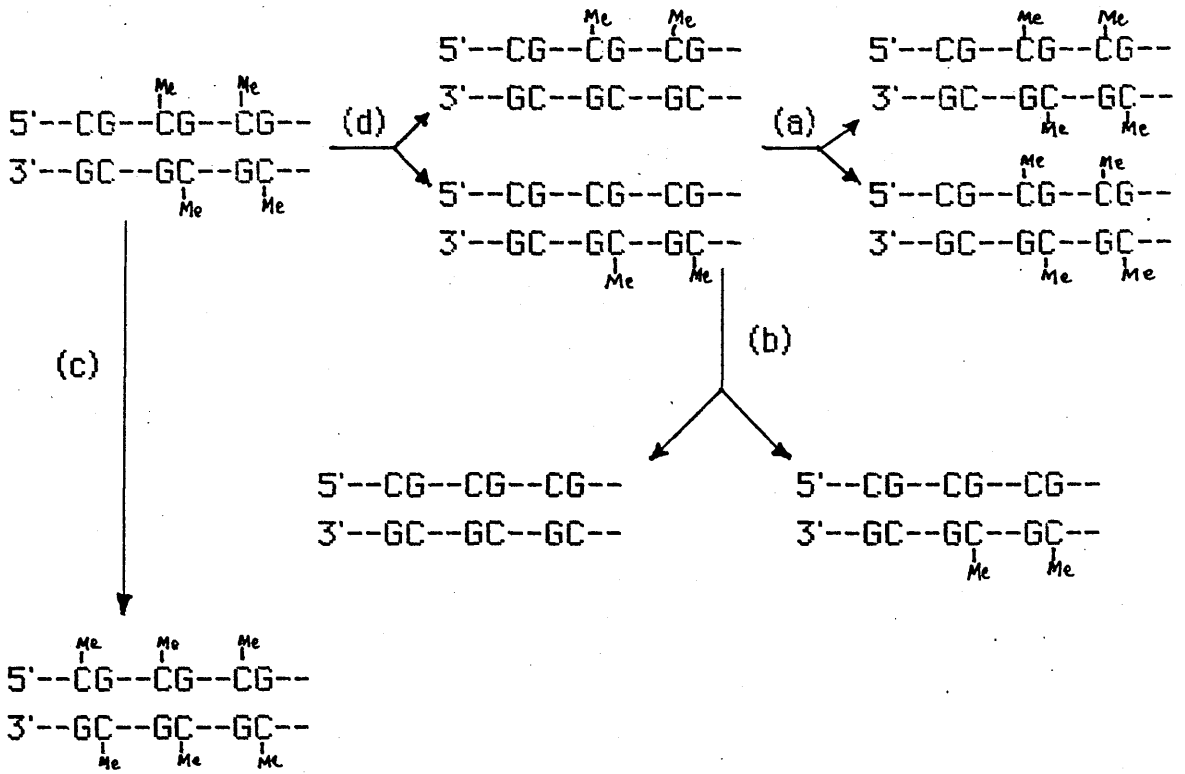


Figure 4. Mechanism for the maintenance of the methylation pattern and ways in which the pattern can be changed (Adams et al., 1986b)

(a) = Methylation of hemimethylated DNA by DNA methylase

(b) = If during two rounds of replication, methylation is blocked, then half of the daughter cells will be unmethylated

(c) = De novo methylation of unmethylated CG sites as a result of specific developmental signals.

(d) = Replication

Methylation of the daughter strand in post-replicative DNA is known as maintenance methylation (Razin and Riggs, 1980). Each strand of the parental DNA serves as a template for a maintenance methylase. Hemimethylated sites present immediately following replication act as the specific sequences recognised by the DNA methylases. Methyl groups are added to the bases in the new chain only when a symmetrically positioned one is already present in the parental chain. In this way methylated sites remain methylated and unmethylated sites remain unmethylated, thus preserving the parental pattern of methylation from generation to generation (Wigler et al., 1981; Gruenbaum et al., 1982; Stein et al., 1982). It has been postulated (Adams and Burdon, 1985) that the stable inheritance of the methylation pattern of the parental DNA is the major function in vivo of DNA methylases. The mechanism of maintenance methylation and the ways in which the pattern can be changed are illustrated in Figure 4.

1.3.2.3. **De Novo methylation**.

Methylation of previously unmethylated regions of the DNA is known as de novo methylation (Figure 4). Somatic tissues are more highly methylated than extraembryonic tissues which are hypomethylated. The sequential changes in the level of DNA methylation which arise as a result of development are the result of a de novo DNA methylation activity (Holliday and Pugh, 1975; Razin et al., 1984). De novo DNA methylation has been demonstrated in a mouse cell line transfected with proviral DNA by Jahner et al. (1983). These workers found that the insertion of proviral DNA into the genomic DNA of cells from a mouse germ line influences the methylation pattern of the host DNA. The sequences flanking the proviral DNA at the integration site become hypermethylated.

This change in methylation was shown with methyl-sensitive restriction enzymes. This hypermethylation of proviral DNA flanking sequence, occur even when the proviral DNA integrated into a chromosomal region which has all the characteristics of an active gene (hypomethylated). Methylation of foreign DNA integrated into eukaryotic nuclear genomes as a result of in vitro transfection systems is due to a de novo DNA methylation activity and has been demonstrated in other cell lines by other research workers (Jaenisch and Jahner, 1984; Keshet et al., 1986; Cedar, 1988).

Maintenance methylation occurs more often in somatic cells than de novo methylation (Pollack et al., 1980; Stein et al., 1982). De novo methylation process occur to a significant level only in the early embryonic stages and in the methylation of foreign DNA integrated into the nuclear genome as discussed above (**1.3.2.3**). The results obtained in experiments with purified enzymes from mammalian and plant cells, suggest that both these activities may be carried out by the same DNA methylase protein (Roy and Weissbach, 1975; Simon et al., 1978; Wang et al., 1984; Adams and Burdon, 1985; Theiss et al., 1987; Yesufu et al., 1989).

1.4. Purification and assay of eukaryotic DNA methylases.

DNA methyltransferase has been isolated from a number of tissues and cells in higher animals. The purification of DNA methylase has proved difficult due to the small amounts of the enzyme available and the instability of the enzyme. The first report of eukaryotic DNA methylase activity was made by Burdon (1966), from mouse ascites cells. Subsequently other workers have purified DNA methylase, not only from mouse ascites cells but also from a number of cells from various mammalian tissues as discussed below.

1.4.1. Vertebrate DNA methylases.

Animal cell DNA methylase forms two types of complexes with DNA. The bulk of the enzyme is only loosely associated with the nuclei and can be removed with low salt treatments. The enzyme also forms a strong matrix-bound complex resistant to high salt (Drahovsky and Morris, 1971; Turnbull and Adams, 1976; Burdon et al., 1985). DNA methylase has been purified from a number of mammalian tissues by means of various chromatographic procedures. These include chromatography on phosphocellulose, ammonium sulphate fractionation, gel filtration, chromatography on DEAE cellulose, DNA- and Heparin-Sepharose columns.

DNA methylase has been purified from the following animal cells: rat spleen (Kalousek and Morris, 1968; Kalousek and Morris, 1969); rat hepatoma cells (Sneider et al., 1975), mouse ascites cells (Turnbull and Adams, 1976; Adams et al., 1986a); Hela cells (Roy and Weissbach, 1975); rat liver cells (Drahovsky and Morris, 1971; Morris and Pih, 1971; Simon et al., 1978), bovine thymus (Sano et al., 1983); murine erythroleukemia cells, (Bestor and Ingram, 1983; Bestor et al., 1985; Bestor et al., 1988) and human placenta, (Wang et al., 1984; Pfeiffer, et al., 1985); mouse P815 mastocytoma cells (Pfeiffer and Drahovsky, 1986; Spiess et al., 1988); mouse plasmacytoma cell (Hitt et al., 1988). The purified mammalian enzyme has a molecular mass estimated to be between 150,000-280,000 (Adams and Burdon, 1985).

1.4.1.1. Assay conditions for eukaryotic DNA methylases.

DNA methylase activity is assayed by measuring the transfer of methyl groups from S-adenosyl-[^{3}H]-L-methionine to DNA substrate in the presence of 1mM EDTA, 1mM dithiothreitol and 10% glycerol in 50mM Tris HCl buffer by most workers.

The DNA product is purified free of protein and RNA (contaminant) present in the reaction mixture by a process of deproteinisation (phenol, pronase treatment, or chloroform/isoamyl alcohol) and alkali treatment (Kalousek and Morris, 1968; Sneider et al., 1975; Turnbull and Adams,1976). The DNA is isolated by ethanol precipitation in the presence of carrier DNA, washed in dilute acid and dissolved in perchloric acid for estimation of radioactivity. The extent of radioactive incorporation gives a measure of enzyme activity (Kalousek and Morris, 1969a; Adams et al., 1986a). The specific activity of the enzyme depends on the DNA substrate used. To confirm that the methyl groups are transferred to cytosine, the composition of the DNA product is determined from a base separation analysis of the formic acid hydrolysate of the DNA and shown to be 5-methylcytosine (Adams and Burdon, 1983).

1.4.1.2. Model for eukaryotic DNA methylase activity.

A model of an enzyme with two reactive domains, A and B, was proposed (Adams et al., 1986a). Domain A carries the recognition site for the daughter strand in post-replicative DNA and the active site which will methylate unmethylated CG dinucleotides. Domain B recognises the parental strand and carries an allosteric site which modifies the activity of domain A. The loss of domain B with limited proteolysis of the enzyme would result in an increase in the de novo DNA methylase activity of the enzyme. Spiess et al., (1988) purified a single polypeptide of DNA methylase from mouse P815 mastocytoma cells with a molecular weight of 190K, similar to that of Adams et al. (1986a). From the results of the structural analysis of the enzyme by electron microscopical techniques, they proposed a hemi-elliptical globular structure with a small appendix at the flat side.

The structure has two reactive domains similar to the structure proposed by Adams et al. (1986a). They allocated the recognition site for hemimethylated DNA, to the 'handle' and the active site of the enzyme to the 'cup' like structure. The 'handle' of their model was not removed by limited proteolysis and proteolysis leading to an increase in de novo activity was thought to occur elsewhere in the polypeptide.

A report has been made of the cloning and sequencing of a cDNA encoding DNA methylase of mouse cells (Bestor et al., 1988). Bestor and coworkers (1988) obtained a nucleotide sequence containing an open reading frame sufficient to encode a polypeptide of amino acid sequences large enough to give a molecular weight of about 180,000. They also proposed two reactive domains for their enzyme, a catalytic site near the carboxyterminus of the polypeptide and a regulation site, containing a potential metal-binding region, in the longer amino terminal portion of the polypeptide structure.

Although the details involved in the actual mechanism of methyl transfer are not the same in all models, they are all based on the common model of two reacting sites in the one enzyme molecule; an active site and an effector site. The correct mechanism still remains to be established.

1.4.2. **Plant DNA methylases.**

The first report of DNA methylase activity in higher plants (pea seedlings) was made by Kalousek and Morris (1969b). Their assay for methylase was identical to that developed for the mammalian enzyme. They however obtained low methylase activity. The plant enzyme has a a different temperature optimum (30^0C) from the mammalian DNA methylases (mouse enzyme has a temperature optimum of 37^0C). No detailed studies have since been made on the pea enzyme.

DNA methylases of two different molecular weights have been purified and characterised from the green algae, Chlamydomonas reinhardii (Sano and Sager, 1980; Sano et al., 1981). My project was to isolate, purify and characterise DNA methylase from pea seedlings. Since the start of my project, DNA methylase has been purified from wheat embryo by Theiss et al. (1987). They obtained an enzyme of native molecular mass of 50,000-55,000 in contrast to the high molecular weight enzyme from mammalian cells, which has a molecular mass of about 200,000. Theiss et al. (1987) also assayed the wheat enzyme in conditions similar to those used for the mammalian enzymes, except for the assay temperature of 30^0C. This is the same temperature optimum for pea DNA methylase (Kalousek and Morris, 1969b).

More work is still to needed on plant DNA methylases to obtain enzymes of reactivity comparable to that of the purified mammalian enzymes, so that the mode of action can be investigated.

1.4.3. Properties of eukaryotic DNA methylases.

Unlike the prokaryotic enzyme, eukaryotic DNA methylases have no cofactor requirements, are not stimulated by ATP or Mg^{2+}, and EDTA seems to be slightly stimulatory. Dithiothreitol is similarly required in the reaction which has a pH optimum of between 7.0 and 8 (Adams and Burdon, 1983). The specific activity of the enzymes depends on the DNA substrate used. The highest obtained so far is 70,000 units per mg for the human placenta DNA methylase when hemimethylated DNA substrate is used (Pfeiffer et al., 1985). The assays for the purified mammalian enzymes and the plant DNA methylases have been designed with these requirements in mind (**1.4.1.1**).

1.5. Patterns of eukaryotic DNA methylation.

1.5.1. General.

The modification reaction of DNA methylase, occurs at specific sites in polymeric DNA (Shapiro, 1976; Bird and Southern, 1978). Navey-Many and Cedar (1981) analysed the distribution of 5-methylcytosine in animal and higher plant cells using methyl-sensitive restriction enzymes, and gel electrophoretic analysis. They obtained a bimodal distribution pattern for mouse liver DNA, indicating the existence of clusters of methylated and unmethylated HpaII (CCGG) sites in the DNA. They also obtained a similar bimodal pattern of distribution for clusters of methylated and unmethylated EcoRII (CNG)$_n$ (CCA/TGG) sites in the DNA from wheat, cauliflower and tobacco. They concluded from their results that the methyl groups in higher plants and animal cells are not placed randomly on the DNA. They appear to be arranged in long clusters of fully methylated or unmethylated residues, most probably representing the inactive or active fraction of the genome. These findings were subsequently confirmed by other workers (Bird et al., 1985; Bird, 1986; Gardiner-Garden and Frommer, 1987; Antequera and Bird, 1988). Gardiner-Garden and Frommer (1987) using information from the GenBank DNA sequence data bank, screened vertebrate genomic sequences containing genes transcribed by RNA polymerase II (Insulin, GAPDH, Histone 4 and Opsin). A large number of these genes contain CpG islands associated with their 5' ends (all housekeeping and many tissue-specific genes) and the 3' ends of some tissue-specific genes. In the case of the tissue-specific genes where these islands occur at both ends, they are separated by several base pairs of CpG-depleted DNA. Similarly, Antequera and Bird (1988), using methyl-sensitive restriction endonucleases (HpaII/MspI, HinPI and EcoRII/BstNI) found

CpG islands in the genomic DNA from several plants (rye, tobacco, maize and wheat) and some tissue-specific genes in maize. They then concluded that both higher plants and animals have specific patterns of methyl group distribution in their genomic DNA.

1.5.2. Distribution of 5-methylcytosine in vertebrate DNA.

Most 5-methylcytosine which occurs in the DNA of higher animals is present in the dinucleotide sequence CpG (Cedar et al., 1979; Razin and Riggs, 1980). In vertebrate DNA the level of 5-methylcytosine is of the order of 2-8% (Shapiro, 1976). Although vertebrate genomes are considerably more methylated compared with insects and nematodes, in no cell type has there been found complete methylation of the CpG sequences and the distribution among the various sites is uneven. CpG sequences are arranged in clusters of methylated CpG sequences and unmethylated CpG sequences (known as CpG islands) many of which are associated with active genes. The experimental evidence for this has been discussed (**1.5.1.**).

1.5.3. Distribution of 5-methylcytosine in plant DNA.

Plant DNA has a high content of 5-methylcytosine (Vanyushin et al., 1960). 5-methylcytosines are found in the dinucleotide sequence, CpG, which is also the major modified site in vertebrate cells DNA (Burton et al., 1979; Gruenbaum et al., 1981). They are also present in the trinucleotide sequence CpNpG (Gruenbaum et al., 1981; Navey-Many and Cedar, 1981). The level varies among DNA from different plant tissues and also shows species variation as in the DNA from mammalian cells. In higher plants, 5-methylcytosine can account for up to 45% of the cytosine content (Follman et al., 1989). In spite of this high level of methylcytosine in the DNA of higher plants, compared with the DNA of

animal cells and other organisms, the level of methylation at methylatable sequences in plant is less than 100%. The plant genome, like that of mammalian cells is unevenly methylated (Antequera and Bird, 1988). The extent of methylation of CpG and CpNpG sites is not even, but depends on the genomic location of the particular sequence. Clusters of non-methylated CpG and CpNpG groups have also been associated with a number of expressing plant genes. The experimental evidence is discussed above (**1.5.1.**) and this has been confirmed by the more recent report of Antequera and Bird (1988).

1.6. Biological role of DNA methylation.

1.6.1. Biological role of DNA methylation in prokaryotes.

Methylation of DNA in prokaryotes has been assigned a role in the restriction-modification system which protects the organism from the consequences of invasion by foreign DNA (Wantanabe et al., 1966; Arber, 1974 and 1979; Yuan and Hamilton, 1984; Smith and Kelly, 1984). Only a small proportion of the methylation of these organisms, (e.g E.coli) is involved in this defence mechanism. The majority of the methylation products are the result of the activities of the dam and dcm methylases (**1.3.1.**). The function of the dcm methylase is unknown (Barras and Marinus, 1989). The dam methylase has a recognition sequence, GATC, present in genes involved in a variety of cellular processes.

DNA methylation in prokaryotes also plays a role in asymmetric mismatch repair (Pukkila et al., 1983; Wagner et al., 1984) and regulation of transposition in bacteria (Roberts et al., 1985). The newly replicated or repaired strand is transiently unmethylated and can be distinguished from the old strand by the mismatch repair system,

resulting in preferential correction to the sequence of the unmethylated strand (Figure 5). In wild type E.coli cells, most of the GATC sites are methylated at residue A on both strands and shortly after replication, the hemimethylated DNA is subjected to mismatch repair when the need arises. MutH protein (a component of the mismatch repair system) recognises the methyl group on one strand, binds to it and cleaves the DNA 5' to G in the GATC site in the daughter strand. The complex of proteins of the mismatch repair system (MutH, MutS, MutL) recognises the mispair and nicks the DNA and replaces the base. Helicase and an endonuclease activity create a gap in which the Ssb protein protects the methylated single strand and DNA ligase seals the nick.

DNA methylation has also been implicated in the control of prokaryotic gene expression (Plasterk et al., 1984; Roberts et al., 1985; Seiler et al., 1986). The presence of the recognition sequence of dam methylase in the promoter regions of some genes influences DNA-protein interactions, since the presence of a methyl group on residue A was found to lower gene expression. This could be due to the decreased stability of mA-T base pairs (Barras and Marinus, 1989) see Figure 2. DNA methylation is also linked with DNA replication in prokaryotes. Experimental evidence indicates that methylation in E.coli is required for efficient chromosome replication. Barras and Marinus (1989) reported that in E.coli, minichromosomes with one site of replication origin (oriC) are unstable in dam$^-$ mutant cells and over-production of dam methylase results in a more frequent rate of replication. Initiation of DNA replication is dependent on the binding of DNA protein A, which acts to separate the two strands of a DNA double helix. Methylation would thus facilitate the formation of an open complex catalysed by DNA protein A (Bramhill and Kornberg, 1988).

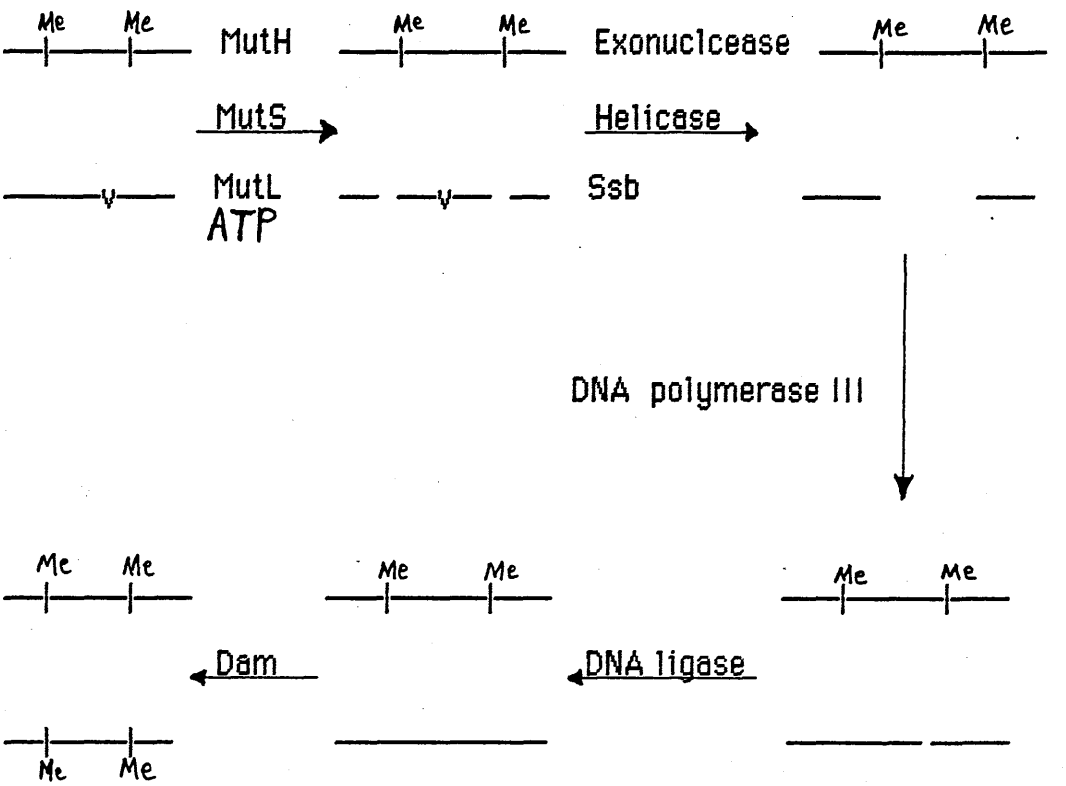


Figure 5. Repair of base mismatch in hemimethylated DNA in E.coli. (Barras and Marinus, 1989). In the presence of ATP, a complex of MutH, MutL and MutS proteins binds to hemimethylated DNA containing a mispair, nicks the unmethylated DNA and DNA polymerase III fills in the gap with the correct base.

However dam⁻ mutants are known to be viable, able to initiate and replicate their chromosomes hence the role of methylation still remains to be solved (Barras and Marinus, 1989).

1.6.2. Methylation and DNA conformation.

In addition to the right-handed double helix conformation (B-DNA), DNA can exist as a left-handed double helix (Z-DNA), at high salt concentrations. This is also the preferred conformation of DNA with alternate residues of 5-methylcytosine and guanine (Behe and Felsenfeld, 1981; Van Lier et al., 1983; Klysik et al., 1983; Adams and Burdon, 1985). The low frequency of CpG in eukaryotic DNA (Adams and Eason, 1984; Adams et al., 1987) would tend to exclude the possibility of Z-DNA formation. The control region of an inactive gene might contain a short region of methylated DNA in the Z-configuration stabilised by methylation and by association with Z-DNA binding proteins (Zacharias et al.,1988). Demethylation of DNA, as a result of gene activation, might be associated with the conversion of Z-DNA to B-DNA. DNaseI-sensitive sites associated with active genes are delimited by non-B-DNA, such as Z-DNA. It seems that nucleases recognise these junctions between B-form and non-B-DNA. The boundary between the Z-DNA and B-DNA may be the DNaseI-hypersensitive sites. That DNA methylation may be an important switch mechanism for influencing B-Z equilibrium in DNA topology, has been supported by the experimental findings of Zacharias et al. (1988). They investigated the structural consequences of cytosine methylation on the supercoil dependent B-Z equilibrium in alternating dC-dG sequences cloned into recombinant plasmids. They found that methylation of the dC-dG sequences results in a two fold decrease of the unwinding at B-Z junction regions.

1.6.3. Methylation and chromatin conformation.

DNA methylation is known to be involved in the regulated expression of nuclear genes by the influence on the local configuration of a gene (**1.1.3**). It was suggested that methyl groups play an active part in controlling transcription of genes either directly by steric hindrance of sequence specific DNA binding proteins, or indirectly by the changes in the chromatin structure of methylated DNA. The tight packing of any gene and its regulatory sequences will prevent the approach of RNA polymerase proteins. During the activation of such an inactive gene, its chromatin has to adopt a relaxed, more "open" configuration and this could arise through demethylation of the DNA (Keshet et al., 1986). This is illustrated by an increase in sensitivity of the gene to nucleases such as DNaseI and the appearance of DNaseI hypersensitive sites (Elgin 1981; Nickol and Fensenfeld, 1981; 1984; Eisenberg et al., 1986; Cartwright and Elgin, 1987; Klysik et al., 1988). Some experimental evidence has been obtained in some plants and animal systems to support the inverse correlation of DNA methylation, chromatin conformation and gene expression.

1.6.3.1. Chromatin structure and gene expression in animals.

Most data on chromatin structure and its changes before gene activation during expression and before gene inactivation, have been obtained from animal and viral systems (Dawid et al., 1970; Keshet et al., 1986). The changes in chromatin conformations are indicated by the changes in sensitivity to DNaseI, an inactive conformation being associated with DNAseI resistance. An increase sensitivity to DNAseI and localised sites of DNaseI hypersensitivity are associated with potential gene expression (Weisbrod, 1982; Eissenberg et al., 1985). Support for this has been obtained in a number of experimental systems as shown in these examples.

An enhanced sensitivity to DNAse I of transcriptionally competent genes in animal genomes, was first shown for the β-globin gene, by Weintraub and Groudine (1976). This has since been supported by the experimental results of other research workers (McGhee and Ginder, 1981; Saluz et al., 1986).

Weisbrod (1982) using electron microscopy showed that the unfolding of tightly packed chromatin fibres may be the first step in gene activation. Buschhausen et al. (1987), from their studies with mock methylated and methylated herpes simplex virus, thymidine kinase gene, showed that the inhibition of the HSV, tk gene by DNA methylation is an indirect one. This occurs through the formation of an inactive chromatin conformation shown by a delay in inhibition with methylated tk gene.

Benevisty and Reshef (1987) carried out studies with phosphoenol-pyruvate carboxykinase to examine the chromatin conformation of the gene and its relationship with DNA methylation. The changes in conformation was indicated by DNAse I sensitivity. Phosphoenol-pyruvate carboxykinase is partially hypomethylated in the foetal liver but also undergoes sequential loss of methyl groups prior to and after gene expression until the final state of hypomethylation in the adult liver. They were able to demonstrate that the sensitivity to nuclease digestion is acquired before the onset of transcription and persists after the initiation of gene expression. They were able to induce premature expression and hypomethylation with the use of 5-azacytidine, hence showing a direct relationship between DNA methylation, chromatin conformation and expression of this gene.

These examples show the possibilities that exist between DNA methylation and the resulting change of conformation on active gene expression in animal systems.

1.6.3.2. Chromatin structure and plant gene expression.

Research on the chromatin architecture of various plant genes started only recently (Spiker et al., 1983; Kahl et al., 1987). An example of a system that has been studied is the chlorophyll a/b protein (Cab) genes in leaf chromatin. The active form of the gene in leaf is more sensitive to DNaseI than the inactive form of the gene which exist in endosperm tissue.

Most of the ribosomal DNA genes in plants were found to be resistant to DNaseI and were thought to be probably inactive (Flavell et al., 1986; Blundy et al., 1987). rRNA genes at different nucleolar organiser loci are expressed at different levels and this preferential expression of certain loci has been associated with increased sensitivity to DNaseI (Thompson and Flavell, 1988).

Kahl and his group (Kahl et al., 1987), were able to demonstrate the organisation of integrated T-DNA genes into nucleosome like structures with the same repeat pattern as the bulk chromatin of the host cell. Constitutively transcribed T-DNA genes showed blurring of this pattern, indicative of an increased sensitivity to nuclease activity. A direct correlation of T-DNA gene expression and DNaseI sensitivity has not, however been established.

Increased DNase I sensitivity has been demonstrated in other active plant genes, the activity of which are controlled by developmental and environmental stimuli. Such examples are the small subunit of ribulose-1,5-bisphosphate carboxylase (rbcS), alcohol dehydrogenase gene and the sucrose synthase gene. They all showed an increase sensitivity to DNase I during active gene transcription.

1.6.4. Biological role of DNA methylation in eukaryotes.

There is no evidence for any restriction endonuclease activity in eukaryotes that is affected by DNA methylation (Watabe et al., 1983).

There are several pieces of experimental evidence that support the hypothesis that the state of DNA methylation is critical for gene expression in eukaryotic genomes (Doerfler, 1983; Cedar, 1988; Dynan, 1989). DNA methylation has been assigned an epigenetic role in the regulation of gene expression during aging, differentiation and development (Holliday, 1989). Balanced against its regulatory usefulness for active gene expression, at some sites, 5-methylcytosine imposes a mutational risk. Deamination of 5-methylcytosine can lead to a replacement by thymine. Hare and Taylor (1985) suggested that methylation may be able to compensate for this error by serving as a mechanism for strand discrimination in mismatch repair. A random repair mechanism would result in a replacement of the GC pair by an AT base pair. The CpG deficiency in eukaryotic DNAs (Bird, 1980; Adams and Eason, 1984; Adams et al., 1987) implies that this mismatch repair mechanism in eukaryotes, over evolutionary times, has not maintained the CpG at the expected level in the genome. At sites where CpG frequency is normal, some specific repair system might have developed to protect 5-methylcytosine from loss by deamination to thymine. Hare and Taylor (1985) suggest that 5-methylcytosine serves as the criterion for discriminating the two strands. They obtained evidence for such a role from the results of their experiment with hemimethylated, single base pair mismatch, SV40 DNA transfected into a monkey cell line. The single mispair was corrected.

Several research workers have reported that developmentally regulated genes remain fully methylated until they are expressed in the relevant somatic tissue, whereupon they undergo demethylation (Singer et al., 1979; Razin et al., 1984; Young and Tilgham, 1984; Wilks et al., 1982; Benevisty et al., 1985; Doerfler, 1983).

One of the clearest links between hypomethylation and gene expression comes from studies of the deactivation of foreign DNA integrated into random sites in animal and plant cells (Pollack et al.,1980; Amasino et al.,1984). Recent work of several groups using a variety of experimental approaches (use of methylation-sensitive restriction enzymes, and genomic sequencing) have established that there is a negative correlation between DNA methylation and the expression of eukaryotic genes (Bird and Southern, 1978; Razin and Riggs,1980; Wilks et al.,1984; Cedar et al.,1988; Saluz and Jost,1989).

1.6.4.1. Transcriptional regulation.

1.6.4.1.1.Transcriptional regulation of genes in animal cells.

Mapping of methylated sites with methylation sensitive restriction endonuclease has shown a general correlation between the transcriptional activation of developmentally regulated genes and the disappearance of methylated sites in or around the genes in mammalian cells (Doerfler, 1983; Razin and Cedar, 1984; Cedar, 1988). Such studies include examples discussed below.

In vitro methylation studies with methylated hamster aprt and herpes virus tk genes show inhibition of their expression by an inhibition of active transcription of the gene (Ehrlich et al., 1981; Cedar et al., 1983). Cedar's group concluded from their results that the small unmethylated region near the 5' end of the aprt gene may represent the methylation-responsive domain of the gene since restriction enzyme mapping of the natural hamster aprt gene convincingly showed that undermethylation near the 5' end of the gene played a critical role in the active transcription of the gene.

Christman et al. (1977) and Shen and Maniatis (1980) demonstrated a direct correlation between the expression of the rabbit β-globin gene and hypomethylation of the DNA. Active transcription of the β-globin gene was induced in erythroleukemia cells grown in the presence of ethionine instead of methionine. The inhibition of active transcription of the β-globin gene by methylation was shown to be due to the methylation of a subset of CpG sites near the 5' end of the gene, whereas methylation of the body of the gene had no effect on gene activity (Busslinger et al., 1983). A correlation between under-methylation and the expression of chicken α and β globin genes (McGhee and Ginder, 1979) and the ovalbumin gene (Mandel and Chambon, 1979) has also been demonstrated.

Some exceptions to the rule were detected. In some cases the relationship between under methylation and gene activity is difficult to sustain. Examples of such cases include genes for the human growth hormone (Hjelle et al., 1982). Also, Macleod and Bird (1983) reported that fully methylated ribosomal DNA isolated from Xenopus laevis was transcribed when injected into Xenopus oocytes

1.6.4.1.2. Transcriptional regulation of plant genes.

Most of the information on plant genes has been obtained from a few intensively investigated systems (Higgins, 1984; Kreis et al., 1985; Kuhlemeier et al., 1987; Walker et al., 1987) such as the studies with seed protein genes, genes induced by hormones and environmental cues, such as anaerobic stress and light. Several of these plant genes are thought to be regulated at the transcriptional level (Evans et al., 1984; Silverthorne and Tobin, 1984; Hagen et al., 1984; Eckes et al., 1985). Correlation between cytosine methylation and inactivation of these genes has been observed in plants in some cases.

Light-induced changes provide the basis for much of plant development. The increase in synthesis of nuclear encoded proteins, regulated by light was correlated with transcriptional regulation. Quantitative mRNA estimation showed an increase in total mRNA levels with increase in the enzyme activity of proteins involved in photosynthesis. A prominent example of a light-induced gene is the small subunit of ribulose-1,5-bisphosphate carboxylase (rbcS) (Berry-Lowe and Meagher, 1982; Gallagher and Ellis, 1982; Gallagher et al., 1985). The rbcS gene is controlled by light-induced phytochrome system and regulation is at the transcriptional level. These results confirmed the link between increase in active transcription and enzymes activity involved in photosynthesis.

There is evidence that the expression of the maize alcohol dehydrogenase I gene is transcriptionally regulated under conditions of anaerobic stress and this is associated with the DNA methylation level of the gene (Nick et al., 1986). Bianchi and Viotti (1988) have provided the first evidence that in higher plants, as in animal cells, under-methylation of certain tissue specific genes (maize zein and glutelin genes) correlates with active transcription of these genes. Other plant genes have been shown to be transcriptionally regulated (Evans et al., 1984; Beach et al., 1985; Hagen et al., 1984; Eckes et al., 1985). The vast number of nuclear ribosomal RNA genes in the plant genome are more than is needed to supply rRNA to the cytoplasmic pool (Long and Dawid, 1980; Flavell et al., 1985). Plant cells must possess a mechanism which determines which copies are transcriptionally active and which will be silent. Correlation between the level of cytosine methylation in the non transcribed regions, and the activation of the rRNA genes in peas (Blundy et al., 1987; Watson et al., 1987) has been demonstrated, and the regulation of the activity of these genes is at the transcriptional level.

1.6.5. Inactivation of foreign DNA.

1.6.5.1. Inactivation of foreign DNA in animal cells.

The first direct evidence that integrated genes had been silenced by methylation came from studies with avian and animal viral transformed cell lines (Pollack et al., Groudine et al., 1981; Hoffman et al., 1982). This is similar to the sequence of events described for de novo DNA methylation activity (**1.3.2.3**). Gautsch and Wilson (1983), from studies on the transfection of viral DNA into a mouse cell line, showed a repression of the viral DNA transcription and methylation of the viral DNA in the embryo carcinoma cells but not in the differentiated cells. However methylation of the foreign DNA seem to be secondary to the actual inactivation process but was responsible for maintaining the inactive state of the viral DNA. In some systems however the inhibitory effects of DNA methylation can sometimes be reversed by treatment of these cells with 5-azacytidine. This indicates a direct correlation between inactivation of the foreign DNA and DNA methylation (Taylor and Jones, 1979; Venolia et al., 1982; Taylor and Jones, 1982; Adams and Burdon, 1985).

1.6.5.2. Inactivation of foreign DNA in plant cells.

The 5-methylcytosine content of certain plant genes sequences change upon infection with viruses and tumour inducing bacteria (Amasino et al., 1984). In plants, the expression of foreign DNA inserted at random locations in the genome of plant tumour lines, can be suppressed by DNA methylation. An example of such a suppression of foreign DNA expression is the inhibition of the expression of a nopaline synthase reporter gene in a flax tumour (Hepburn et al., 1983). T-DNA from the Ti plasmid of Agrobacterium tumefaciens can integrate into plant nuclear genomic DNA on infection with the bacterium.

Phenotypic variation in the host plant and the ability to regenerate normal plant from a crown gall tumour cell line, has been shown to depend on the methylation level of the T-DNA (Amasino et al., 1984). All T-DNA in all tissue types was susceptible to methylation after integration into the plant genome (Amasino et al., 1984; van Slogteren et al., 1984). Methylation of the previously unmethylated plasmid DNA on integration into the host genome indicates the existence of a de novo methylation system in the plant. The acquired methyl groups stably maintain the T-DNA sequences in an inactivated state. This silent T-DNA is reactivated when the level of T-DNA methylation is reduced by treatment of transformed cells with the methylation inhibitor, 5-azacytidine (Hepburn et al., 1983; Blundy et al., 1986; Matze et al., 1989; Zambryski et al., 1989).

1.6.6. Other roles of DNA methylation in animal cells.

1.6.6.1. Genomic imprinting.

The expression of certain genes is determined by whether the gene is inherited from the male or female parent. This concept is called parental imprinting (Cattanach and Kirk, 1985; Swain et al., 1987). This predetermined developmental gene expression is observed in all tissues in the adult animal, thus it must have occurred either during gametogenesis in the parent or early embryogenesis in the offspring. This was observed from studies with mice (Swain et al., 1987). Surani et al. (1988) using genetic studies with mice have identified chromosomal regions, the functions of which are dictated by their parental origin. Such regions influence phenotypic characteristics such as shape, behaviour and size. These workers (Surani et al., 1988), deduced that about a quarter of the mouse genome is imprinted and the rest is unaffected by the parental origin.

DNA methylation has been associated with genomic imprinting in mammals (Sapienza et al., 1987; Swain et al., 1987; Holliday, 1987a and 1989). The methylation state of the gene in each individual is present in the germ line of the parent. The selective gene transcription as a result of genomic imprinting, is thought to be determined by the developmental DNA methylation status of the genome (Benevisty and Reshef, 1987). Jones and Taylor (1980) showed the induction of a differentiated state in cultured mouse embryo cells with 5-azacytidine, an inhibitor for DNA methylation. This is evidence for the involvement of DNA methylation in the initiation of differentiation. DNA methylation is said to be an "epigenetic" mechanism by which gene activity patterns, as opposed to genes per se, are passed from one generation of cells to another during development.

1.6.6.2. Aging.

The mature animal generally has a body which remains quite stable for a certain length of time until ultimately the body ages and the tissues function less efficiently. This is thought to be due to a gradual failure of cell maintenance mechanisms, such as DNA repair, replacement of lost cells and regulation of gene transcription. If the heritability of methylation pattern by cells is important to the long term functioning of tissues (imprinting) then a gradual loss of methyl groups could contribute to aging (Holliday, 1989). In animal cells, the content of 5-methyl cytosine decreases with aging (Vanyushin et al., 1973). The loss of methyl groups could arise as a result of an occasional failure of maintenance methylation activity during mitosis or failure to replace methyl groups after repair of DNA in nondividing cells. The loss of methylation would cause activation of silent genes and thus derangement of cellular function which could lead to cell death.

Experimental evidence for this was obtained from studies with human cells (Holliday, 1987b; 1989). A decline in the number of methylated sites in the cell DNA was found with increasing number of cell divisions and the cultured cells eventually lost their ability to proliferate. It was therefore concluded that the life span of human cells in cellular culture depended on the number of cell divisions and not on the chronological time per se. A similar observation had earlier been reported from studies with mouse and human cells (Wilson and Jones, 1983). Wilson and Jones (1983) observed that the genomic 5-methylcytosine level decline with the age of the animals (different species of mice) and that the rate of decline is inversely proportional to the maximum life span potential. This was supported with experimental results which showed that cultured human cells have more stable DNA methylation patterns than the rodent cells (Wilson and Jones, 1983).

These observations (imprinting, modification of expression during development and aging) all point to methylation of DNA as an important epigenetic factor in the regulation of gene expression in eukaryotes. Only time and more research will tell whether this factor is responsible for developmental control in all species, and the mechanism by which it is effected.

1.6.6.3. X chromosome inactivation.

Dosage compensation of X-linked genes in mammalian females is achieved by inactivation of one X chromosome early in embryogenesis. It becomes genetically inert over much of its length. It is extremely stable, and strong experimental evidence links the establishment of this inactive state to DNA methylation (Adams and Burdon, 1985; Monk, 1986; Lock et al., 1987; Hansen et al., 1988).

The state is maintained in the somatic cells by the maintenance methylation activity. In germ cells reactivation occurs just before meiosis (Kratzer et al.,1981; Migeon et al., 1986).

The initiation of X-inactivation is little understood at the molecular level. It has been suggested that the primary inactivation of X chromosome takes place in the early embryo followed by progressive methylation of the genes on the inactive X chromosome by the process of de novo methylation (**1.3.2.3**). The perpetuation of the inactive state of the X chromosome is thus a result of the DNA methylation level of the chromosome. Results of experiments with the use of methyl-sensitive restriction enzymes and transfection experiments, (Venolia and Gartler, 1983; Yen et al., 1984; Lock et al., 1986; Keith et al., 1986; Toniolo et al., 1988) show differential methylation between active and inactive X-linked genes. These workers (Lock et al.,1986; Hansen et al., 1988) showed the presence of hypomethylated clusters of CpG islands with some X-linked genes, (Hprt, Pgk, G6pd and α-galactosidase) on reactivation, while the same regions of these genes were methylated in the inactive X chromosome.

Hansen et al. (1988) used 5-azacytidine to reactivate Hprt, Pgk, G6pd and α-galactosidase genes from the inactive state,thus indicating the involvement of DNA methylation. Also on activation there was an increased sensitivity to such enzymes as MspI (Toniolo et al., 1988). The same CpG islands in these genes also exhibited nuclease hypersensitivity on reactivation (Wolf and Migeon, 1985; Riley et al., 1986; Hansen et al. 1988).

All these results strongly support the involvement of methylation in the maintenance of an inactive X chromosome. It has however been shown that methylation per se is a post-inactivation event occurring several days after the initiation of inactivation (Monk, 1981). since the process of inactivation does not occur in all cells at the same time.

1.6.7. Other roles of DNA methylation in higher plants.

Evidence for the correlation of DNA methylation and gene activity in higher plants is scarce (Nick et al., 1986; Walling et al., 1986). Not much is known about the character of methylation of functionally different DNA fractions of higher plants. In the past few years there has been a burst of information generated on gene expression processes in higher plants (Schell, 1987; Kuhlmeier et al., 1987).

In general the overall principles governing plant gene expression and the structure and organisation of plant genes are thought to be similar to those studied in animals (Goldberg, 1988). The precise mechanism responsible for controlling plant gene expression and the detailed events by which plant genes programme specific processes are still not known. It is suggested that the presence or absence of methyl groups may act as a signal.

1.6.7.1. Regulation of transposable elements.

In 1950, McClintock reported that unstable mutations of a gene in plants can be caused by the insertion of and/or excision of certain mobile genetic components (transposable elements). Transposable elements have since been discovered as components of other eukaryotic genomes (Swartz et al., 1962). It has been suggested that DNA methylation, may play an active role in the genetic activity of plant transposable elements, (Schwartz and Dennis, 1986; Chomet et al., 1987; Chandler and Walbot, 1986). Schwartz and Dennis (1986) and Chomet et al. (1987) reported their observations that methylation of certain DNA sequences is associated with the reversible phase changes of the transposable element, Ac. Hypomethylation of the DNA at the border sequences seems to be involved in the ability of Ac, to transpose from the waxy locus to other positions in the genome and its ability to destabilise unlinked Ds elements.

Methylation of the border sequence causes suppression of movement of the transposable element. Hypermethylation of DNA has also been associated with the inactive state of other plant transposable elements such as Mu (Chandler et al, 1986) and Spm (Fedoroff,1983 and 1989; Chomet et al., 1987).

1.6.7.2. Incidence of somaclonal variation.

Plant cells are totipotent and successful methods for the in vitro propagation of whole plants from cultured plant tissues such as tobacco and carrots were described over 50 years ago (White, 1939). As tissue culture technology advanced, other plants such as tomatoes, maize and other small grains have since been included in the list (Green and Phillips, 1975). Variations which often occur among plants regenerated from tissue culture is termed "somaclonal variation". Departure from the normal chromosome number and structure have been associated with somaclonal variation. This represents a response by the plant genome to the imposed "stress", as a result of tissue culture. This may arise as a result of preexisting genetic differences in the somatic cells, or could be induced by specific components of the culture medium. It has been suggested that both factors contribute to the incidence of somaclonal variation (Lee and Phillips, 1988; Evans, 1989). The changes in phenotypic expression in the regenerants are epigenetic as they are not expressed in the sexual progeny of the regenerated plants.

DNA methylation has been assigned a possible role in somaclonal variation. Brown and Lorz (1986) reported differences in the methylation levels of the DNA in the tissue culture induced regenerants They suggested that this could be a contributing factor in the preferential expression of certain genetic components.

They were unable to show a direct correlation between the degree of methylation and the variations in the phenotypic expression of the regenerants and there is no other report of a similar finding. Further investigations on the molecular basis for somaclonal variations as an epigenetic controlling factor are necessary.

1.6.7.3. Differentiation and development.

The establishment of specific developmental states in higher plants requires a differential gene expression programme. As in animal cells, these developmental differences are associated with variations in the levels of specific mRNAs. Certain gene products are present only in certain cell types at specific stages of development or following the application of distinct environmental stimuli. Example include the maize alcohol dehydrogenase gene which is only expressed under conditions of anaerobic stress (Walker et al., 1987). Under anaerobic conditions, the maize alcohol dehydrogenase gene is actively expressed and this is associated with hypomethylation of the DNA sequence. The expression of seed protein genes is another example of developmental and tissue specific regulation of plant gene expression. The increase in the mRNA levels of zein genes in Zea mays, was associated with hypomethylation of the DNA in the endosperm tissue compared to the methylation status of this gene sequence in tissues where it is not expressed (Spena et al., 1983).

1.7. Justification of the research project.

The advantages of methylation as a mechanism for gene inactivation are still to be established. The mechanism of action of DNA methylation is not fully understood at the molecular level. A considerable amount of work is still needed to completely purify and characterise eukaryotic DNA methylases.

Plant DNA has a much higher level of methylcytosines than DNA from higher animals, suggesting a possible difference in the regulation of plant and animal genomes. Despite the abundance of 5-methylcytosine in plants, its biosynthesis has not received much attention. Some properties of the purified plant DNA methylase (Theis et al., 1987) resemble those for the animal cell DNA methylases (**1.4.2**). It remains to be established whether these facts reflect a convergent evolution of similar mechanisms in the control of nuclear genes in both higher plants and animals. Further studies with plants may provide the experimental systems for the study of DNA methylation.

The work reported in this project is the purification and characterisation of pea DNA methylase. Purification of DNA methylase from pea seedlings would form the basis for future characterisation of 5-methylcytosine formation in plant DNA. It would serve as a source of a plant enzyme for in vitro methylation studies similar to that carried out with purified mouse ascites DNA methylase (Bryans, 1989).

1.7.1. Plants as research tools.

Many plant cells are totipotent and retain the ability to regenerate into fully differentiated fertile plants. The development of gene transfer technology allows the introduction of genes into plant cells that retain the capacity to differentiate. Such transgenic plants would provide a much better background for the study of gene regulation in its full complexity. Vertebrates and higher plants are alike in having comparatively large genomes most of which are not transcribed; hence studies with plants would contribute to a greater understanding of animal systems as well. Pea has been chosen because of its long association with studies in molecular biology, and it provides viable seedlings under suitable laboratory conditions, to provide material for study.

CHAPTER TWO

Materials and Methods

Section A. Materials.

2A.1. Biological materials.

2A.1.1. Plant: Pea seeds (Pisum sativum, variety : Feltham first) were purchased from Booker Seeds Ltd., Sleaford, Lincs., U.K.

2A.1.2. Sera: Rabbit antiserum against mouse Krebs II ascites DNA methylase, and horse serum, were kindly supplied by Dr. Adams.

2A.1.3 Nucleic acids: DNA from salmon testis, calf thymus (bovine in text), M. luteus, phage DNA, (ϕX174 RF DNA) and plasmid DNA, (ColE1) were obtained from Sigma Chemical Co. Ltd., Poole, Dorset, England. Plasmid pVHC I was kindly donated by M. Bryans; λH *ind III* and M13 mp 18 *Hinf1* DNA fragment size markers were kindly donated by D. Bourn and T. Carr from this department. Pea DNA was purified from pea seedling shoot tips by an adaptation of the method of Marmur, (Marmur J., 1961). Radioactive labelled pea DNA was prepared as described in Methods section by A. Rinaldi.

2A.1.4. Proteins and enzymes: Pancreatic DNase (DNase 1), ribonuclease A, trypsin, Proteinase K and spleen phosphodiesterase were obtained from Sigma Chemical Co. Ltd., Poole, Dorset, England; micrococcal nuclease from Boehringer Co. Ltd., Mannheim House, Lewes, E. Sussex and E.coli DNA polymerase 1 from Anglian Laboratories, Whitehall House, Colcester, Essex. Restriction enzymes, HpaII, EcoRII, and SstII were obtained from Northumbria Biologicals Ltd., Cramlington, Northumberland, U.K. PstI and MspI, were obtained from GIBCO BRL Ltd, Paisley, Scotland.

BstNI was obtained from C.P. Laboratories Ltd., Bishops Stortford, Herts, England distributors for Biolabs, New England. Myosin, α_2-macroglobulin, glutamate dehydrogenase, phosphorylase b, alcohol dehydrogenase, were obtained from Sigma Chemical Co. Ltd., Poole, Dorset, England.

2A.2. Radioactive Compounds .

Radiochemicals, $\alpha[^{32}P]dATP$, $\alpha[^{32}P]dCTP$, $\alpha[^{32}P]dGTP$, $\alpha[^{32}P]dTTP$ (3000Ci per mMole) and S-adenosyl-L-[^{3}H-methyl]-methionine (AdoMet) were obtained from Amersham International plc, Lincoln Place, Aylesbury Buckinghamshire, England. AdoMet was diluted to a specific activity of 4.64 mCi/µmole and stored at -20^{0}C. ^{125}I- protein A (1 x 10^{6} cpm/100µl) was prepared by A. Rinaldi in this department.

2A.3. Chemicals.

All chemicals used were of analytical grade except the following which were as specified by the suppliers.

TEMED (N,N,N',N'-Tetramethylethylene diamine) was obtained from BDH Chemicals Ltd., Poole England. Triton X-100, Ficoll type 400, and scintillation fluid (Ecoscint) were obtained from Koch-Light laboratory Ltd., Colnbrook, England. Dithiothreitol was obtained from Boehringer, Manheim house, Lewes, E. Essex. Phenylmethylsulphonylfluoride (PMSF), ethidium bromide, 8-hydroxyquinoline, bovine serum albumin (BSA), m-cresol, Coomasie Brilliant Blue R (65% dye) and Coomasie Brilliant Blue G (65% dye), EGTA (ethyleneglycol-bis-β-aminoethylether),

bromophenol blue, xylene cyanol, Tween 20 (polyoxyethylene sorbitan monolaureate), deoxyribonucleoside-5'-triphosphates, (dATP, dCTP, dGTP and dTTP) were obtained from Sigma Chemical Co. Ltd., Poole, Dorset, England.

2A.4. Chromatograhic material.

Heparin Sepharose CL- 6B, CNBr activated Sepharose 4B, DEAE Sephacel, phosphocellulose PII, blue dye matrix (Blue Sepharose), Superose 6 (prepacked column), Mono Q HR 5/5 column, Mino RPC column were obtained from Pharmacia, Pharmacia House, Milton, Keynes. Aminex A6 resin was obtained from Bio-Rad laboratory, Caxton Way, Watford, England. Agarose (Ultra pure, electrophoresis grade) was obtained from GIBCO Ltd., Paisley, Scotland. Acrylamide (electrophoresis grade) and NN'methylenebisacrylamide were obtained from FSA Laboratory Supplies, Bishop Meadow Road, Loughborough, England.

2A.5. Other Materials.

Whatman No. 1 filter paper and 3MM sheets were obtained from Whatman International Ltd. Maidstone, England. Nitrocellulose (Hybond-C extra) was obtained from Amersham International plc, Lincoln Place, Aylesbury, Buckinghamshire, England. Centriprep concentrator 30 was obtained from Amicon division, W.R.Grace & Co., Cherry Hill Drive, Danvers M.A. USA. MILLEX-GS sterilising filter units were obtained from Millipore S.A. Zone Industrielle, Molsheim, France.

2A.6. Composition of Standard Solutions.

2A.6.1. Buffer M^+ (pH with 5M HCl).

Tris-HCl	pH 6.0, 6.4, 7.0, 7.8, 8.4	50mM
EDTA		1 mM
Dithiothreitol		1 mM
Sodium azide		0.02%
Glycerol		10.0%
Phenylmethylsulfonylfluoride		0.06 %

2A.6.2. Buffer A.

Tris-HCl	pH 7.0	10mM
Calcium chloride		1mM
Magnesium chloride		2mM

2A.6.3. Nick translation buffer. (x 10)

Tris-HCl	pH 7.2	0. 5M
Magnesium chloride		0.1M
Dithiothreitol		10 mM
Bovine serum albumin		0.05%

2A.6.4. Aminex A6 running buffer. (pH with NH_3)

Ammonium carbonate	pH 10.2	20mM

2A.6.5. Mino RPC running buffer (Buffer B). (pH with 1M H_3PO_4)

Tetrabutylammonium phosphate	pH 6.0	25mM
Sodium chloride		80mM

2A.6.6. SDS / PAGE electrode buffer.

Tris	25 mM
Glycine	191mM
SDS	0.1%

2A.6.7. SDS / PAGE sample buffer.

Tris-HCl	pH 6.8	0.1875M
SDS		6 %
Glycerol		30 %
Mercaptoethanol		15 %
Bromophenol blue		0.1%

2A.6.8. SDS / PAGE casting buffers.

Resolving gel buffer.

Tris-HCl	pH 8.8	1.486M
SDS		0.4%

Stacking gel buffer

Tris-HCl	pH 6.8	0.5M
SDS		0.4%

2A.6.9. Buffer C.

Tris	pH 6.8	25.0mM
Glycine		0.192M
Methanol		20 %
SDS		0.02 %

2A.6.10. Buffer D.

Tris-HCl	pH 7.2	20 mM
NaCl		0.15M
Tween 20		0.5%
NaN_3		0.05%

2A.6.11. Non denaturing PAGE : electrode buffer (Buffer E).

Tris	pH 8.7	50 mM
Glycine		120 mM
Glycerol		17 %
Mercaptoethanol		0.1%
NaCl		0.2%

2A.6.12. Agarose gel electrode buffer (STE). (Maniatis et al., 1982).

Tris-HCl	pH 8.0	10mM
NaCl		100mM
EDTA	pH 8.0	1mM

2A.6.13. Agarose gel sample buffer. (Maniatis, et al., 1982).

Ficoll type 400	15 %
bromophenol blue	0.25%
Xylene cyanol	0.25%

2A.6.14. Phosphate Buffered Saline (PBS).

Na_2HPO_4	pH 7.2	10 mM
KH_2HPO_4	pH 7.2	1.84mM
$CaCl_2.6H_2O$		5.0mM
$MgCl_2.6H_2O$		4.6mM
NaCl		0.172M
KCl		3.35mM

2A.6.15. Polyacrylamide gel casting solutions (for DNA gels)

Solution A.

Tris-HCl pH 8.8	1.5M
TEMED	0.2%
Salmon DNA	0.5mg/ml

Solution B.

Acrylamide	20.0%
NN'methylenebisacrylamide	0.4%

Solution C.

Ammonium persulphate	0.2%
Glycerol	40.0%
NaCl	0.4M

2A.6.16. SDS Stop Solution.

SDS	1%
EDTA	2mM
Butanol	5%
4-Aminosalicylic acid	3%
Salmon DNA	0.25mg/ml
NaCl	0.125M

2A.6.17. Phenol : m-Cresol : Hydroxyquinoline (Phenol Mix).

Phenol	88%
m - cresol	12%
Hydroxyquinoline	0.1%

2A.6.18. Phenol : Chloroform (1:1) pH = 7.6. (Maniatis et al., 1982).

"Chloroform" = 24 : 1 (v/v) chloroform : isoamylalcohol

"Phenol" = phenol pre-equilibrated with 1.0 M Tris-HCl (pH 8.0) and 0.2% β-mercatoethanol, containing 0.1% hydroxyquinoline.

2A.6.19. Diphenylamine reagent

Diphenylamine	1.5%
Sulphuric acid	1.5%
Glacial acetic acid	97 %
Acetaldehyde (add just before use)	0.009%

2A.6.20. Bradford's reagent.

Coomasie brilliant blue G	0.1mg/ml
Ethanol	4.75%
Phosphoric acid	8.5%

Section B. METHODS

2B.1. Distribution of DNA methylase activity in pea seedlings.

2B.1.1. Growing pea seedlings.

Peas (200g dry weight per tray) were swollen in water for 4-6 hours and then seeded out fairly thickly in a monolayer, into a garden tray (21 x 33cm) of compost and covered with a layer of compost to about 1 inch deep. Except for the experiment reported in Table 2, they were grown at 26^{0}C under continuous white light illumination and kept moist by watering with just enough water to keep reasonably moist. The shoots were ready for harvest on the 7th day, except where otherwise indicated. The 1.5-2.0cm apical shoot tips (or other portions where stated) were removed and stored overnight at -20^{0}C.

2B.1.2. Preparation of pea nuclei.

The procedure adopted for the preparation of pea nuclei is an adaptation of the method of Ts'O and Sato, 1959. All steps in the preparation of pea nuclei were done at 0-4^{0}C. The apical shoot tips fraction or other portions of pea seedlings were homogenised in buffer M^+ pH 7.0 (**2A.6.1**) either by grinding in a morter (100µl buffer per gram material) or with a few short bursts (5 x 10 seconds) in a Philips blender (1.5-2.0 ml buffer per gram material). Care was taken to avoid homogenising too vigorously otherwise excessive nuclear damage occurred. The homogenate was diluted with buffer M^+, filtered through 6 layers of muslin and centrifuged at 2500 rpm for 15 minutes, 4^{0}C in a Beckman bench top centrifuge, model TJ-6.

The pellet was resuspended in buffer M^+ and centrifuged at 2000 rpm, 10 minutes, 4^0C and then resuspended in1% Triton X-100 in buffer M^+ and left standing on ice for 15 minutes. This causes lysis of contaminating plastids in the nuclear suspension. The nuclei were pelleted by centrifugation at 2000 rpm for 10 minutes at 4^0C and washed in buffer M^+ containing 1% Triton X-100 until no longer green. The isolated nuclei were stored at -20^0C in buffer M^+, pH 7.0 containing 50% glycerol (25µl per gram starting material).

2B.1.3. Assay for DNA concentration.

The assay for DNA in isolated nuclei was carried out by the colorimetric method of Burton, 1956. Salmon sperm DNA was used as standard. 0-50µg DNA in a volume of 50µl was treated with 0.5ml perchloric acid by heating for 30 minutes at 70^0C. 3 ml of diphenylamine reagent (**2A.6.19**) was added to the cooled mixture and left to stand overnight in the dark. The absorbance was measured at 600nm and used to plot a standard curve. 100µl of pea nuclei in buffer M^+ pH 7.0, containing 50% glycerol was hydrolysed in an Eppendorf tube with 0.9 ml of 0.5M PCA. This was centrifuged at 6,500 rpm for 10 minutes in an MSE Eppendorf centrifuge. 3 ml of diphenylamine reagent was added to 0.5 ml of the supernatant and left to stand overnight in the dark and the absorbance measured at 600nm. The concentration of DNA was then calculated from the standard curve.

1.4. Assay for protein concentration.

The protein concentration in all samples were assayed by Bradford's method, (Bradford, 1976). Bovine serum albumin was used as standard protein. 0-100µg of bovine serum albumin was taken in 100µl of buffer M^+ and 3 ml of Bradford's reagent (**2A.1.4**) was added, mixed and allowed to stand for 5 minutes. The absorbance at 595nm was measured in a Cecil Linear readout UV Spectrophotometer and used to plot a standard curve. 10-35µl samples of pea DNA methylase fractions were diluted to 100µl with buffer M^+ and 3 ml of Bradford's reagent was added, mixed and left to stand for 5 minutes and the absorbance was measured at 595nm. The protein concentration was estimated from the standard curve.

2B.1.5. Protocol for silver staining of protein gels.

All solutions were made with distilled and deionised water. Solution A containing 0.8g of $AgNO_3$ in 4 ml of water was added dropwise to solution B (1.4 ml 14.8M NH_3 liquor + 21 ml 0.36% NaOH) whilst stirring vigorously. The solution was made up to 100 ml with water and used immediately and referred to, as solution C (0.8% $AgNO_3$, 0.07% NaOH, 0.12% NH_3). The gel was fixed in 50% methanol for at least 2 hours before staining in solution C with constant agitation on a shaker, at room temperature for 15 minutes. The gel was rinsed with water for 5 minutes with constant agitation and developed by immersing it in solution D (5 x 10^{-3}% citric acid in 0.019% formaldehyde) until protein bands begin to appear. The gel was rinsed in water and photographed immediately with a Polaroid camera.

2B.1.6. Staining of gels with Coomasie brilliant blue R.

The stain was a 0.1% (w/v) Coomasie brilliant blue R in 50% (v/v) methanol and 10% (v/v) acetic acid. The gel was immersed in the coomasie stain for 1 hour at 37^0C and excess dye was poured off. The gel was destained for 12 hours at 37^0C with several changes of destaining solution [10% (v/v) acetic acid and 10% (v/v) methanol] and photographed with a Polaroid camera.

2B.1.7. DNA : Protein ratio in isolated pea nuclei.

The concentration of protein in 10µl of the nuclear preparation was assayed by Bradford's method, as described in **2B.1.4.** and the protein concentration estimated for 50µl of pea nuclei.

The DNA concentration in 50µl of the nuclei was assayed by the colorimetric method with diphenylamine reagent, using salmon DNA as standard, as described in **2B.1.3.** The concentration of DNA was calculated from the standard curve.

2B.1.8. Methylase assay.

Nuclei from the Triton insoluble fraction of the nuclei/chloroplast pellet were used as a source of DNA methylase, and were found to contain all recoverable DNA methylase activity. DNA methylase was assayed in the nuclear preparation by an adaptation of the method for assay of mouse ascites DNA methylase, (Adams et al, 1986a). The reaction was carried out in a final volume of 70µl, using 10µl S-adenosyl-L-[^{3}H-methyl] methionine (AdoMet; 1.08µCi per assay) 20µl buffer M^+, 35µl nuclei (about 70µg protein; 1.0mg/ml) and 5µl bovine serum albumin (0.7mg/ml).

The reaction mixture was incubated for 2 hours at 30^{0}C. The reaction was stopped by the addition of 0.5ml of SDS stop solution (**2A.6.16**). It was then extracted with phenol mix (**2A.6.17**). The aqueous phase was precipitated with two volumes of absolute ethanol and the pellet was resuspended in 50µl of 0.3M NaOH. It was incubated at 37^{0}C for 1hour and transferred onto Whatman 3MM filter strips and the DNA precipitated with 5% TCA. The filter strips were washed three times in 5% TCA, twice in methylated spirits, once in diethylether and air dried in a fume cupboard. The DNA was dissolved in 600µl of 0.5M perchloric acid by incubating at 60^{0}C for 1 hour and counted with 5 ml of scintillation fluid in an LKB 1209 Rackbeta, liquid scintillation counter with a 10% counting efficiency. The background reading was about 20cpm.

2B.1.9. <u>Product of pea DNA methylase</u> .

For unequivocal characterisation of the enzyme activity in the pea nuclei as DNA cytosine methyltransferase, the product was shown to be 5-methylcytosine. The methylated DNA product (10µg) was isolated after alkali hydrolysis, by acid precipitation with 50% TCA and washed twice with 5% TCA and left to dry. The dried samples were pyrolysed with formic acid by method of Pollock et al, 1978 as described by Adams and Burdon, 1979. The DNA was taken up in 100µl 98% Formic acid and hydrolysed by heating in sealed tubes for 1 hour at 170^{0}C. The tubes were dried down and the samples were taken up in 100µl of ammonium carbonate buffer, pH 10.2.

50µl of the sample was injected into a 1 x 50cm HPLC Aminex A6 ion exchange column (Bio-Rad) and the free bases were eluted with 20mM ammonium carbonate buffer, pH 10.2. The column was operated at a flow rate of 1 ml per minute and a back pressure of 1.5 Mpa. The elution profile was monitored with a PYE Unicam UV detector at 260nm and recorded on a chart at a speed of 12 cm/hr and a full scale deflection of 0.16. 25 drop fractions (1 ml) were collected, 5 ml scintillation fluid was added and the radioactivity was counted in the scintillation counter.

2B.1.10. <u>Location of DNA methylase in cellular organelles</u>.

Presoaked peas were grown either in continuous light or continuous dark (100g per tray) for 7 days and the apical shoot tips were harvested and frozen overnight at -20^{0}C. The homogenising was performed in an ice cold mortar (100µl buffer M^{+}/g) and a portion of the homogenate was kept for methylase assay. Nuclei were prepared from the rest as described above except that lysis was carried out in a reduced volume and the supernatant containing lysed chloroplasts or etioplasts was kept (plastids). The homogenate, isolated pea nuclei and the plastid fractions were assayed for DNA methylase activity by incubating 35µl of each in an assay volume of 70µl at 30^{0}C in the presence of 1.08µCi of AdoMet and 0.7mg/ml BSA as described for methylase assay in isolated pea nuclei (**2B.1.8**).

2B.1.11.Variation of methylase activity with location and age.

Nuclei were prepared as described above from various fractions of growing pea seedlings of different ages, 5, 7, 9 and 11 days growth post imbibition. The top 1-2 cm from the growing shoot tips was referred to as **shoot apex;** the next 2 cm (2-4 cm from the shoot tips) was referred to as the **subterminal portion.** The top 2 cm from the cotyledon was called **nearest pea portion** and the first free leaves were referred to as **leaf. Root tip** was the 2 cm portion from the growing root tips. The nuclei were assayed for DNA methylase activity as described above (**2B.1.8**).

2B.2. Preparation of DNA substrates.

2B.2.1. Native pea DNA.

DNA was prepared from isolated nuclei by an adaptation of the method of Marmur (1955) as described by Maniatis et al., 1982. Nuclei from 100g each of 5, 7, 9 and 11 day pea seedlings were resuspended in 3ml PBS buffer and 3ml of a 2% SDS solution containing 4mM EDTA and 10% butanol. The nuclear fractions were incubated at 37^0C with heat treated RNase A (50µg/ml) for one hour. Proteinase K, (50µg/ml) was added to the RNase A digest and incubation was continued at 37^0C for another one hour. The reaction was stopped by heating at 60^0C for 5 minutes and phenolised (2x) with an equal volume of phenol/chloroform (1:1) with gentle mixing and centrifugation at 3000rpm, 20^0C for 20 minutes. The aqueous supernatant was extracted with 6ml of chloroform and finally with 6ml of water saturated ether.

Any traces of ether left were dried off in a fume cupboard and the DNA precipitated with 2 volumes of precooled absolute ethanol (-20^{0}C). The DNA was spooled out, dried and dissolved in 1ml of 50mM KCl. The absorbance at 260nm was measured and used to estimate the DNA concentration (Ohlenbusch et al., 1967). One mg/ml native DNA was considered to have an absorbance at 260nm of 20.9.

2B.2.2. Heat denatured single stranded DNA substrates.

DNA from M.luteus, calf thymus, salmon sperm and 5 day pea seedling shoot tips were dissolved in 50mM KCl. Heat denatured DNA from each of these sources was prepared by heating 1mg per ml solutions of these DNA at 100^{0}C for 5 minutes. The heated DNA solutions were allowed to cool at room temperature and were designated single stranded DNA substrates.

2B.2.3. Hemimethylated DNA substrates.

The reaction was carried out by the method of Rigby et al., 1977. 5µg M. luteus DNA and 5µg calf thymus DNA were nick translated in 100µl of a reaction mixture containing, 10µl of nick translation buffer, pH 7.2 (**2A.6.3**), 10µl of a 4mM solutions of each of dATP, dGTP, dTTP and 5-mdCTP (or dCTP for calf thymus DNA; 400µM each) and enough distilled water to make up the volume of 100µl in 4 Eppendorf tubes. The mixture was incubated at 15^{0}C for 90 minutes in the presence of 0.01 unit of DNase I and 10 units of E.coli DNA polymerase I and the reaction was stopped by the addition of EDTA to a concentration of 10mM. The mixture was extracted with an equal volume of phenol/chloroform.

The supernatant was precipitated with 2 volumes of absolute ethanol (-20^{0}C) in the presence of 0.6M sodium acetate and left to stand in dry ice for 15 minutes before centrifugation at 14,000 rpm, 4^{0}C for 10 minutes in an Eppendorf centrifuge 5415. The precipitate was washed by resuspending in 70% alcohol (-20^{0}C) and left to stand in dry ice for 15 minutes before centrifugation at 14,000 rpm, for 10 minutes at 4^{0}C. The nicked translated DNA product was drained and left to dry. The DNA was dissolved in 0.5 ml of distilled water and the absorbance at 260nm was measured and used to estimate the DNA concentration and recovery.

2B.3. Extraction of DNA methylase.

2B.3.1. Isolation of "free" enzyme by sonication.

Pea nuclei prepared from 7 day seedlings were resuspended in buffer M$^+$ containing 50% glycerol and subjected to 5 second treatment at 4^{0}C in an MSE Ultrasonic power unit for different durations (15, 30 and 60 seconds) each at full voltage setting. The broken nuclei were subjected to centrifugation at 10,000 rpm for 20 minutes at 4^{0}C in a Beckman J2-21 Centrifuge. The pellets were resuspended in buffer M$^+$ containing 50% glycerol and the supernatant and pellet were assayed in the absence and presence of 10µg exogenous native pea DNA.

2B.3.2. Extraction of pea nuclei with sodium chloride.

Pea nuclei were subjected to centrifugation at 2000 rpm for 20 minutes at 4^0C in a Beckman TJ-6 bench top centrifuge and the nuclear pellet was resuspended in buffer M^+. An equal volume of buffer M^+ containing 0.4M NaCl was added slowly with continuous stirring on ice. It was stirred for another 30 minutes and then centrifuged at 2,000 rpm for 20 minutes at 4^0C in the Beckman bench top centrifuge. The supernatant is referred to as 0.2M extract I. The pellet obtained was resuspended in buffer M^+ to which $CaCl_2$ (1.5µmole per ml) was added. The nuclear suspension was incubated for 10 minutes at 37^0C with microccocal nuclease, (400 units per ml) and the reaction stopped with the addition of EGTA (10 µmole per ml). The partially digested nuclei were reextracted with an equal volume (to the new volume) of 0.4M NaCl in buffer M^+ as described in the first extraction above and the supernatant referred to as 0.2M extract, fraction II. A portion (1ml) of each extract was saved and the extracts were pooled and 1ml of this was also reserved. Occasionally it was found necessary to subject the 0.2M NaCl extracts to further centrifugation at 15,000 rpm, 2^0C for 20 minutes to exclude fine pieces of broken nuclei. The pooled extract was used for further purification. The 1ml fractions were dialysed against 50% glycerol in buffer M^+ at -20^0C (100ml buffer/ml of 0.2M NaCl extract). 35µl of these dialysed extracts were assayed for DNA methylase activity in a 70µl reaction mixture in the presence and absence of added 10µg pea DNA, with 10µl (1.08 µCi) AdoMet, 10 or 20µl buffer M^+ and 5µl of BSA. The reaction was incubated for 2 hours at 30^0C.

The reaction was stopped by the addition of 0.5ml of SDS stop solution and the methylated DNA product was isolated and monitored for ^{3}H-methyl group incorporation as described in section **2B.1.8.**

2B.4. Chromatographic purification of nuclear extract.

2B.4.1. Heparin Sepharose and Mono Q.

Heparin Sepharose was washed in buffer M^{+} (200ml per gram) and a 1.5 x 10 cm column was poured by gravity settling. The column was transferred to 4^{0}C, washed through with 10 volumes of 0.2M NaCl in buffer M^{+} and the combined nuclear salt extract (Fraction II, **2B.3.2**) from nuclei obtained from 350g 7day apical shoot tips was pumped on to the column at 3 ml per hour. The column was washed through with 2 column volumes of 0.2M NaCl in buffer M^{+} and the column eluted with 0.6M NaCl in buffer M^{+}, after previously establishing this as the required salt concentration which gives the best yield of the bound enzyme from Heparin Sepharose. The protein peak obtained with 0.6M NaCl was pooled and a portion (1 ml) was reserved for methylase assay (Fraction III) and the remainder purified further on a Mono Q HR 5/5 column (Pharmacia) after desalting as described below.

Fraction III was desalted by ultrafiltration through a membrane by centrifugation in a Centriprep 30 concentrator. The protein fraction was diluted (2 x or more) each time and centrifuged for 40 minutes, 4^{0}C at 3000 rpm in the Beckman bench top centrifuge, to a final volume of 1-2 ml. At least three centrifugation steps with dilution were found necessary for complete desalting.

The desalted protein peak was injected into a pre equilibrated Mono Q HR 5/5 column (Pharmacia). Pump A was filled with buffer M^+ and pump B with buffer M^+ containing 1M NaCl. The 1-2 ml Fraction III concentrate was injected into the Mono Q HR 5/5 column, the column was washed for 5 minutes with buffer M^+, at a pump rate of 1 ml per minute before starting a 25 minutes gradient run (0-1M NaCl). The column was operated at a pump rate of 1ml per minute and a back pressure of 1.5 Mpa. 1ml fractions were collected and the elution profile monitored by absorbance at 280nm with a UV detector (Pharmacia). The 1ml fractions and the reserved 1ml of Fraction III were dialysed against 50% glycerol in buffer M^+ (100ml buffer/ml fraction) and assayed for DNA methylase activity in the presence of 10µg pea DNA as described for soluble pea DNA methylase (**2B.3.2**).

2B.4.2. <u>Gel permeation chromatography.</u>

The 0.2M NaCl nuclear extract obtained from nuclei prepared from 300g of pea seedling shoot tips, was concentrated by ultrafiltration in a Centriprep 30 concentrator by centrifugation at 3000 rpm, 4^0C for 3 x 40 minutes. The concentrated fraction had a volume of 2ml and this was injected into a precalibrated 100ml FPLC Superose 6 column (Pharmacia) and the column eluted with 0.2M NaCl in buffer M^+ at a flow rate of 1ml per minute and a back pressure of 1.5 Mpa. 1 ml fractions were collected and dialysed against buffer M^+, pH 7.0 containing 50% glycerol and the dialysates were assayed for DNA methylase activity in the presence of native pea DNA as described for soluble pea DNA methylase in section **2B.3.2**.

The 100 ml Superose 6 column was calibrated by injecting alcohol dehydrogenase (1mg/ml) and mouse ascites DNA methylase (about 1mg/ml) in two separate runs and the column eluted with buffer M^+ containing 0.2M NaCl at a flow rate of 1ml per minute.

The elution volume was plotted against the reciprocal of the established molecular mass of the two proteins and this was used to estimate the molecular mass of the pea enzyme from its elution volume. In a similar way the 0.6M NaCl protein peak (Fraction III) from a Heparin Sepharose column was concentrated down to 1ml and purified by gel filtration on the FPLC Superose 6 column.

2B.4.3. DEAE Sephacel and pea DNA Sepharose.

The 0.6 M NaCl fraction (Fraction III) from a Heparin Sepharose column obtained as described in section **2B.4.1**, was desalted by ultrafiltration in a Centeriprep 30 concentrator with centrifugation, at 3000rpm, 4^0C and dilution with buffer M^+. The desalted concentrate was diluted with buffer M^+ and pumped onto an equilibrated 1.5 x 10 cm DEAE Sephacel column at a flow rate of 3 ml per hour at 4^0C. The column was washed with 2 volumes of buffer M^+ and eluted with 0.2M NaCl in buffer M^+ and the protein fractions were pooled. A 1 ml portion (Fraction IV') was reserved and the rest of it was purified by affinity chromatography on a pea DNA Sepharose column.

The eluted protein fraction was desalted in a Centriprep 30 concentrator and diluted with buffer M^+. This was applied onto an equilibrated column of pea DNA Sepharose (1.5 x 5 cm) at a flow rate of 2ml per hour and the column was washed through with 4 volumes of buffer M^+ before eluting with 0.2M NaCl in buffer M^+.

One ml fractions were collected and these and the 1ml fraction from the DEAE Sephacel column were all dialysed against buffer M^+ containing 50% glycerol (100ml buffer/ml sample). One ml samples of the unbound protein fractions from both columns were also dialysed against buffer M^+ containing 50% glycerol and assayed for DNA methylase activity. Previously 1ml fractions of the 0.2M NaCl eluted proteins from the DEAE Sephacel column were collected, dialysed and assayed separately for DNA methylase activity. The peak tubes were analysed on a 7% SDS polyacrylamide gel after dialysis and after 48-72 hours of storage at -20^0C.

2B.4.4. Use of phosphocellulose and blue dye matrix.

Phosphocellulose was washed with 0.5M NaOH (25ml/g) and rinsed with water by filtration in a buchner funnel until the pH of the filtrate was below 10. This was neutralised by an acid wash with 0.5 M HCl (25ml/g). It was rinsed with water until the pH of the filtrate was above 5. The phosphocellulose was saturated with bovine serum albumin in buffer M^+ (100µg/ml) by stirring it in the buffer for 20 minutes at 0^0C. Excess bovine serum albumin was washed away in a buchner funnel with buffer M^+ until no protein was detected in the washes. The phosphocellulose was then equilibrated in buffer M^+ containing 0.2M NaCl. Blue dye matrix resin (0.5ml) was taken in an Eppendorf tube and washed 3 times in buffer M^+ containing 0.2M NaCl. 100µl of phosphocellulose and 100µl of equilibrated blue dye matrix resin were resuspended separately in Eppendorf tubes with 1ml of 0.2M NaCl nuclear extract and left standing in ice for 30 minutes with occasional stirring.

The suspensions were spun down in an MSE Eppendorf centrifuge at 14,000 rpm, 4^{0}C for 5 minutes and the supernatants were saved. The pellets were extracted with 1ml each of increasing concentrations of NaCl in buffer M$^+$ (0.4M, 0.6M and 1.0M NaCl). In each case the supernatants and the supernatants of unbound methylase at 0.2M NaCl were dialysed against buffer M$^+$ containing 50% glycerol and 35µl of these dialysates were assayed for DNA methylase activity in a reaction mixture of 70µl in the presence of 10µg pea DNA as described in section **2B.3.2.**

2B.5. Characterisation of pea DNA methylase.

2B.5.1. Molecular weight determination.

The peak fractions from Heparin Sepharose, Mono Q HR 5/5, DEAE Sephacel and pea DNA Sepharose columns were subjected to electrophoresis in a one dimensional SDS-polyacrylamide slab gel. This was performed essentially according to Laemmli's method (Laemmli, 1970).

2B.5.1.1 SDS/polyacrylamide gel electrophoresis.

The resolving gel was a 40 ml solution composed of 9.5ml of a solution of acrylamide/NN'methylenebisacrylamide (30% / 0.8%), 10ml resolving gel buffer (**2A.6.8**), 50µl of TEMED, 200µl of a 10% (v/v) ammonium persulphate solution, and 20.2ml of distilled water. This was stirred and poured into a pair of clamped gel plates to a height of about 13cm.

The stacking gel was a 10.23 ml solution made up of 2.5 ml stacking gel buffer (**2A.6.8**), 5.5 ml of distilled water, 2 ml of a solution of acrylamide/NN'methylenebisacrylamide (30%/0.8%), 150µl of a 10% (v/v) ammonium persulphate solution, and 75µl of TEMED. This was stirred and poured on the resolving gel to a height of about 3 cm and a comb was inserted.

The protein concentrations in each fraction from the chromatographic steps in the purification of pea DNA methylase, were estimated by Bradford's method (**2B.1.4**) and 2 x 5µg of each was taken in an equal volume of sample buffer (**2A.6.7**) and subjected to electrophoresis in duplicate in the SDS/polyacrylamide gel at 50mA until the dye front reached four fifth of the length. The electrode buffer was 25mM tris/glycine buffer (**2A.6.6**). The gel was run with protein markers (myosin 200K, α_2-macroglobulin 170K, phosphorylase b, 95.5K and glutamate dehydrogenase 55K) of known molecular weights and the gel was cut in half at the end of the run. One half was fixed in 50% methanol and used for the detection of the protein bands in the gel by the silver staining procedure as described in section **2B.1.5**. The second half of the gel was used for electrophoretic blotting.

2B.5.2. pH of optimum activity.

Isolated pea nuclei (1ml portions) were taken in Eppendorf tubes and centrifuged at 6,500 rpm for 5 minutes at 4^0C. The pellets were resuspended in buffer M^+ containing 50% glycerol at different pH values (6.0, 6.4, 7.0, 7.8 and 8.4, **2A.6.1**) and they were assayed for methylase activity at these different pH values with the corresponding buffer M^+ as described in section **2B.1.8.**

One ml each of the 0.2M NaCl nuclear extracts were also dialysed against buffer M^+ containing 50% glycerol at different pH values (6.0, 6.4, 7.0, 7.8, and 8.4) and 35 µl of each were assayed for DNA methylase activity in the presence of 10µg pea DNA as described in section **2B.3.2**, with the corresponding buffer M^+.

2B.5.3.<u>Dependence of activity on DNA substrate concentration</u>.

35µl of dialysed 0.2M NaCl extract of pea nuclei (27.5µg protein), were assayed for DNA methylase activity in a reaction mixture of 70µl, containing 1.08µCi of AdoMet, 0.7mg/ml BSA in the presence of 0, 5, 10, and 20µl of native pea DNA, (0, 71, 142 and 280µg per ml) and enough buffer M^+ to make up the volume of 70µl. The reaction was incubated for 2 hours at 30^0C, stopped with 0.5 ml SDS stop solution and the DNA product was isolated and monitored for tritium incorporation as described in section **2B.1.8**.

2B.5.4. <u>Effect of protein concentration on enzyme activity</u>.

10, 20, 30 and 40µl of the solubilised enzyme (0.2M NaCl extract) was assayed for DNA methylase activity in the usual reaction cocktail of 70µl in the presence of 10µg DNA at the different enzyme protein concentrations (0.5, 0.75, 1.0 and 1.5 mg/ml) in the presence of 1.08µCi AdoMet, 0.7mg/ml BSA and 20, 10, 5, or 0 µl of buffer M^+. The reaction was carried out as described in section **2B.3.2**. This reaction was repeated at the different protein concentrations (0.5-1.5 mg/ml) with the different volumes of 0.2M NaCl nuclear extract, in the presence of 0.6µg pea DNA (0.01 mg / ml).

2B.5.5. Time course for in vitro DNA methylase activity.

35µls of isolated nuclei were assayed for DNA methylase activity under assay conditions as described in section **2B.1.4,** by incubating the reaction mixtures at 30^0C for different lengths of time (1, 2, 3 and 4 hours) before the addition of 0.5ml SDS stop solution and the isolated methylated DNA products were monitored for tritium incorporation. The reaction was repeated in isolated nuclei in the presence of 200mM concentrations of NaCl in the reaction mixtures, for 1, 2, 3, and 4 hours of incubation respectively.

2B.5.6. DNA substrate specificity.

2B.5.6.1. Requirement for double and single stranded DNA

35µls of soluble DNA methylase were assayed for DNA methylase activity at the assay conditions described in section **2B.3.2,** with 5µg each of DNA substrates (72µg/ml), from different sources (M.luteus DNA, salmon sperm DNA, calf thymus (bovine) DNA and pea DNA from 5 day seedling shoot tips). The reaction was repeated with 5µg each of heat denatured single stranded DNA from these organisms prepared as described in section **2B.2.2.** All reactions were incubated for two hours at 30^0C.

2B.5.6.2. Requirement for hemimethylated DNA substrate.

Hemimethylated DNA derivatives from M.luteus and calf thymus DNA were prepared as described in section **2B.2.3.** Control nick translation of 5µg of each of these DNA samples were also carried out as described, in the presence of 400µM dCTP instead of 5-mdCTP.

The nick translated DNAs were used as substrates at 72µg per ml for DNA methylase assays with 35µl of soluble pea DNA methylase. The reactions were carried out as described in **2B.3.2,** by incubating with 1.08µCi of AdoMet, for 2 hours at 30^0C.

2B.5.6.3. <u>Requirement for unmethylated DNA substrate.</u>

4 x4µg of ϕX174 RF DNA in Eppendorf tubes were used as substrates for DNA methylase assay with 3.7 units of soluble pea DNA methylase (fraction III) in a starting volume of 100µl with 1.08µCi of AdoMet in the presence of 2% BSA. The reaction mixtures were incubated for a total period of 48 hours. Tube 1 was a reaction mixture of 100µl and after 2, 4, 6, 12, 36 and 48 hours, 10µl (400ng DNA) samples were withdrawn and taken in 0.5 ml of SDS stop solution for isolation of DNA product and monitoring of ^{3}H-methyl group incorporation as described in section **2B.3.2.** To Tubes 2, 3 and 4 were added another 2.9 units of enzyme and 0.54µCi of AdoMet after 12 hours of incubation. More enzyme (1.49 units) and 0.54µCi of AdoMet, were added to Tubes 3 and 4 after 24 hours. 1.49 units of enzyme and 0.54µCi of AdoMet were added to Tube 4 after 36 hours. A volume of sample containing 400ng of phage DNA was withdrawn from these tubes at the specified time point, (Tube2; 24, 36, and 48 hours; Tube 3; 36 and 48 hours; Tube 4; after 48 hours).

The reaction was repeated in a reaction mixture of 200µl (Tube 5) containing 4µg ϕX174 RF DNA, 10.3 units of enzyme, 3.24µCi of AdoMet. and 1% BSA.

This was incubated for 48 hours and a volume of sample containing 400ng DNA was taken up in 0.5 ml SDS stopper solution after 2, 4, 6, 12, 36 and 48 hours.

All DNA samples withdrawn from Tubes 2, 3, 4 and 5 were taken up in 0.5 mls of SDS stop solution containing carrier DNA, as in Tube 1. The DNA products were isolated and monitored for incorporation of ^{3}H-methyl groups. The reaction left in each tube was stopped by heating to 60^{0}C for 5 minutes and the mixture was phenolised with an equal volume of phenol/chloroform and the methylated DNA in the supernatant isolated by ethanol precipitation. This was stored for use in sequence specificity studies, with methyl-sensitive restriction enzymes (**2B.8.2.**).

2B.6. <u>Stimulatory effect of a glycerol soluble fraction.</u>

The supernatant fraction obtained from pea nuclei stored in buffer M^{+} containing 50% glycerol at -20^{0}C, on centrifugation at 2000 rpm, 4^{0}C, 20 minutes as described in **2B.3.2**, is referred to as the glycerol soluble fraction. This fraction is subjected to further centrifugation at 15,000 rpm, 4^{0}C, for 20 minutes to exclude any fine pieces of broken nuclei. 25µl of stored extracted pea nuclei I (11.25 µg protein; specific activity of 5.5 cpm/mg) were incubated for 2 hours at 30^{0}C in a volume of 70µl containing 1.08 µCi of AdoMet, 0.7 mg/ml BSA and 0, 5, 10, 15 and 20 µl of the glycerol soluble fraction and buffer M^{+} to make up the volume of 70µl. The reaction was stopped by the addition of 0.5 ml of SDS stop solution and the DNA product monitored for tritium incorporation as described in **2B.1.8.**

20µl of the glycerol fraction was assayed for DNA methylase activity in the presence and absence of 10µg pea DNA substrate as described for soluble pea DNA methylase in **2B.3.2.**

Similarly 25µl of soluble pea DNA methylase was incubated with 0, 5, 10 and 20µl of the glycerol fraction in a 70µl reaction mixture containing 1.08µCi of AdoMet, 0.7 mg/ml BSA, 10µg pea DNA and buffer M^+ for 2 hours at 30^0C. The reaction was stopped with the addition of 0.5 ml of SDS stop solution and the DNA product isolated and monitored for tritium incorporation as described in **2B.3.2.**

2B.7. Immunoreaction with antibody against mouse ascites DNA methylase.

2B.7.1. Direct effect on in vitro pea DNA methylase activity.

35µl samples of Fraction III (14.8 units) were assayed for DNA methylase activity in a reaction volume of 70µl in the presence of 142µg per ml native pea DNA, 1.08µCi AdoMet, 0.7mg/ml BSA, by incubating for 2 hours at 30^0C in the presence of 0, 3, 5 and 10µl of antiserum no.401, raised against mouse ascites DNA methylase. Control assays were also carried out without the addition of antiserum no.401 but in the presence of equal volumes of horse serum.

This reaction was also repeated with a different batch of antiserum (0, 2.5 and 10µl) under similar reaction conditions.

2B.7.2. Immunoblot reaction of protein bands on nitrocellulose.

The second half of the SDS polyacrylamide gel, obtained as described in section **2B.5.1,** was subjected to "Western" blotting. The method used was as described by Towbin et al., 1979.

2B.7.2.1. Electrophoretic transfer of proteins to nitrocellulose sheet.

A sheet of nitrocellulose (Hybond-C extra, pore size 0.45µm) just large enough to cover the gel, was thoroughly wetted by cappillary action with electrophoretic blotting buffer (buffer C, **2A.6.9**) and laid on the gel which has been placed on a pre soaked sheet of 3MM Whatman paper care being taken to avoid air bubbles. Another sheet of 3MM Whatman paper soaked in buffer C was placed on the nitrocellulose sheet and the set up was enclosed in a pair of scouring pads soaked in buffer C and supported in a perspex cassette. The cassette was fitted into a transblot tank filled with buffer C so that the nitrocellulose sheet was facing the cathode. The electrophoretic blotting was carried out at constant voltage at 400 mA at room temperature for 4 hours. After stopping the reaction the gel was stained by the silver stain procedure described in section **2B.1.5** and no protein bands were detected due to a complete transfer of all proteins to the nitrocellulose sheet.

2B.7.2.2. ^{125}I-Protein A detection method.

The electrophoretic blot was soaked in 3% 'Marvel' (skimmed milk powder) in buffer D (**2A.6.10**) overnight at 4^0C.

Excess 'Marvel' was washed away by incubating in buffer D with constant agitation on a shaker at room temperature. The nitrocellulose blot was incubated with antiserum no.401 diluted 1 to 500 (v/v) with buffer D containing 2% horse serum as carrier serum, for 90 minutes on a shaker at room temperature. The sheet was washed five times in buffer D on a shaker for a period of 5 x 5 minutes and incubated with the indicator, ^{125}I-protein A (1 x10^6 cpm) diluted in buffer D, for 90 minutes at room temperature on a shaker. The nitrocellulose blot was rinsed in buffer D at least five times by 5 x 5 minutes washes on a shaker at room temperature and then dried between filter paper before exposure to a Kodak X-Omat R film, for 10 days at -70^{0}C with an intensifying screen.

2B.8. Sequence specificity of pea DNA methylase.

The sequence specificity of methyl group incorporation in vitro by pea DNA methylase was determined by two methods, nearest neighbour analysis and studies with methyl sensitive restriction enzymes.

2B.8.1. Nearest neighbour analysis.

The sequence specificity of methyl group incorporation in vitro, of the pea enzyme was determined by an adaptation of the method of Rigby et al 1977, as previously described, (Adams et al, 1986). Four 100µl reaction mixtures containing 5µg pea DNA in 50mM Tris-HCl buffer, pH 7.2, 0.05% BSA, 10mM $MgCl_2$, 10mM dithiothreitol, 10 units E.coli DNA polymerase I, 0.1 unit DNAse I and 400µM each of all four deoxy-ribonucleoside-5'-triphosphates (dATP, dCTP, dGTP and dTTP) one of

which was labelled, [α-^{32}P]dNTP (specific activity; 30µCi/10pmole) were incubated for 90 minutes at 15^{0}C, in Eppendorf tubes and the reactions were stopped with the addition of 2µl of 0.5M EDTA. The labelled DNA was isolated, by ethanol precipitation (Maniatis et al, 1982) as described in **2B.2.3.** The DNA product was dissolved in 10µl H_2O and methylated with 14.8 units of pea DNA methylase. The methylated labelled DNA products were isolated as described in **2B.3.2**, by ethanol precipitation in the presence of 0.6M sodium acetate at -70^{0}C. The DNA was washed with 70% ethanol, drained and left to dry. The DNA samples were taken up in 80µl of buffer A (**2A.6.2**) and incubated at 37^{0}C with 2 x 5µl micrococcal nuclease, (10 units/µg DNA) for 2 hours (2 x 1hr). 2 x 5µl of spleen phosphodiesterase, (2 units/µg DNA) was added to the hydrolysate for another 2 x 1 hour incubation. The reaction was stopped by heating at 60^{0}C for 10 minutes, 10µl samples were taken in 5 ml scintillation fluid and counted for ^{32}P and ^{3}H incorporation. 50µl samples were injected into a Mino RPC column (Pharmacia) and the deoxyribonucleoside 3'-monophosphates were eluted from the column with Buffer B (**2A.6.5**). The column was operated at a flow rate of 1 ml per minute, 40^{0}C, an absorbance range of 0.16 and a chart speed of 12 cm per hour. 8 drop samples (25 drops = 1 ml) were collected and 5 ml of scintillation fluid added and the ^{32}P and ^{3}H counts for each base was monitored. The average crossover between channels was determined for ^{3}H and ^{32}P. Labelling with each of the four ^{32}Pd.NTP, allows the estimation of methylation at all possible dinucleotide sequences.

2B.6.2. Restriction enzyme studies.

Plasmid DNA (pVHC1; M. Bryans, 1989 and ColE1) and phage DNA ϕX174 RF were used as substrate in methylation with pea DNA methylase in three separate reactions and digested with methyl sensitive restriction enzymes.

2B.6.2.1. Separation of DNA by agarose gel electrophoresis.

The electrophoretic separation of DNA fragments, was carried out by the method of Maniatis et al., 1982. A 1% (w/v) agarose gel was made in electrode buffer (**2A.6.12**) containing 0.5µg/ml ethidium bromide. A horizontal gel was poured and a comb inserted. The DNA samples were digested in a reaction volume of 10µl. 5µl of sample buffer (**2A.6.13**) was added to the reaction mixture before the reaction is stopped by heating at 60^0C (100^0C, for BstNI). The samples were applied into the wells. 5µl each of λH_3 DNA fragments and M13 mp18 HinfI DNA fragments, size markers, in sample buffer were applied also to separate wells in the gel. The gel was subjected to electrophoresis in the electrode buffer (**2A.6.12**) at constant voltage, 30mA for 3 hours or until the dye front reached 3/4 of the length of the gel. The gel was soaked in distilled water for 2 hours, drained, illuminated with a UV lamp and photographed with a Polaroid camera.

2B.6.2.2. Salicylate fluorography.

A 1M solution of sodium salicylate was made by dissolving 13.8g salicylic acid in 100ml of 1M NaOH and the pH was adjusted to read between 5 and 7.

The gel was soaked in the 1M sodium salicylate solution for a minimum of 2 hours. It was transferred onto a Whatman 3MM filter paper, covered with a peice of cling film and dried in a Slab gel dryer (Bio-Rad) for 2 hours at 60^{0}C and a pressure of 0.5 torr. The gel was exposed to a Hyper film-MP (Amersham) for 10 days at -70^{0}C without the use of an intensifying screen.

2B.8.2.3. <u>Restriction pattern of methylated pVHCI.</u>

2 x 1µg samples of pVHCI were methylated separately in 100µl reaction mixture with 1.9 units of Fraction II of pea DNA methylase by incubating at 30^{0}C for 4 hours. A sample of 100ng of DNA was removed and added to 0.5 ml of SDS stop solution for monitoring of tritium incorporation as described in section **2B.3.2**. The DNA in the remainder was isolated as described in section **2B.2.3**. It was dissolved in 8µl of distilled water and digested in a 10µl volume containing 1µl of <u>Hpa</u>II or <u>Msp</u>I and 1µl of the reaction buffers as specified by the suppliers. The reaction mixtures were incubated at 37^{0}C for 2 hours and the reaction stopped by heating at 60^{0}C for 10 minutes after the addition of 5µl of sample buffer (**2A.6.13**).

The DNA fragments were separated by electrophoresis in a 1% agarose gel in the presence of 0.5µg per ml ethidium bromide as described in section **2B.8.2.1** and photographed with a Polaroid camera. To see if faint higher molecular weight tritiated DNA bands were present, the gel was fluorographed as described in section **2B.8.2.2**.

2B.8.2.4. Restriction pattern of methylated ColEI.

4µg of ColEI was methylated in a reaction mixture of 100µl by incubating at 30^{0}C with 15.8 units of Fraction III of pea DNA methylase for 24 hours. A 400ng DNA (10µl) sample was taken in 0.5 ml SDS stop solution and monitored for ^{3}H incorporation as described in section **2B.3.2**. The DNA in the remaining sample was isolated as described in section **2B.2.3**, dissolved in 32µl of distilled water. This was divided into 4 x 8µl samples and digested with 1µl of each of these enzymes (10 units each), HpaII, MspI, PstII and SstII, by incubating at 37^{0}C for 2 hours in the appropriate 10 x reaction buffers (1µl each) as specified by the suppliers. The DNA fragments were separated by electrophoresis as described in section **2B.8.2.1** and photographed. To see if faint higher molecular weight tritiated DNA bands were present, the gel was fluorographed as described in section **2B.8.2.2**.

2B.8.2.5. Restriction pattern of methylated ϕx174 RF DNA.

4µg of ϕX174 RF DNA was incubated with 10.3 units of enzyme in a reaction mixture of 200µl at 30^{0}C for 48 hours as described in section **2B.5.6.3**, (Tube 5). The methylated DNA was isolated as described in section **2B.2.3**. The DNA was dissolved in 32µl of H_2O and divided into 4 x 8µl in 4 Eppendorf tubes. 1µl of the appropriate React buffers as specified by the suppliers for each restriction enzyme (HpaII, MspI, EcoRII and BstNI) was added and 1µl of each restriction enzyme (10 units) was added and incubated at 37^{0}C (60^{0}C for BstNI) for 2 hours.

5µl of sample buffer (**2A.6.13**) was added to the digested DNA fragments and they were separated by electrophoresis in a 1% agarose gel as described in section **2B.8.2.1**. The gel was photographed with a Polaroid camera and fluorographed as described in section **2B.8.2.2**.

2B.9. Specificity of enzyme activity of the protein bands in the purified fractions.

2B.9.1. Electrophoretic separation in a one dimensional nondenaturing polyacrylamide gel.

A 5% polyacrylamide slab gel in 37.5mM Tris-HCl pH 8.8, containing 0.14 mg/ml salmon sperm DNA, 0.2M NaCl, and 10% glycerol was made with "native" gel casting solutions A, B and C [A : B : C ; 1 : 1 : 2 (v/v)] (**2A.6.15**). This was poured into clamped gel plates to a height of about 13 cm. A 3.5% polyacrylamide stacking gel in 35mM Tris-HCl, pH 8.8, containing 0.14mg/ml salmon sperm DNA, 0.2MNaCl, 23% glycerol was made with "native" gel casting solutions A, B and C [A : B : C; 1 : 0.75 : 2.5] and layered onto the resolving gel. The gel was transferred to 4^0C. The gel was run at 150V for 30 minutes in the electrode buffer (buffer E, **2A.6.11**) before applying samples of pea DNA methylase fractions (35µl each) and mouse ascites DNA methylase (20µl) in duplicate to the gel. The gel was run at 120V for 26 hours at 4^0C and the gel was cut in two halves.

One half of the gel was stained in 0.1% (w/v) Coomasie brilliant blue R in 50% (v/v) methanol and 10% (v/v) acetic acid as described in section **2B.1.6.** and photographed.

2B.9.2. <u>In situ assay for DNA methylase activity.</u>

The second half of the gel was desalted by 3 x 1 hour washes in buffer M^+ at 4^0C. The gel was resuspended in 25 ml buffer M^+ pH 7.0 containing 30µCi S-adenosyl-L-(^{3}H-methyl)-methionine (TRK 581, 60 Ci/mmol) and incubated for 12 hours at 30^0C. The reaction was stopped by washing the gel for 5 minutes in 10 changes of a solution of 10% (v/v) methanol and 10% (v/v) acetic acid.

The gel was fluorographed as described in section **2B.8.2.2** except that the gel was exposed to Hyper Film-MP (Amersham) for 20 days at -70^0C.

CHAPTER THREE

RESULTS

3.1. Distribution of DNA methylase activity.

3.1.1. Isolation of nuclei.

The isolation procedure used to obtain nuclei from the pea seedling homogenate, as described in section **2B.1.2** is an adaptation of the method of Ts'O and Sato for the quantitative separation of subcellular components (Ts'O and Sato, 1959). Centrifugation at 2500 rpm for 15 minutes, sediments most of the nuclei but they are heavily contaminated with chloroplasts. These are lysed by treatment with Triton X-100. The washed nuclei were found to be free of other cellular organelles when examined under the phase contrast microscope. The nuclei are intact but lack an outer membrane due to the use of the detergent Triton X-100, to remove thylakoids. The DNA:protein ratio in the nuclei was 1 : 2.2 - 3.0 as shown in Table 2.

3.1.2. Methylase assay.

Nuclei prepared as above from the Triton insoluble fraction of the nuclei/chloroplast pellet were used as a source of DNA methylase, and werefound to contain all recoverable DNA methylase activity. The assay measures the incorporation of ^{3}H-methyl group from S-adenosyl-L-[^{3}H-methyl] methionine into material which survives deproteinisation, alkali treatment, is acid precipitable in cold 5% TCA and is soluble in hot PCA. This is DNA. It is a slight modification of the method for the detection of DNA methylase activity from the mouse ascites cells (Adams et al.,1986a).

Table 2. DNA : protein ratio in isolated pea nuclei.

The concentration of DNA in 50µl of 4 different samples of pea nuclei from 7 day apical shoot tips was estimated by the colorimetric estimation of the deoxyribonucleoside content by reaction with Burton's reagent as described in Methods section **2B.1.3**. The protein concentration in 50µl of the pea nuclei samples was estimated by Bradford's method as described in Methods section **2B.1.4.**

Pea nuclei	µg DNA	µg Protein	DNA : Protein
1	53	145	1 : 2.7
2	56	125	1 : 2.2
3	58	180	1 : 3.1
4	49	150	1 : 3.1

Deproteinisation with phenol and alkaline hydrolysis at 37^0C, for an hour removes protein and RNA which could act as acceptors for the radioactive methyl group in the nuclear extracts. A unit of enzyme activity is that amount which catalyses the incorporation of one picomole of methyl group into DNA per hour at 30^0C. The specific activity of a seven day homogenate is 0.09 units/mg protein, (Table 3).

3.1.3. Identification of the methylated base in the DNA product.

The product of pea DNA methylase was further characterised by formic acid hydrolysis of the DNA product and separation of the bases on an anion exchange Aminex A6 column as described in section **2B.1.9.** The methylated base was shown to be 5-methylcytosine, (Figure 6). No radioactivity was detected in thymine or any other base. By a similar procedure, the base composition of DNA from 5 day pea seedlings was determined. The relative amount of each base was estimated from the integrated area of each peak of absorbance corresponding to each base multiplied by a correction factor derived from the relative molar absorbance ratio of each base at 260nm at the pH of 10.2 (Ford et al., 1980). The proportion of cytosine methylated in pea DNA was found to be 26%, the G+C content was 37.1 and the A+T content was 62.9%.

3.1.4. Location in organelles.

Table 3 shows the results from previous work done in our laboratory by A. Rinaldi (Yesufu et al.,1989) as described in section **2B.1.10.** Nuclei isolated from pea shoot tips contain all the recoverable DNA methylase activity.

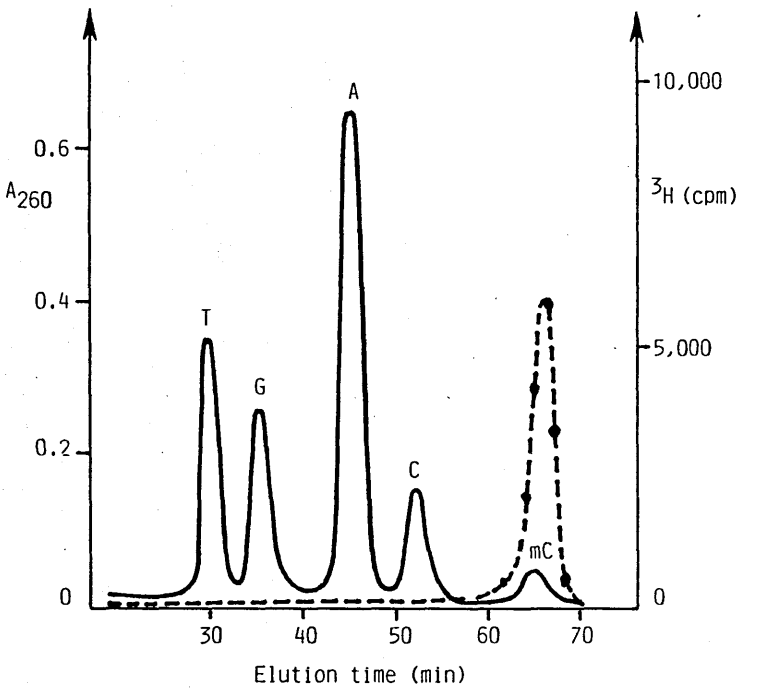


Figure 6. Base composition of DNA product.

Exogenous

Pea DNA was methylated using pea DNA methylase (fraction II) and then hydrolysed to the bases which were separated on a column of Aminex A6. The solid line shows the absorption profile at 260nm of acid hydrolysed pea DNA. The broken line shows the elution of tritium incorporated from ^{3}H-methyl group of AdoMet.

See p.96

Table 3. Location of DNA methylase activity.

Plants were grown, either in continuous light or continuous dark, for 7 days prior to harvest. The nuclei were prepared as described in the Methods section **2B.1.10.** The plastids were the meterial solubilised in the wash with Triton X-100

Illumination:	LIGHT		DARK	
Source of enzyme	total act. (units)	spec.act. (u/mg)	total act. (units)	spec.act. (u/mg)
homogenate	112.8	0.09	30.0	0.04
nuclei	76.4	28.6	28.8	12.7
plastids	0.1	0.04	0.2	0.06

Essentially no activity was found in plastids from plants grown either in continuous light (chloroplasts) or in continuous dark (etioplasts).

3.1.5. Location in seedling portions.

The nuclei with the highest specific activity were obtained from 5-day old shoot tips (Table 4). However, at this stage only about half the seedlings have emerged sufficiently through the compost to be harvested, and most of the subsequent work was done with 7-day old pea shoot tips as the yield was highest. At all ages the most active nuclei were those obtained from the apical shoot tips i.e. the top 1.5 to 2.0cm. A lower level of activity is found in root tips (1.5-2.0cm), first free leaves and in subterminal shoot portions (regions 2.0 to 4.0 cm from the growing tip) of the same age (Table 4). Those regions of stalk further than 4cm from the shoot tip and the nearest pea portions showed little or no activity.

3.2. Extraction of unbound pea DNA methylase.

3.2.1. Sonication of pea nuclei.

Attempts to obtain a DNA-free, soluble pea methylase by sonication as described in section **2B.3.1.** failed. Brief pulses of sound caused disruption of nuclei as shown in Figure 7a. The methylase activity in the supernatant was independent of external addition of DNA substrate. All activity observed was associated with bound endogenous DNA, even after sonication for 100 seconds. This conclusion was reached as there was no difference in the methylase activity of the supernatant in the presence or absence of exogenous pea DNA (Figure 7a).

Table 4. Variation in nuclear DNA methylase activity with location and age.

DNA methylase was assayed in nuclei isolated from the indicated part of pea seedlings, harvested at increasing age from the time of swelling.

Age (days)	DNA methylase (units/mg protein)			
	Shoot (apex)	Shoot (subterm)	Leaf	Root tip
5	93 (33.5)*	-	-	23
7	64 (67.2)	25	-	4
9	24 (9.3)	10	10	2
11	15 (4.9)	13	5	1

* The figures in brackets indicate the yield of enzyme present in shoot apices from one tray of peas.

_ The indicated portions were not available for assay at the specified ages.

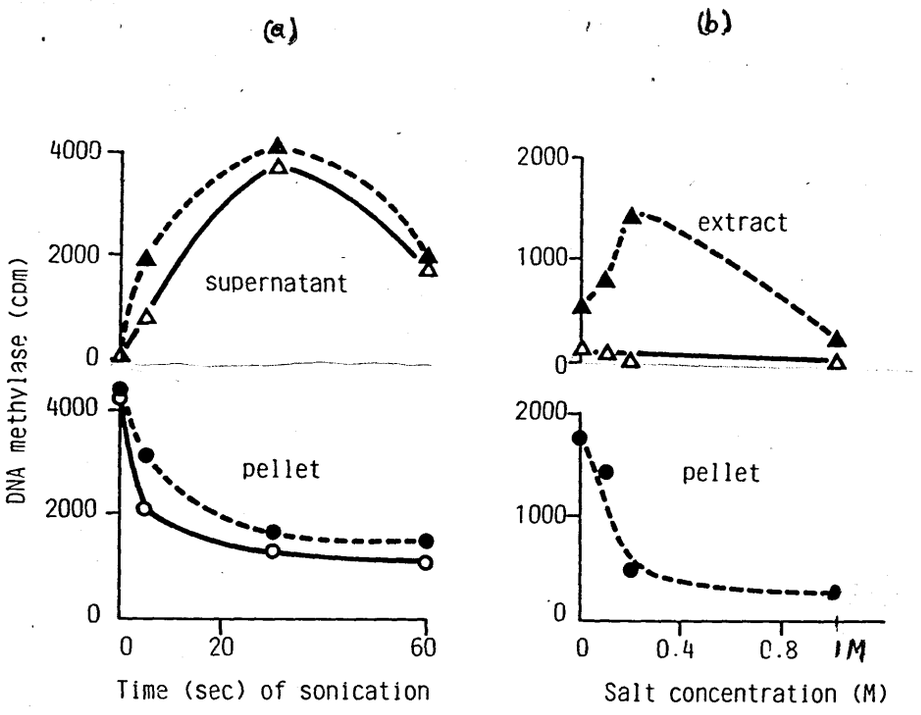


Figure 7. Extraction of unbound DNA methylase.

(a). Effect of sonication for different durations on pea methylase activity in the supernatant and pellet of fractionated nuclei.

(b). Extraction of pea nuclei with increasing salt concentration.

The solid lines (**a** and **b**) indicate methylase activity in the absence of exogenous pea DNA and the broken lines indicate activity in the presence of exogenous pea DNA.

3.2.2. Extraction of pea nuclei with Sodium Chloride.

The effect of increasing concentration of NaCl in the extraction of soluble pea DNA methylase is shown in Figure 7b. A portion of the DNA methylase activity can be obtained free of DNA by extracting the nuclei with buffer M^+ containing 0.2M NaCl. Higher salt concentrations did not improve the yield. This is a similar finding to that obtained for the mouse ascites enzyme except that only about 20-40% of the activity is extracted with the first 0.2M NaCl treatments in buffer M^+. On partial digestion of the pellet of extracted nuclei with micrococcal nuclease, (400units/ml) as detailed in section **2B.3.2**, up to 80% of the activity could be obtained in the combined extract when followed with a second 0.2M NaCl extraction (Tables 5.1 and 5.2). The dialysed fractions were reduced to a third of the starting volume and can be stored in this concentrated form. The DNA methylase activity in the dialysed combined 0.2 MNaCl extract (0.8mg / ml protein) was fairly stable for a period of one week at -20^0C in buffer M^+ containing 50% glycerol as shown in Figure 8.

3.3. Chromatographic purification of nuclear extract.

DNA methylase has been purified from a number of animal cells; mouse ascites cells, rat liver cells, rat hepatoma cells, bovine thymus, and human placenta, (Turnbull and Adams, 1976; Kalousek and Morris, 1978; Adams et al.,1986; Morris and Pih, 1971; Sano et al., 1983; Pfeifer et al., 1983) by different chromatographic procedures. The purified enzyme has a molecular mass estimated between 150,000-200,000. DNA methylase has been purified from wheat embryo, with a molecular mass of 50,000-55,000 (Theiss et al., 1987).

Table 5.1. Extraction of soluble pea DNA methylase.

Pea nuclei were extracted with 0.2M NaCl in buffer M^+, before and after partial digestion with micrococcal nuclease, to obtain soluble DNA methylase, as detailed in section **2B.3.2.**

Fraction	**Activity**		
	cpm/µg	Total units	%
Pea nuclei	50	2314	100
Extracted nuclei(I)	48	1746	75
Extracted nuclei(II)	18	580	25
Salt extract (I)	60	462	20
Salt extract (II)	84	1177	51
Combined Salt extract (I & II)	78	1639	71

Table 5.2. Extraction of soluble pea DNA methylase.

Pea nuclei were extracted with 0.2M NaCl in buffer M^+, before and after partial digestion with micrococcal nuclease, to obtain soluble DNA methylase, as detailed in section **2B.3.2.**

Fraction	**Activity**		
	cpm/µg	Total units	%
Pea nuclei	33	1618	100
Extracted nuclei (I)	31	1023	63
Extracted nuclei (II)	7	132	6
Combined salt extract	56	1329	82

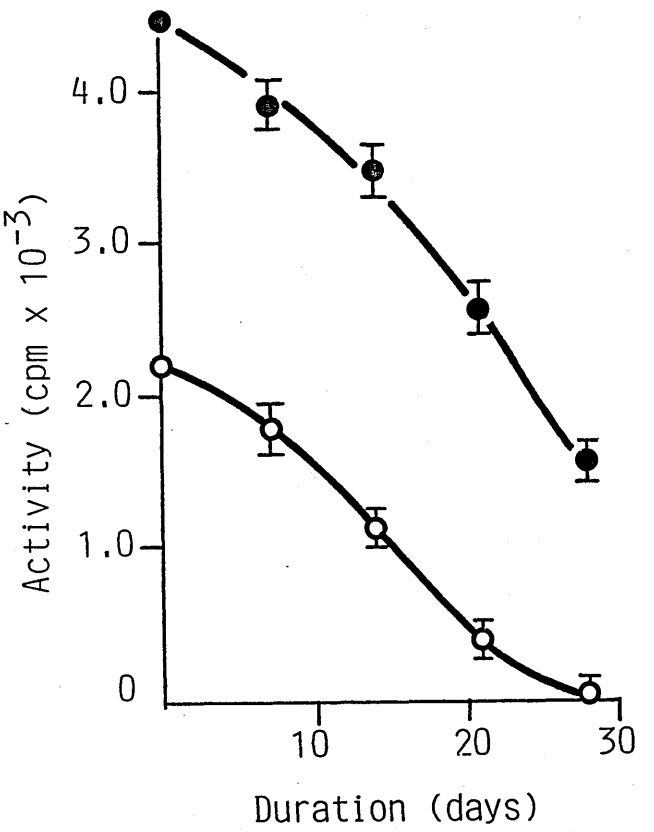


Figure 8. Stability of DNA methylase activity with time.

DNA methylase activity was assayed in isolated pea nuclei and in 0.2M NaCl nuclear extract stored at -20^{0}C in 50% glycerol in buffer M^+ for varying length of time. (●) = methylase activity in pea nuclei. (0) = methylase activity in 0.2M NaCl extract.

DNA methylase has not been purified from pea, but there is a report of the enzyme activity in crude nuclear preparation from this plant, (Kalousek and Morris, 1969[6]). Similar chromatographic steps to those described for mouse ascites DNA methylase (Adams et al.,1986a) were tried in the purification of DNA methylase from the pea nuclear preparation and the most active fractions were obtained using columns of Heparin Sepharose and FPLC Mono Q HR 5/5 (Pharmacia). Fractionation with ammonium sulphate led to major activity losses, hence the use of Centriprep concentrator 30.

3.3.1. Heparin Sepharose and Mono Q.

The result for the purification procedure starting with whole nuclei is summarised in Table 6 and detailed in section **2B.4.1**. The 0.2M NaCl extract, (Fraction II) was applied onto a Heparin Sepharose column which binds pea DNA methylase at this salt concentration. The active protein fraction, (Fraction III) was eluted with 0.6M NaCl in buffer M^+. This was desalted using a Centriprep concentrator 30 and injected into an FPLC Mono Q HR 5/5 column, (Pharmacia). The column was eluted with a salt gradient of 0-1.0M NaCl and the fraction containing active methylase (Fraction IV) was obtained at 0.35M NaCl. The fraction had a specific activity of 1738, and a 50 fold purification over the nuclear fraction. This represents a 19,000 fold purification with respect to the original homogenate, since the nuclear fraction itself is an enrichment over the homogenate. The enzyme elutes as a single peak from each of these columns (Figures 9a and 9b).

Table 6. Purification of pea DNA methylase

Step	Fraction	Total activity (units)	Protein (mg)	Specific activity (units/mg)	Purific. (fold)
Pea nuclei	I	905	26.00	35	1
0.2M NaCl extract	II	871	13.20	65	2
Heparin Sepharose (0.6M NaCl peak)	III	422	0.64	660	19
Mono Q (0.35M peak)	IV	113	0.06	1880	50

Figure 9. Chromatographic purification of pea DNA methylase.

The enzyme elutes as a single peak in each of these steps.

(a). Elution profile of 0.2M NaCl nuclear extract on a Heparin Sepharose column.The points indicate absorbance at 280nm. |—| indicates the active fractions pooled.

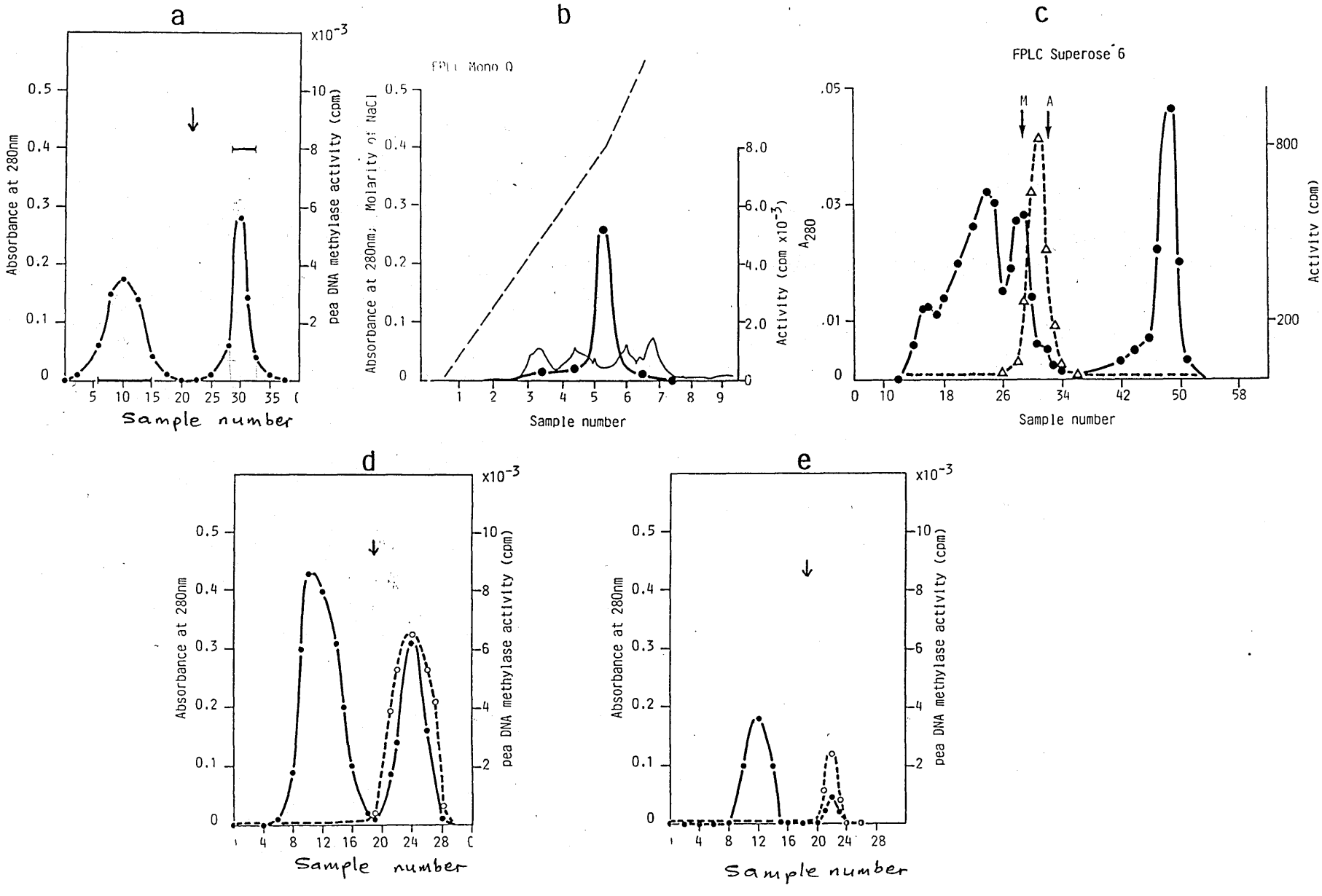
(b). Purification of the active methylase fraction from a Heparin Sepharose (4 ml) on FPLC Mono Q HR 5/5 column (Pharmacia). The solid line indicates the absorption profile at 280nm; the solid circles show DNA methylase activity and the broken line indicates the NaCl gradient.

(c). Gel filtration of the nuclear extract (2 ml) on a calibrated FPLC column of Superose 6 (Pharmacia). The solid line indicates the absorption at 280nm; the broken line indicates the activity of DNA methylase. M = Mouse ascites DNA methylase (190,000); A = Alcohol dehydrogenase (150,000), used as markers.

(d). Purification of the active methylase fraction from Heparin Sepharose (5 ml) on a DEAE Sephacel column. The solid line indicates absorbance at 280nm and the broken line indicates methylase activity.

(e). Purification of the active methylase fraction from DEAE Sephacel on a pea DNA Sepharose column. The solid line indicate the absorbance at 280nm and the broken lines indicates methylase activity.

In **a, d** and **e** sample the fractions collected before the arrow were 3ml and represent unbound material. After the arrow 1 ml samples were collected and the enzyme eluted with buffer M^+ containing 0.6M NaCl (**a**) or 0.2M NaCl (**d** & **e**)


a
b
c
d
e
Absorbance at 280nm
pea DNA methylase activity (cpm)
x10-3
Sample number
FPLC Mono Q
Absorbance at 280nm; Molarity of NaCl
Activity (cpm x10-3)
FPLC Superose 6
M
A
A280
Activity (cpm)

3.3.2. Gel filtration.

The 0.2M NaCl extract, (Fraction II) and the 0.6M NaCl protein peak from Heparin Sepharose, (Fraction III) were also purified by gel filtration on a calibrated Superose 6 column (Pharmacia). Only one peak of enzyme activity was obtained with the 0.2M NaCl nuclear extract (Figure 9c). Activity was detected in fractions from 28-32 ml elution volume with the highest activity in the 30 ml fraction which corresponded to a molecular mass of about 160,000. Similarly only one peak of DNA methylase activity was obtained with the purification of the 0.6M NaCl protein fraction from a Heparin Sepharose column on the FPLC Superose 6 column. This procedure is not included in the scheme shown in Table 6 as the incorporation of more than two chromatographic steps in the purification leads to loss of enzyme activity and was found to be unnecessary.

3.3.3. DEAE Sephacel and pea DNA Sepharose.

The 0.6M NaCl extract from a Heparin Sepharose column, (Fraction III) after desalting in a Centriprep 30, was also purified by anion exchange on a DEAE Sephacel column, to which it binds at zero salt concentration. The active DNA methylase fraction was obtained by elution with 0.2M NaCl from this column (Fraction IV'). This was further purified by affinity chromatography on a pea DNA Sepharose column to which it binds at zero salt concentration and active DNA methylase fraction is obtained with 0.2M NaCl in buffer M^+, as detailed in section **2B.4.3**. No methylase activity was detected in the unbound fractions from either column. The enzyme also eluted as a single peak from these columns (Figures 9d and 9e).

Chromatographic purification with the incorporation of DEAE Sephacel and pea DNA Sepharose did not improve the specific activity even though all purifications were done at 4^0C. The pattern of peptide bands obtained on immediate and delayed electrophoretic separation of proteins in the active fractions from these columns are shown in Figure 10.2.

3.3.4. Phosphocellulose and blue dye matrix.

The enzyme binds to phosphocellulose at a salt concentration of 0.2M NaCl but the activity was not released with higher salt concentrations. Phosphocellulose has been a good purification step with mouse ascites DNA methylase. Pea DNA methylase (Fraction II) also binds to blue dye matrix resin at 0.2M NaCl but no DNA methylase activity was obtained in extracts with higher salt concentrations (Table 7). The absence of most of the enzyme activity from the unbound 0.2M NaCl fractions showed that the enzyme bound to these resins at 0.2M NaCl but the enzyme activity was not recovered even with the highest salt concentrations hence these chromatographic materials were not employed in the purification of pea DNA methylase.

Table 7. Purification with phosphocellulose and blue dye matrix.

1 ml of 0.2M NaCl extract was applied onto a preequilibrated pellet of phosphocellulose or blue dye matrix resin in the volume ratio of 10 : 1 and the pellets with adsorbed pea DNA methylase were extracted with different salt concentrations, as detailed in section **2B.4.4**. The total activity applied was 106.7 units

Fraction	**Activity in extract** (% of total activity applied)	
(supernatant)	phosphocellulose	blue dye matrix
0.2M NaCl	10.6	14.34
0.4M NaCl	0	0.02
0.6M NaCl	0	0.02
1.0M NaCl	0	0

3.4. Characterisation of pea DNA methylase.

3.4.1. Molecular weightof pea DNA methylase.

The peak fractions with the most active DNA methylase activity from DEAE Sephacel and pea DNA Sepharose were subjected to electrophoresis on an SDS polyacrylamide gel, one major protein band of molecular weight of about 160,000, is seen on silver staining (Figure 10.1). However, the enzyme is unstable and, when subjected to a second gel electrophoresis (within 48h), the high molecular weight protein was replaced by bands at 80,000, and 40,000. Often other protein bands are observed with some active fractions (Figure 10.2).

3.4.2. Optimal assay conditions.

DNA methylase was assayed in isolated nuclei with, or without added DNA or in soluble extracts in the presence of exogenous native pea DNA. Previous work in our laboratory showed that pea DNA methylase has a sharp temperature optimum at 30^0C rather than at 25^0C or 37^0C, when the enzyme activity was assayed in isolated nuclei. All assays for DNA methylase activity were subsequently carried out at 30^0C. An optimum pH of about 7.0 was obtained for both the nuclear and the solubilised enzyme. The result is shown in Figure 11b. A DNA concentration below 0.3mg per ml does not saturate the soluble enzyme (Figure 11c). A pea DNA concentration of 142µg per ml was used in most assays for pea DNA methylase activity. For efficient methylation a protein concentration of about 1mg per ml is required (Figure 11d). The enzyme concentration curve is non linear with native pea DNA as substrate (Figure 11d).

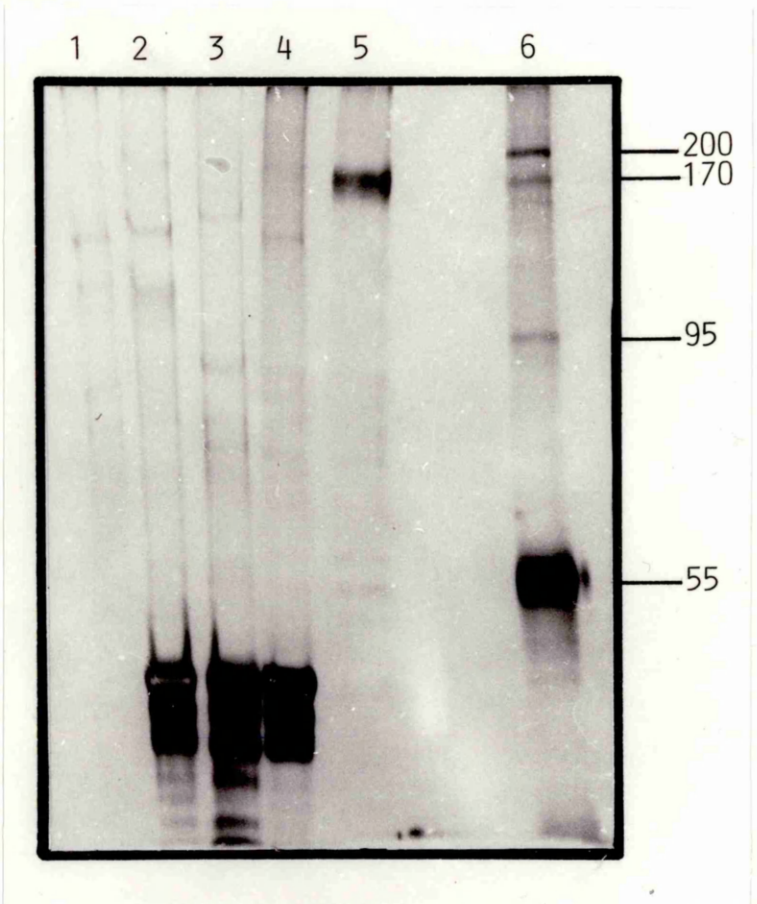


Figure 10.1. SDS/polyacrylamide gel electrophoresis of pea DNA methylase.

The figure shows the silver-stained SDS polyacrylamide gel on which samples from various stages in the purification were separated. Lane 1 = first 0.2M NaCl extract (no nuclease treatment). Lane 2 = 0.2M NaCl extract after micrococcal nuclease digestion. Lane 3 = pooled 0.2M NaCl extract (1+2). Lane 4 = 0.6M NaCl fraction from a Heparin Sepharose column. Lane 5 = 0.2M NaCl peak fraction from a DEAE Sephacel column.

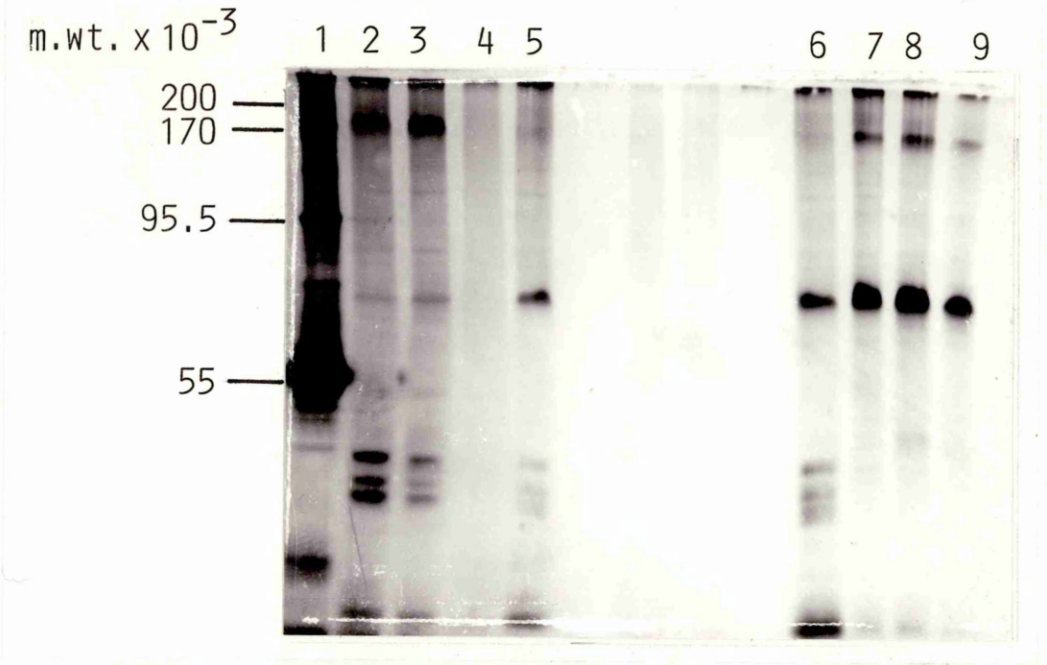


Figure 10.2. SDS/polyacrylamide gel electrophoresis of fractions of pea DNA methylase.

The figure shows the silver-stained SDS polyacrylamide gel of the 0.2M NaCl extract and purified fractions from DEAE Sephacel and pea DNA Sepharose columns. Lane 1= marker proteins, (myosin, 200K; α_2-macroglobulin,170K; phosphorylase b, 95.5K; glutamate dehydrogenase, 55K). Lane 2 = 0.2M NaCl extract. Lane 3 = peak fraction from a DEAE Sephacel column after an overnight dialysis. Lanes 4 and 5 = DEAE Sephacel methylase fractions after 48 hours storage at -20^{0}C. Lane 6 = pooled DEAE Sephacel fraction. Lanes 7, 8 and 9 = methylase fractions from a pea DNA Sepharose column.

Figure 11. Optimum reaction conditions for pea DNA methylase.

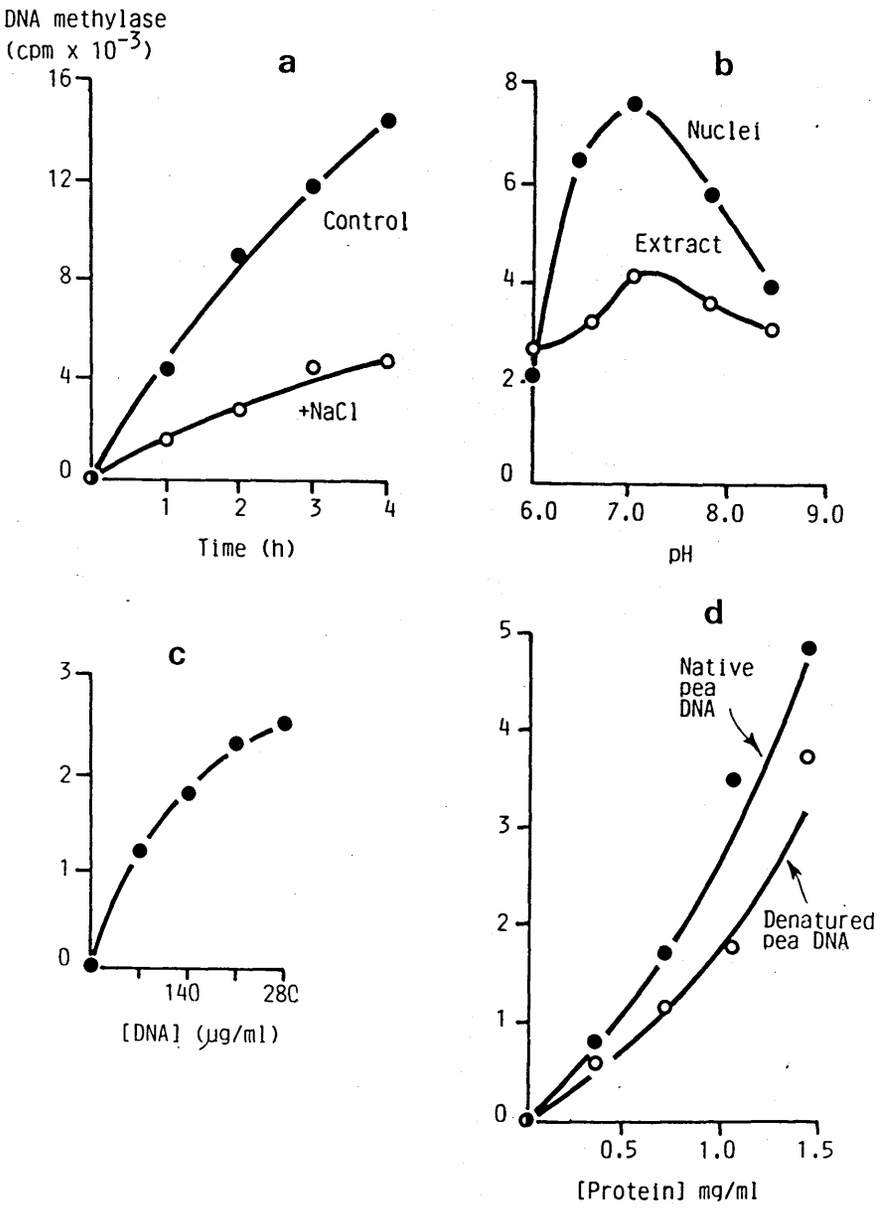
Pea DNA methylase activity was assayed in 35µl of pea nuclei as described in section **2B.1.8** and in 35µl of 0.2M NaCl extract of pea nuclei as described in section **2B.3.2** at 30^0C at varying reaction conditions as indicated below.

(a). Time dependence of the nuclear pea DNA methylase activity and the effect of 200mM NaCl on the nuclear enzyme activity with time.

(b). pH of optimum activity for the nuclear and soluble pea DNA methylase when assayed in buffer M^+ at the indicated pH values.

(c). Dependence of soluble pea DNA methylase activity on pea DNA substrate concentration.

(d). The effect of enzyme concentration on soluble pea DNA methylase activity with native or denatured pea DNA as substrate. The amount of protein in the enzyme preparation is indicated.


DNA methylase
(cpm x 10^{-3})
a
16
12
8
4
0
Control
+NaCl
1
2
3
4
Time (h)
b
8
6
4
2
0
Nuclei
Extract
6.0
7.0
8.0
9.0
pH
c
3
2
1
0
140
280
[DNA] (µg/ml)
d
5
4
3
2
1
0
Native pea DNA
Denatured pea DNA
0.5
1.0
1.5
[Protein] mg/ml

Low concentrations of enzyme give less than the expected amount of product. Similarly DNA methylase activity were assayed at different protein concentrations with limiting concentration of pea DNA substrate (0.01 mg/ml). Such non-linear kinetics are not seen with limiting amounts of DNA as substrate as shown in Figure 12. This may imply a co-operative action of the enzyme on the DNA substrate.

Although pea DNA methylase alone is unstable at temperatures higher than 4^0C, it could catalyse methylation at a constant rate at 30^0C for over 4 hours in the presence of added DNA and in the presence of a high salt concentration. The time course for in vitro methylation is shown in Figure 11a. The reaction is approximately linear for at least 4 hours at the usual assay conditions, but with a high enzyme concentration, even with limiting DNA, methylation continues for much longer (Figure 13). This is similar to the results obtained with the mouse ascites DNA methylase (Turnbull and Adams, 1976).

The addition of more enzyme to an incubation whose rate is slowing leads to further incorporation. The decrease in the initial rate of reaction is thus due to loss of enzyme activity rather than saturation of available sites on the DNA. The initial rate of reaction depends on the initial enzyme concentration and the rate can remain linear for up to 12 hours as shown in Figure 13.

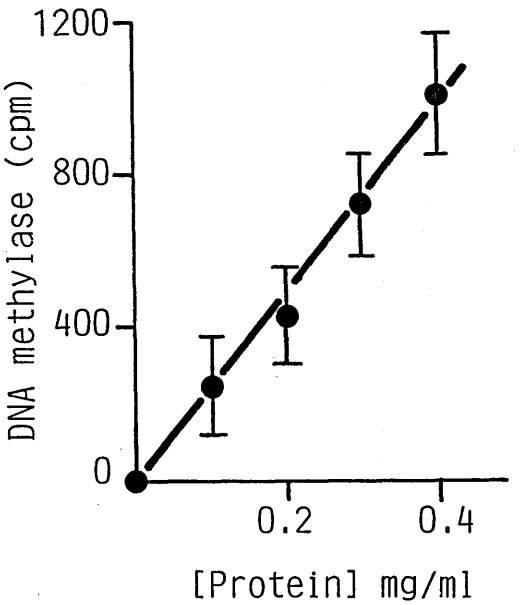


Figure 12. Pea DNA methylase activity with limiting pea DNA substrate (0.01mg/ml at varying protein concentration)

The graph shows the kinetics of (Fraction II) DNA methylase activity under the usual assay conditions except that the DNA concentration was one tenth the normal assay concentration.

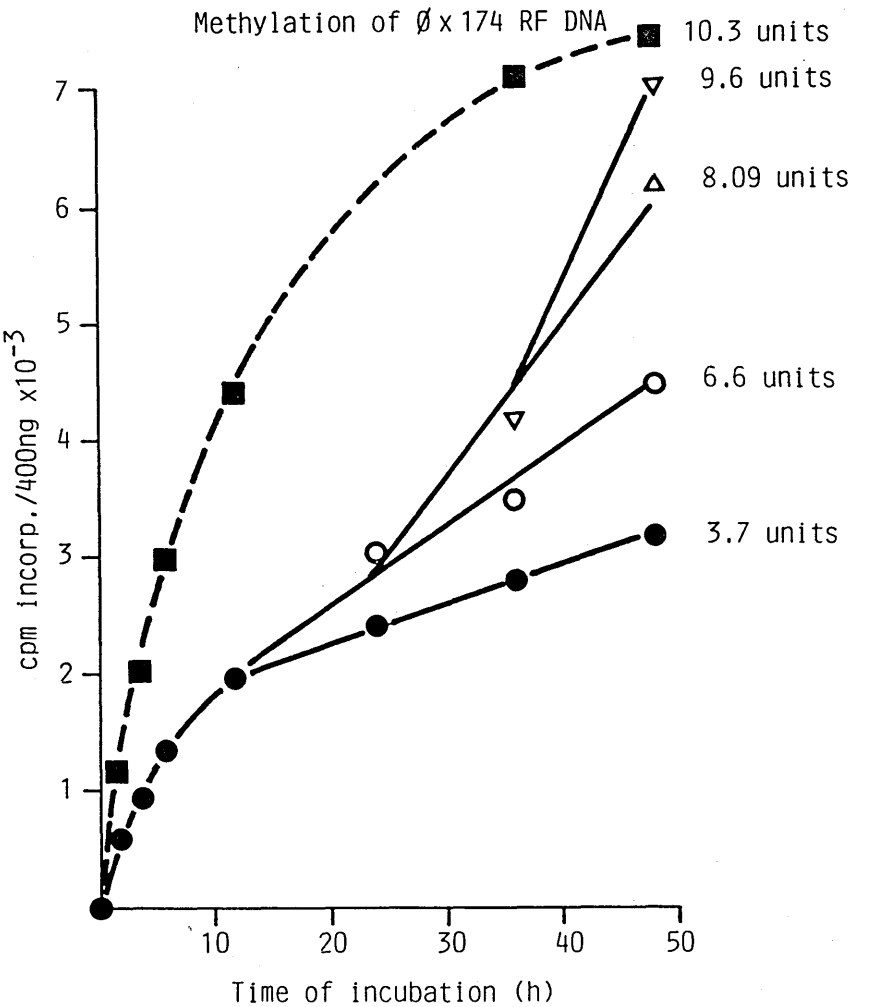


Figure 13. Time course for de novo methylation of ϕX174 RF DNA. The graph shows two incubations initiated with 3.7 units or 10.3 units of pea DNA methylase (fraction III) in a volume of 100µl or 200µl respectively, each containing 4µg ϕX174 RF DNA. To the former additional enzyme (20µl, 1.5 units) and ^{3}H AdoMet (0·5µCi) were added at 6, 12, 24, and 36h. Aliquots, containing 400ng DNA were removed at the indicated times and the extent of methylation analysed.

3.4.3. Substrate specificity.

Pea DNA methylase is active on both single stranded and double stranded DNA substrates. The acceptor activity of the native DNA substrates is reduced by heat denaturation with regard to both rate and extent of methylation in all cases. The best substrate discovered so far for the pea enzyme is native DNA isolated from 5 day old pea shoot tips (Table 8). Native pea DNA is a ten fold better substrate than denatured M.luteus DNA. DNA from salmon or bovine sources is intermediate as a methyl group acceptor and DNA from M. luteus is a poor acceptor (Figure 14a). This is in contrast to the situation with the vertebrate DNA methylases where homologous DNA is usually a very poor substrate and DNA from M. luteus is commonly used (Adams et al., 1986; Pfeiffer and Drahovsky, 1986).

Hemimethylated DNA from M. luteus or calf thymus (nick translated in the presence of methyldCTP) is a much better substrate than the same DNA nick translated with dCTP (Figure 14b). As less than 1% of the DNA was nick translated in this reaction (as calculated by the incorporation of ^{32}P), the preference for the hemimethylated regions must be of the order of 300 times that for the unmethylated DNA. Both these findings support the proposal that the presence of some methyl groups on the DNA substrate is important for activity. Pea DNA methylase, in spite of this preference for hemimethylated DNA, is also capable of de novo methylation as shown by its ability to act on unmethylated M.luteus and ϕX174 RF DNA (Figure 13).

Table 8. Pea DNA methylase activity with endogenous DNA substrates.

35µl of 0.2M NaCl extract were incubated with with 5µg native pea DNA prepared from seedlings of 5, 7, 9 and 11 day growth separately under the usual assay conditions as described in section **2B.3.2.**

DNA (age, days)	Activity (cpm)*
5	4388
7	3447
9	1479
11	867

* Average standard deviation = ± 100

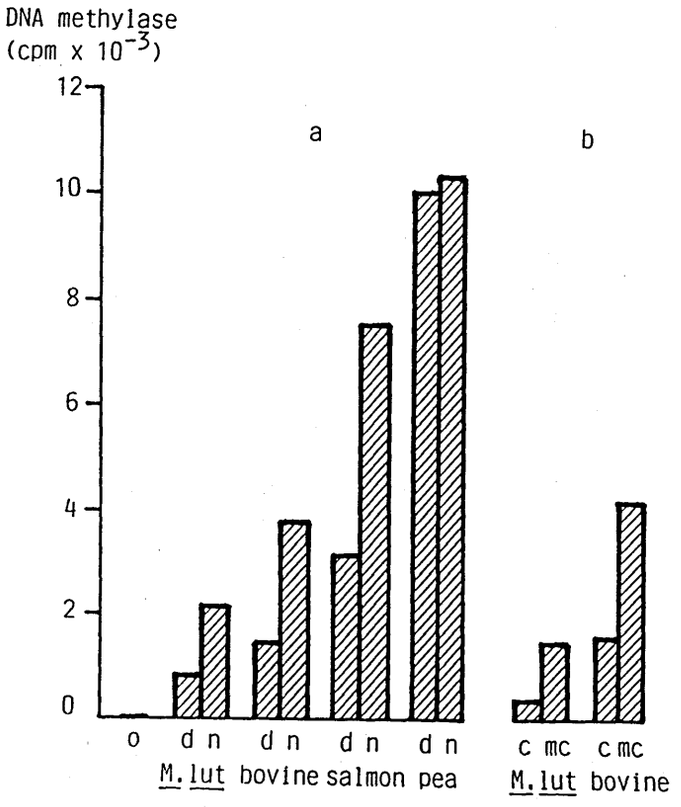


Figure 14. Substrate specificity of pea DNA methylase.

(a). One unit of fraction II was incubated with the indicated DNAs at 70µg/ml. d.= heat denatured, single stranded DNA. n = native, double stranded DNA. M.lut.= DNA from Micrococcus luteus.

(b). Hemimethylated DNA (section **2B.2.3**) was compared with control, unmethylated DNA as substrate for pea DNA methylase (fraction II). The DNA concentration was 42µg/ml. mC = hemimethylated DNA, C = control DNA.

3.5. Mechanism of action of pea DNA methylase.

3.5.1. Effect of sodium chloride.

The dialysed 0.2M NaCl extract, the isolated nuclei and the first pellet obtained after the first 0.2M NaCl extraction (35µl each) were assayed for methylase activity in the assay conditions described in the presence of different concentrations of NaCl (0-300mM). The soluble enzyme is very strongly inhibited by salt; 100mM NaCl concentration causes 85% inhibition of DNA methylase activity (Figures 11a and 15). In contrast the nuclear enzyme is only 49% inhibited by 100mM NaCl. This is also true for the enzyme which remains bound in the nuclei after the first extraction with 0.2M NaCl (Table 8). Two proposals have been put forward as possible mechanisms of DNA methylation by the mammalian enzyme: **(i).** The enzyme binds to a particular site on the DNA, methylates it and then dissociates from the DNA (Turnbull and Adams, 1976). **(ii).** The enzyme binds to DNA and travels along the molecule methylating available sites and stays bound (Drahovsky and Morris, 1971). The present observation may result from the nuclear enzyme's being associated with DNA in a resistant complex prior to the addition of salt. To test this hypothesis, 200 mM NaCl was added to the soluble enzyme after 10 min of incubation with DNA. In this situation only 72% inhibition was obtained compared with the 98% inhibition obtained when the NaCl was added at zero time (Figure 15). This could be interpreted to mean that the binding of the enzyme to DNA is prevented by NaCl concentrations in excess of 100mM. This, however, can be only a partial explanation. Figure 11a shows the effect of 200mM NaCl on the time course of nuclear methylase activity.

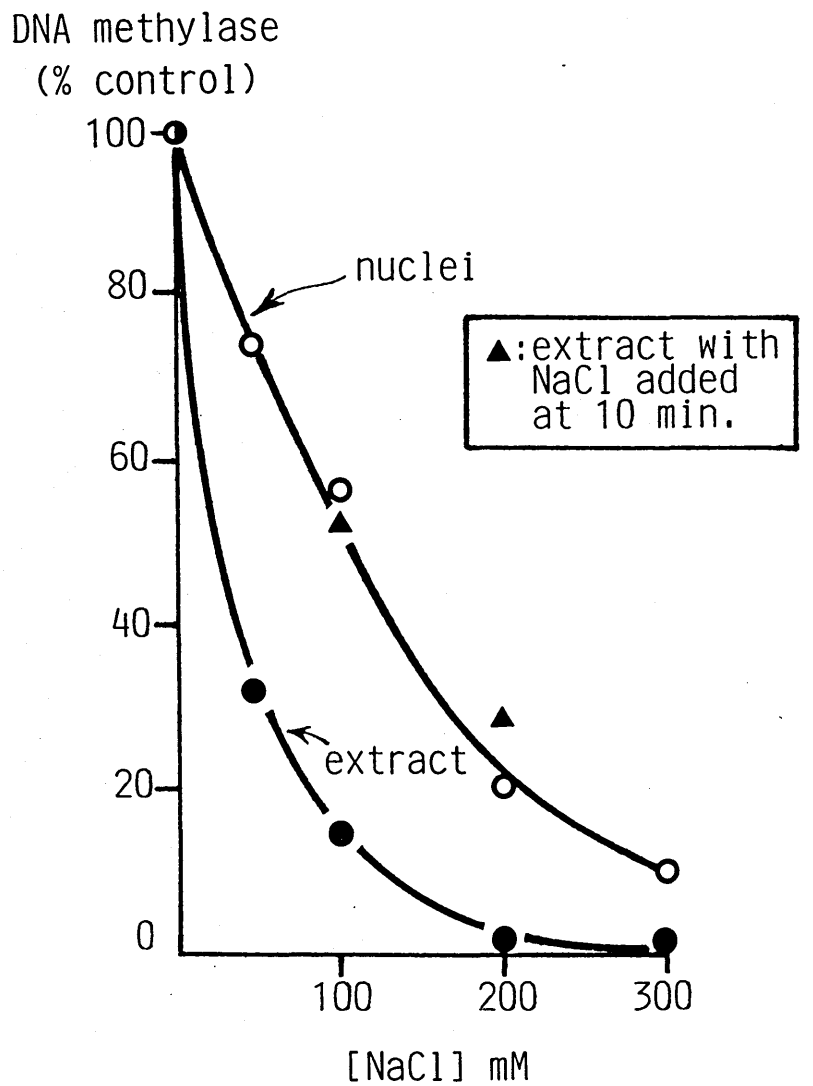


Figure 15. Effect of NaCl on nuclear and soluble pea DNA methylase. Nuclei (66µg protein) or soluble enzyme (25µg fraction II) plus 10µg pea DNA were incubated for 2 hours at 30^0C, with the indicated concentration of NaCl. The NaCl was added at zero time (0 and ●) or after 10minutes of incubation (▲), to the soluble enzyme.

Table 9. Effect of sodium chloride on pea DNA methylase activity.

Methylase activity was assayed in the various fractions, pea nuclei, first extracted nuclear pellet and 0.2M NaCl extract, in the presence of varying salt concentration as detailed in section **2B.6.1**.

[NaCl]	Activity in various fractions (cpm)		
	pea nuclei	extracted nuclei (I)	0.2M NaCl extract
0 mM	14724 (100)*	11835 (100)*	1718 (100)*
50mM	11152 (76)*	10051 (85)*	572 (33)*
100mM	7148 (49)*	5808 (49)*	272 (16)*
200mM	2966 (20)*	1345 (11)*	44 (3)*
300mM	868 (6)*	510 (4)*	0 (0)*

( )* = % of activity in the absence of NaCl.

In the presence of salt, the rate is lower by a constant fraction throughout the 4 hour incubation implying either; **(a)** that the methyl transfer reaction itself or enzyme translocation is inhibited by salt; or **(b)** that only about 34% of the nuclear enzyme is initially bound to DNA and this is responsible for the low rate of methylation. In either event the result shows that all enzyme bound to the endogenous DNA at the beginning of the incubation remains bound throughout the 4 hour incubation period. In light of the extraction data (section **2B.3.2.**), about 80% of the enzyme is bound within nuclei and this therefore favours the first proposal above.

The inhibitory effect of NaCl on pea DNA methylase with double and single stranded DNA substrates is shown in Figure 16. 1.18 units of Fraction II was used to methylate 4 x 10µg each of double stranded and heat denatured single stranded pea DNA in four separate reactions with each DNA type in the presence of 0, 50, 100, and 200mM NaCl in the described reaction mixture for soluble pea DNA methylase with a 2 hour incubation at 30^0C. The radioactivity in the ^{3}H-methyl group incorporated into the DNA products were measured. The only difference noticed is in the actual rate of methylation. The same amount of inhibition is shown in both cases at all salt concentrations. This has not been the case with the mouse ascites DNA methylase (Turnbull and Adams, 1976) and other mammalian enzymes (Kalousek and Morris, 1971; Sano et al., 1983; Pfeifer et al., 1986) where the presence of salt is slightly stimulatory with single stranded DNA as substrate at concentrations which inhibit methylase activity with double stranded DNA substrates.

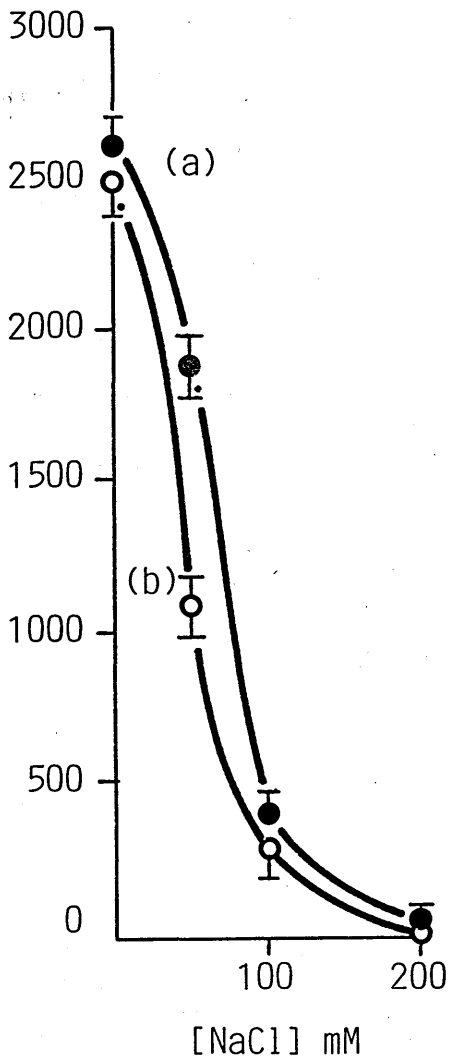


Figure 16. Effect of NaCl on pea DNA methylase activity with double and single stranded DNA substrate.

35µl of Fraction II was incubated for 2 hours with 10µg of pea DNA in an assay for DNA methylase activity in the presence of NaCl at the indicated salt concentrations. **(a)** = DNA methylase activity with double stranded pea DNA. **(b)** = DNA methylase activity with single stranded pea DNA substrate.

3.5.2. Methylation of M.luteus DNA added to isolated pea nuclei.

Table 10 shows a stimulation of DNA methylase activity in pea nuclei which may be due to the methylation of the exogenous M.luteus DNA during the methylation of homologous DNA by bound enzyme in the nuclear preparation. 35µl of pea nuclei were incubated in the usual assay conditions described in section **2B.1.8.** in the presence of 10µg M.luteus DNA. The contribution of added DNA to the methylated DNA product shows the presence of some loosely bound enzyme which binds to any available DNA substrate even in the presence of the much preferred pea DNA substrate. This could be the 20-40% of enzyme which is readily extracted with the first 0.2M NaCl treatments.

3.5.3. Stimulatory effect of a glycerol soluble fraction.

The specific activity of the purest pea DNA methylase fraction is much smaller than that of DNA methylases of animal cells, indicating a possible omission of an essential factor in the enzyme assay. This effect could arise during purification as a result of different fractionation properties of the factors involved.

The stimulation of the rate of methylation of pea nuclei DNA methylase activity in vitro, by the protein fraction in the glycerol rich supernatant from isolated nuclei (Table 11.1) may be similar to the non histone basic protein component of eukaryotic chromatin which has been reported to stimulate the activity of mammalian DNA methylase (Caiafa et al., 1989). Similar stimulatory effect on the in vitro methylase activity of the soluble enzyme was also observed (Table 11.2). Further studies are however required for the identification of the protein and confirmation of this observed stimulatory effect for both bound and soluble enzyme fractions.

Table 10. Methylation of added M.luteus DNA in the presence of bound endogenous pea DNA substrate.

35µl of isolated pea nuclei were incubated for 2 hours in the presence of 10µg M.luteus DNA in the usual assay conditions for nuclear pea DNA methylase as described in section **2B.1.8.**

Incubation Time (hr)	Activity in Fraction (cpm) *	
	Pea nuclei	Pea nuclei + M.luteus DNA
1	962	1209
2	1730	2007
3	2609	2679
4	3236	3571

*** average standard deviation = ± 145**

Table 11.1. The stimulatory effect of a glycerol soluble fraction on pea nuclei DNA methylase activity.

25µl of extracted pea nuclei (I) with a specific activity of 5.5 cpm per µg protein was incubated for 2 hours at 30^0C in the usual assay conditions as described in section **2B.1.8.**, with different amounts of the glycerol soluble fraction. There was no detectable enzyme activity in the glycerol fraction alone in the presence of added pea DNA substrate.

Glycerol Fraction µl	Protein µg/70µl	Activity (cpm)	Specific activity cpm/µg
0	22.7	125	5.5
5	23.4	171	7.2
10	24.1	238	9.4
15	24.8	639	24.0
20	25.5	1464	57.4

Table 11.2. The stimulatory effect of a glycerol soluble fraction on soluble pea DNA methylase activity.

0.725 units of soluble pea DNA methylase were incubated for 2 hours at $30^{0}C$ in the presence of 10µg pea DNA with different amounts of the glycerol fraction in the usual assay conditions as described in section **2B.3.2.**

Glycerol Fraction µl	Protein µg/70µl	Activity (cpm)	Specific activity cpm/µg
0	76	4943	65
5	76.4	6319	83
10	76.8	6782	90
20	77.6	7602	98

Table 12.1. Immunoreaction with mouse ascites DNA methylase antiserum.

6 units of fraction III of pea DNA methylase was incubated for 2 hours under the usual assay conditions with varying amounts of antiserum (no. 401) against mouse ascites DNA methylase (Adams et al.,1986a). The control serum was horse serum.

Control serum (µl)	Mouse methylase antiserum (µl)	Activity (cpm)
-	-	12000
-	1	10756
-	3	9270
-	10	2353
1	-	12302
3	-	11653
10	-	11710

Table12.2. Immunoreaction with mouse ascites DNA methylase antiserum.

Results of incubation of 1.2 units of Fraction II of pea DNA methylase under the usual assay conditions with varying amount of antiserum raised against mouse ascites DNA methylase and horse serum as control.

Control serum (µl)	Mouse methylase antiserum (µl)	Activity (cpm)
-	-	2324
1.0	-	2323
2.5	-	2348
10.0	-	2388
-	1.0	2254
-	2.5	2278
-	10.0	2234

3.6. Immunoreaction with mouse DNA methylase antibody.

3.6.1. Effect on in vitro pea DNA methylase activity.

Purified pea DNA methylase (Fraction III) was assayed in the presence of varying amount of antiserum (no.401) raised against mouse ascites DNA methylase (Adams et al., 1986a). Results are shown in Table 12.1. The inhibitory effect observed, is not due to the presence of other proteins in the serum, as shown by the results with control serum (Table 12.1). The actual significance of this is still to be confirmed with further studies as other batches of antiserum against the same enzyme have failed to inhibit pea DNA methylase activity under identical reaction conditions (Table 12.2).

3.6.2. Immunoblot reaction.

5µg of the protein fractions of purified pea DNA methylase were subjected to electrophoresis in duplicate, on an SDS-polyacrylamide gel, half of which was transferred by Western blotting onto nitrocellulose, (Hybond-C extra, 0.45µm) and incubated with band specific antiserum no. 401. The immune complexes were visualised by ^{125}I-protein A and autoradiography (Towbin et al., 1979) see Figure 17.1b. The indicator for the crossreacting protein complexes, ^{125}I-protein A, showed a nonspecific illumination of all polypeptides present. It might be necessary to incorporate a more specific second antiserum against rabbit serum to highlight specific immunoreaction with antibody present in the first antiserum. Figure 17.1a shows the polypeptide composition of the purified pea DNA methylase fractions on the second half of the gel.

The 160,000 Da polypeptide band (faint) from Mono Q, (Lane 3) and other polypeptides are illuminated which may be the reason for the apparent inhibition of pea DNA methylase activity in vitro by antiserum no.401. It is difficult however to make any conclusions about the recognition of pea DNA methylase polypeptide(s) by the antiserum against mouse ascites DNA methylase for any deduction of any evolutionary similarity or otherwise.

Protein fractions (5µg) obtained from a partial purification of pea DNA methylase on a Heparin Sepharose and an FPLC Superose 6 column (Yesufu et al., 1988) were electrophoretically separated on an SDS polyacrylamide gel in duplicates in the presence of protein markers. One half of the gel was stained with silver nitrate as described in section **2B.1.5.** and shown in Figure 17.2c. The protein bands on the second half was electrophoretically transferred to a nitrocellulose sheet (Hybond-C extra, 0.45µm) and probed as described in section **2B.7.2** with another batch of antiserum against mouse ascites DNA methylase. The gel was dried and the autoradiogram is shown in Figure 17.2d. Crossreactivity studies of pea DNA methylase fractions with this batch of antiserum against mouse ascites DNA methylase, showed the presence of immunoreactive peptides at 40,000 and 80,000d. The Superose 6 fraction had not shown a protein band at 160K corresponding to the calibrated value. Figure 10.2 shows the breakdown of the high molecular weight protein band (Lane 3) with storage at low protein concentration (0.01 mg/ml) and a second protein band at 80,000 in the active fraction from a pea DNA Sepharose column (Lanes 7, 8 and 9).

The crossreacting peptides could therefore be pea DNA methylase or breakdown products of the enzyme or some other protein bands altogether.

Figure 17.1. SDS/PAGE and immunoblot patterns of pea and mouse methylase.

(a). SDS/polyacrylamide gel electrophoresis silver-stain pattern of pea DNA methylase fractions and mouse ascites DNA methylase.
Lane 1 = 0.2M NaCl extract of pea nuclei; Lane 2 = pea DNA methylase from a Heparin Sepharose column; Lane 3 = pea DNA methylase fraction from a Mono Q column; Lane 4 = mouse ascites DNA methylase; Lane 5 = marker proteins (myosin, 200K; α_2-macroglobulin, 170K; phosphorylase b, 95.5K; glutamate dehydrogenase, 55.5K).

(b). Immunoblot pattern, of pea DNA methylase fractions and mouse ascites DNA methylase with antiserum (no. 401) against mouse ascites DNA methylase. The Lanes are the same as in **(a)**

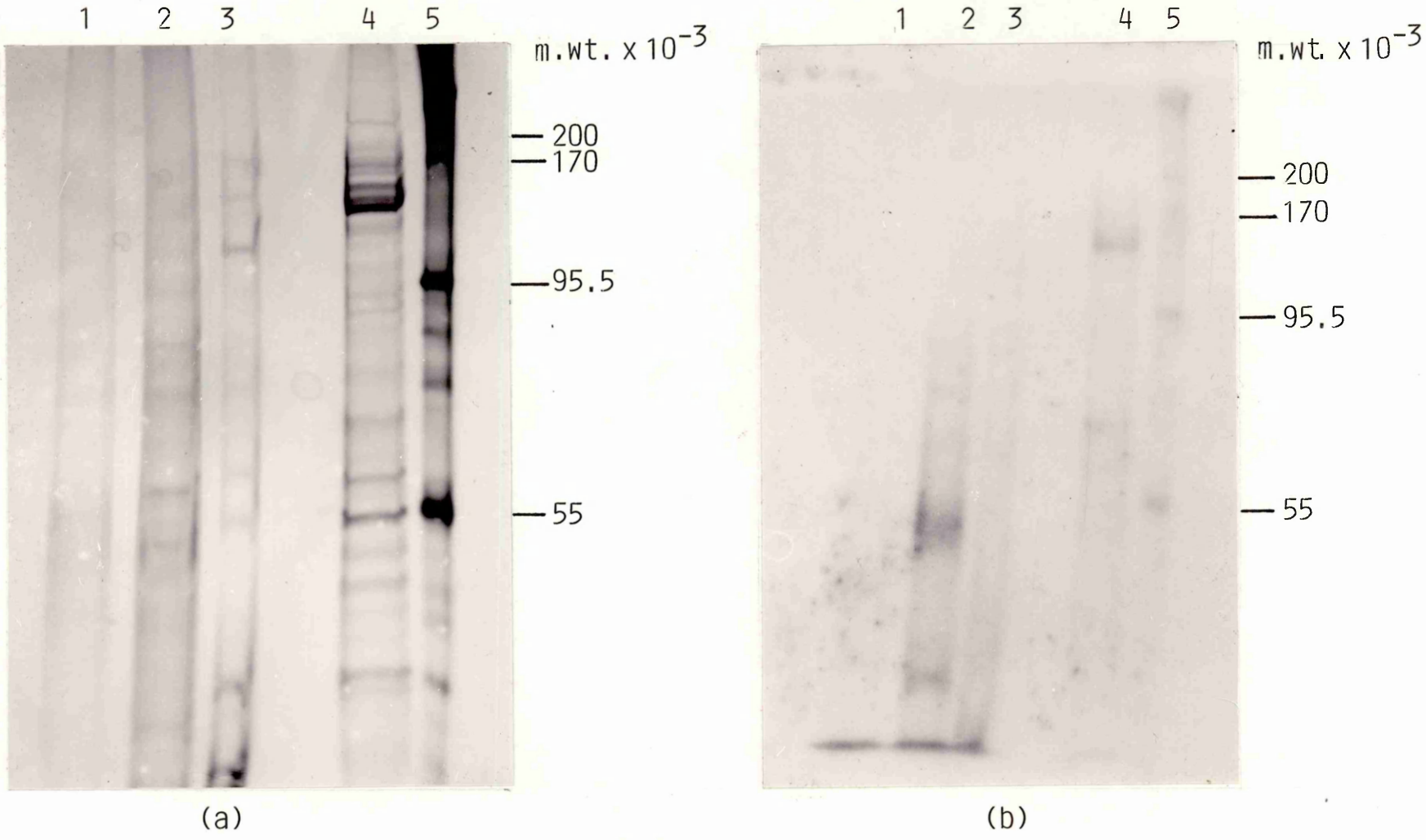
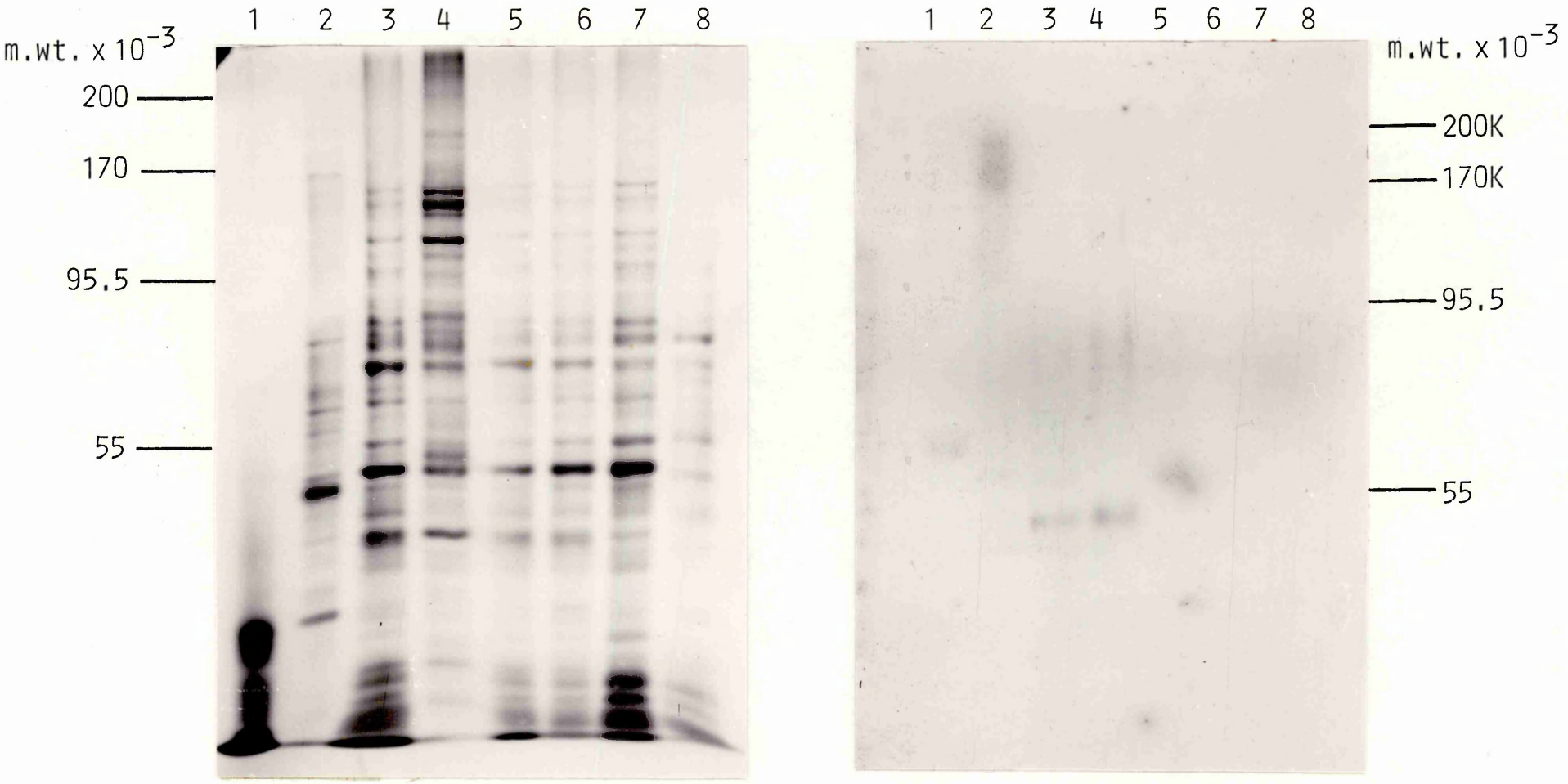

1
2
3
4
5
m.wt. x 10^{-3}
200
170
95.5
55
(a)
1
2
3
4
5
m.wt. x 10^{-3}
200
170
95.5
55
(b)

Figure 17.2. SDS/PAGE and immunoblot pattern of pea and mouse methylase.

(c). Silver-stain pattern of pea DNA methylase fractions from various chromatographic purification steps and mouse DNA methylase.
The estimated position of marker proteins omitted in Lane 1 is shown down the left hand side; Lane 2 = mouse ascites DNA methylase; Lane 3 and 6 = 0.2M NaCl extract; Lane 4 = methylase fraction from a Mono Q column; Lane 5 = excluded fraction from a Mono Q column; Lane 7 = methylase fraction from a Heparin Sepharose column; Lane 8 = methylase fraction from a FPLC Superose 6 column.

(d). Immunoblot pattern of the pea DNA methylase fractions and mouse ascites DNA methylase fraction as in **(c)** with another batch of mouse ascites DNA methylase antiserum. The Lanes are the same as in **(c)**.


1 2 3 4 5 6 7 8
m.wt. x 10^{-3}
200
170
95.5
55
(c)
1 2 3 4 5 6 7 8
m.wt. x 10^{-3}
200K
170K
95.5
55
(d)

3.7. Sequence specificity of pea DNA methylase.

3.7.1. Nearest neighbour analysis.

5µg native pea DNA was labelled by nick translation in four separate reactions each containing a different [α-^{32}P]dNTP as detailed in Methods section. The radioactive pea DNA product was purified by ethanol precipitation (Maniatis et al., 1982) free of unincorporated deoxyribonucleotides, and was used as a substrate for the in vitro methylation with 14.8 units of pea DNA methylase. The methylated DNA product was purified by phenolisation and alkaline hydrolysis and hydrolysed with micrococcal nuclease and spleen phosphodiesterase to deoxyribonucleoside-3'-monophosphates (3'dNMP). The hydrolysed DNA product on separation on an HPLC Mino RPC column, gave a separation profile as shown in Figure 18. The result of the dinucleotide sequence methylation is shown in Table 13. This gives only the relative methylation of cytosine in the dinucleotides in the DNA product. The result shows the methylation of all possible dinucleotides, CpT, CpA, CpC and CpG. The methylation of the first three reflects a possible methylation at CNG sites. To draw such a conclusion that the methylation of the first three dinucleotide sequence is the result of the methylation of the trinucleotide sequence CNG, it would be necessary to show also that the 3' nearest neighbour of T, A, and C is G, in these sequences.

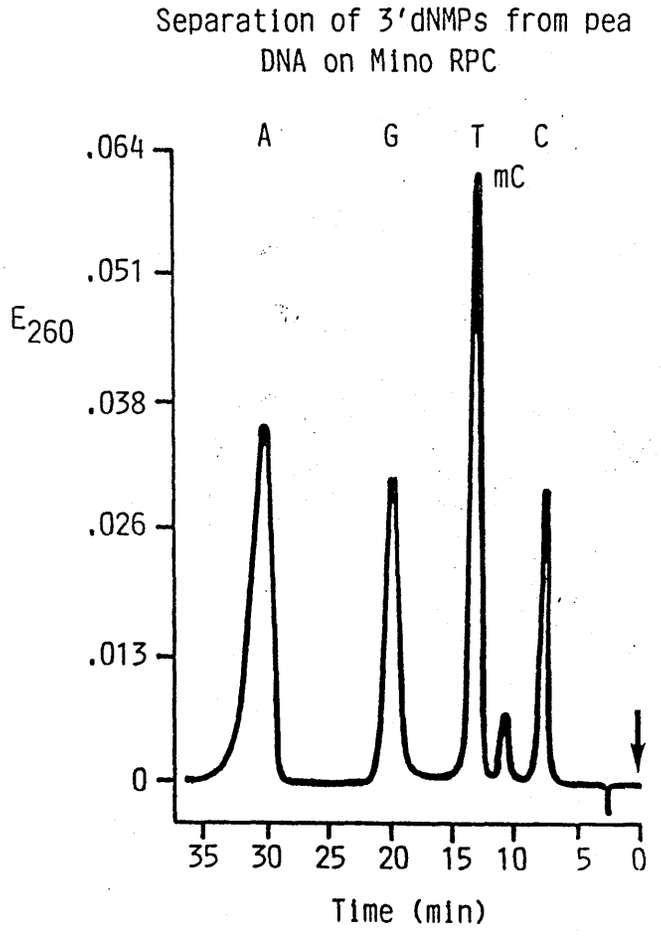


Figure 18. Mino RPC profile of pea DNA deoxynucleoside 3' monophosphates.

Pea DNA was hydrolysed to deoxynucleoside 3' monophosphates as described in Methods section **2B.8.1**. The deoxynucleotides were eluted with tetrabutylammonium phosphate buffer, pH 6.0.The solid line shows absorbance at 260nm. Elution time is given in minutes. 0 = origin; A = deoxyadenosine 3' monophosphate; C = deoxycytidine 3' monophosphate; mC = 5-methyldeoxycytidine 3' monophosphate; G = deoxyguanosine 3' monophosphate and T = deoxythymidine 3' monophosphate.

Table 13. Sequence specificity of methylation of pea DNA methylase.

Pea DNA nick translated with ^{32}P deoxyribonucleoside 5' triphosphates was methylated with 14.8 units of enzyme and the DNA product was hydrolysed to 3' deoxyribonucleotides with micrococcal nuclease and spleen phosphodiesterase as described in Methods section **2B.8.1**. The percentage methylation of cytosine calculated from tritium incorporation, was 4.23% and the distribution between the sequences was as shown below.

^{32}PdNTP	Dinucleotide sequence	% of C methylated in dinucleotide	Max. expected for CNG at 40% G+C and CG deficiency of 75%	
^{32}PdATP	d(C-A)	3.80	20	(19)*
^{32}PdCTP	d(C-C)	0.99	4	(25)*
^{32}PdGTP	d(C-G)	1.50	20	(7.5)*
^{32}PdTTP	d(C-T)	5.30	20	(27)*

( )* = % of CNG methylation obtained from experimental results.

3.7.2. Restriction enzyme studies.

All restriction enzyme digests were carried out in the appropriate buffers. In all cases the DNA fragments were separated by electrophoresis in 1% agarose gels in the presence of ethidium bromide and photographed with a Polaroid camera, as detailed in Methods section **2B.8.2.**

(a). 2 x 1µg of plasmid DNA pVHCI (M. Bryans, 1989) were methylated with 1.9 units pea DNA methylase as described in Methods section **8.2.1** and were digested with HpaII and MspI in two separate reactions. The fluorograph of methylated pVHCI as shown in Figure 19.1b, indicates the incorporation of ^{3}H-methyl group during methylation even though there was no difference observed with the restriction pattern of both methylated and unmethylated plasmid DNA. (Figure 19.1a)

(b) The restriction patterns of methylated ColEI DNA as described in Methods section **2B.8.2.2** are shown in Figure 19.2a. However there was no difference observed in the restriction pattern of unmethylated (19.2b) and methylated ColEI DNA. The fluorograph showed no indication of incorporation of ^{3}H-methyl group from AdoMet during methylation of the DNA.

(c) 4µg of ϕX174 RF DNA was methylated with 10.3 units of pea DNA methylase as described in Methods section **2B.8.2.3.** 4 x 800ng of the methylated DNA was digested in four different reactions with the isoschizomers, HpaII/MspI and EcoRII/BstNI as shown in Figure 19.3. The HpaII and MspI recognise the sequence CCGG, MspI will cleave when the internal C is methylated and HpaII will not. EcoRII and BstNI recognise the sequence CCA/TGG and BstN1 will cleave when the internal C in CCA/TGG is methylated but EcoRII will not cleave.

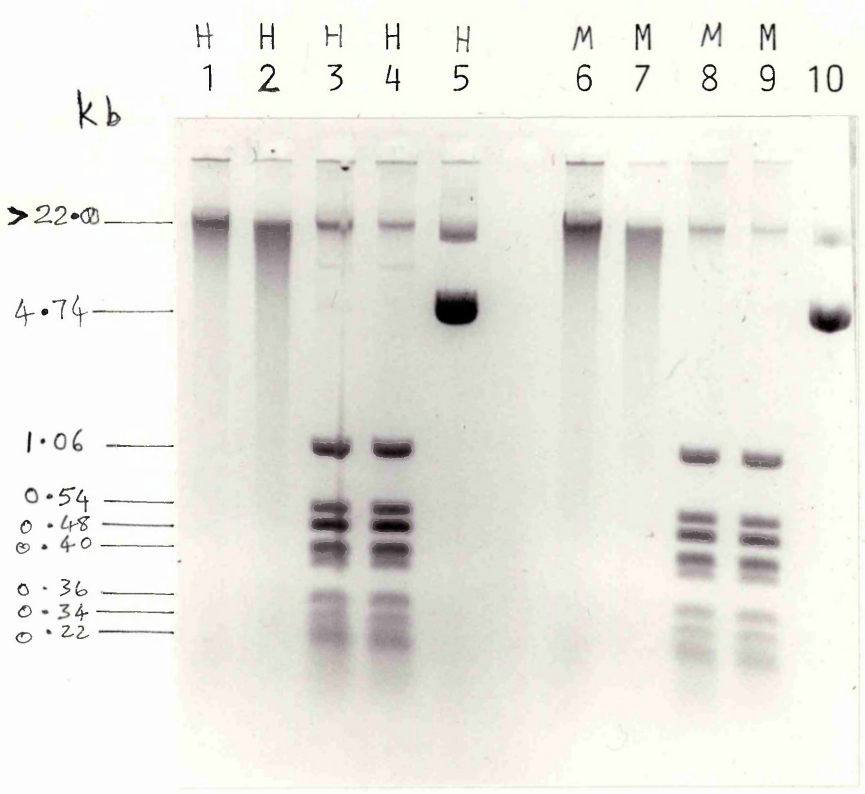
Figure 19.1. Sequence specificity of methylation of pea DNA methylase : study with pVHC1.

Methylated pVHC1 DNA was digested with the indicated enzymes as in Methods section **2B.8.2.**

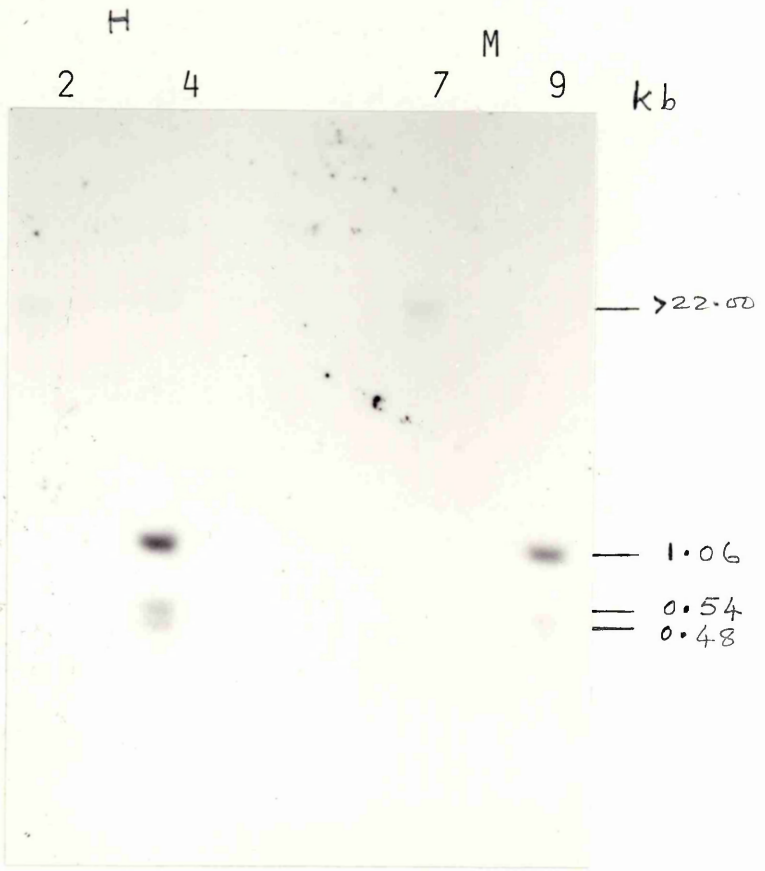
(a) = HpaII and MspI restriction pattern of plasmid DNA, pVHC1 and pea DNA, on a 1% ethidium bromide agarose gel.

(b) = Fluorograph of restriction fragments, showing incorporation of ^{3}H-methyl group from AdoMet into plasmid and pea DNA.

Lanes 1, 6 = unmethylated pea DNA; Lanes 2, 7 = methylated pea DNA; Lanes 3, 8 = unmethylated pVHC1; Lanes 4, 9 = methylated pVHC1; Lanes 5, 10 = plasmid DNA. H = HpaII; M = MspI.


H H H H H M M M M
1 2 3 4 5 6 7 8 9 10
kb
>22.00
4.74
1.06
0.54
0.48
0.40
0.36
0.34
0.22

(a)


H M
2 4 7 9
kb
>22.00
1.06
0.54
0.48

(b)

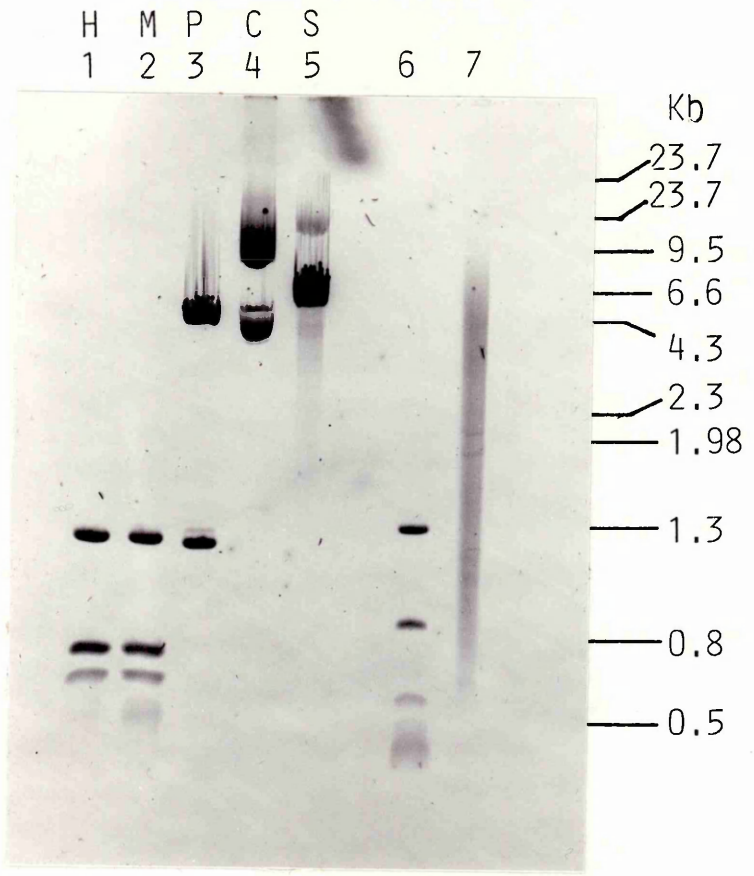
Figure 19.2. Sequence specificity of methylation of pea DNA methylase : study with ColEI

Methylated ColEI DNA was digested with the indicated restriction enzymes as described in Methods section **2B.8.2.2.**

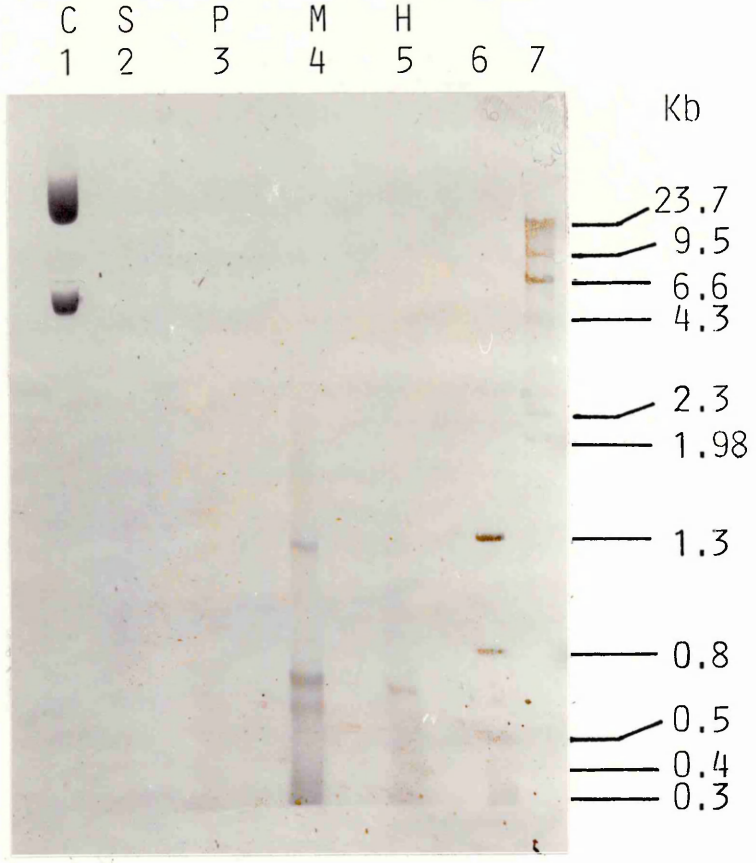
(a) Restriction pattern of unmethylated ColEI. Lanes 1, 2, 3, and 5 are unmethylated ColEI DNA digested with the indicated enzymes. Lane 4 = unmethylated undigested ColEI DNA; Lanes 6 = M13 mp18 $_{\lambda}^{Hin\,f1}$ DNA size markers; Lane 7 = $\lambda H_{\lambda}^{ind\ III}$ DNA size markers.

(b) Restriction pattern of methylated plasmid DNA, ColEI. Lanes 2, 3, 4 and 5 are methylated ColEI DNA digested with the indicated enzymes. Lane 1 = undigested methylated ColEI DNA; Lane 6 = M13 mp18 $_{\lambda}^{Hin\,f1}$ DNA size markers; Lane 7 = $\lambda H_{\lambda}^{ind\ III}$ DNA size markers.

H = HpaII; M = MspI; P = PstI; S = SstII; C = ColEI DNA control.


H M P C S
1 2 3 4 5 6 7
Kb
23.7
23.7
9.5
6.6
4.3
2.3
1.98
1.3
0.8
0.5

(a)


C S P M H
1 2 3 4 5 6 7
Kb
23.7
9.5
6.6
4.3
2.3
1.98
1.3
0.8
0.5
0.4
0.3

(b)

In all three cases the analysis of the methylation of individual sites (CCGG, HpaII/MspI; CCA/TGG, EcoRII/BstNI; CTGCAG, PstI and CCGCGG, SstII) by pea DNA methylase in these DNA substrates could not be detected. Hence conclusions on the trinucleotide sequence, CNG methylation shown in plant DNA in vivo could not be unambiguously attributed to the purified pea DNA methylase fractions. The low percentage methylation (12%, ϕX174 RF DNA; 8.6%, ColEI; and 4.46%, pVHC1) in all cases, does not enhance the chance of methylation of the sites being monitored. These results are discussed in more detail in section **4.7.4.**

3.8. Identification of protein bands in the purified fractions.

35µl samples of the purified fractions shown in Table 6 and 20µl of mouse ascites DNA methylase were electrophoresed on a 5% non-denaturing polyacrylamide gel. The protein profile of the stained half of the gel in Coomasie brilliant blue R-250 is shown in Figure 20a. The second half was subjected to in situ assay for DNA methylase activity as described in section **2B.9.2.** and fluorographed. The result shown in Figure 20b showed evidence of tritium incorporation only in the lane for mouse ascites DNA methylase. This may be a result of the higher specific activity of the mouse ascites enzyme Figure 20a showed the presence of protein in the lanes and the exposure times of 10 and 20 days to a highly sensitive film such as Hyper Film-MP (Amersham) was considered adequate enough to monitor the presence of any tritium incorporation. It would be necessary to obtain a more active pea enzyme preparation for any conclusive identification of the protein bands, by in situ assay for DNA methylase activity.

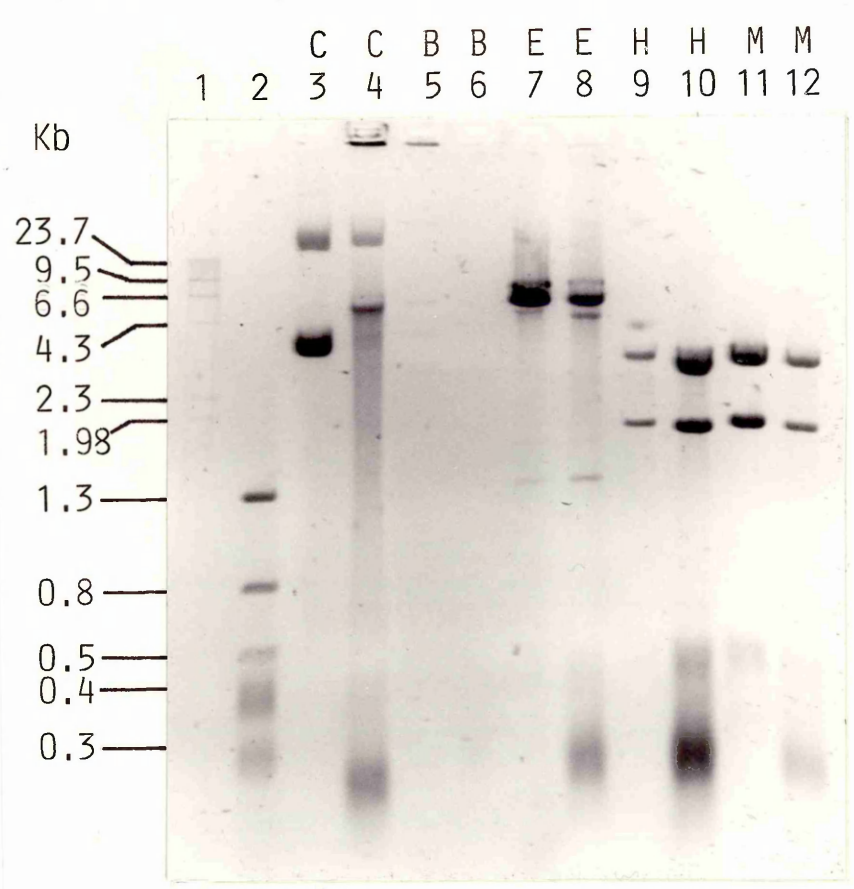


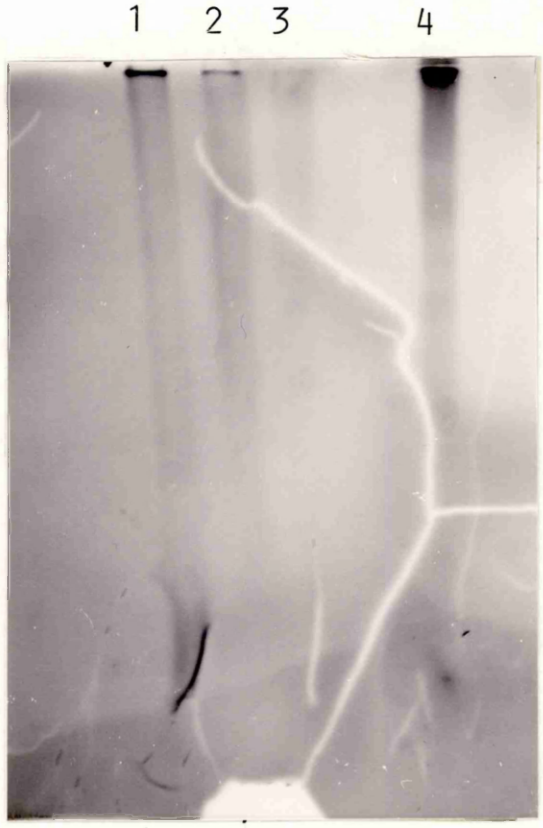
Figure 19.3. Sequence specificity of methylation of pea DNA methylase : study with ϕX174 RF DNA.

Restriction pattern of methylated and unmethylated ϕX174 RF DNA. Methylated ϕX174 RF DNA was obtained as described in Methods section **2B.5.6.3** and digested with the indicated enzymes (Methods section **2B.8.2**). Lane 1= λ$H_{ind\ III}$ DNA size markers; Lane 2 = M13 mp18$_{Hinf\ I}$ DNA size markers; Lane 3= unmethylated ϕX174 RF DNA control. Lane 4 = methylated ϕX174 RF DNA control. Lanes 5, 7, 9 and 11 = restriction fragments of unmethylated ϕX174 RF DNA. Lanes 6, 8, 10 and 12 = restriction fragments of methylated ϕX174 RF DNA. B = BstNI; E = EcoRII; H = Hpa II; M = MspI.

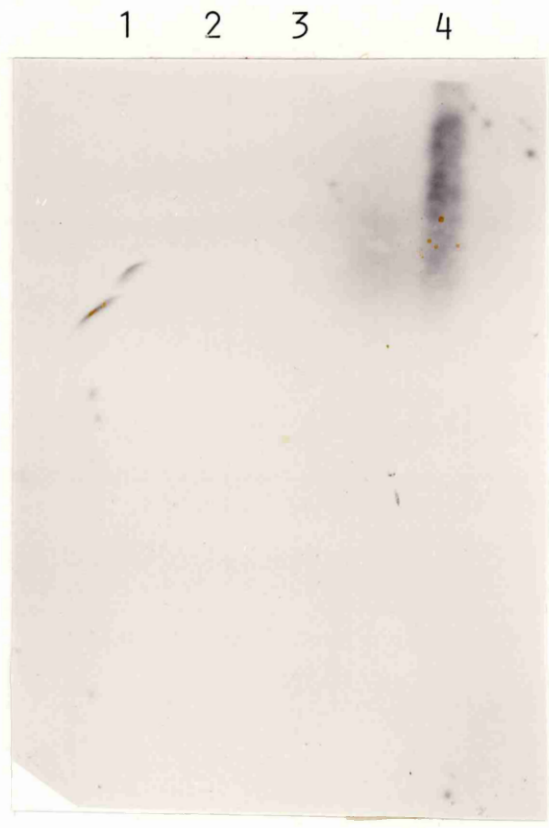
Figure 20. In situ assay for pea DNA methylase activity in protein bands from purified fractions.

(a) Pattern of protein separation of fractions of soluble pea DNA methylase on electrophoresis on a 5% non-denaturing polyacrylamide gel for 26 hours, 120V, at 4^0C.
Lane 1 = 0.2M NaCl extract. Lane 2 = Heparin Sepharose fraction. Lane 3 = Fraction from a Mono Q column. Lane 4 = mouse ascites DNA methylase fraction.

(b) Fluorograph of the second half of the gel after an overnight incubation at 30^0C in buffer M^+ and 30µCi AdoMet (TRK 581, 60Ci/mmol). The visible lane showing tritium incorporation is mouse ascites DNA methylase (Lane 4) as in Figure 20a.



(a)



(b)

CHAPTER FOUR

DISCUSSION

4.1. General.

Characterisation of plant DNA methyltransferases have lagged behind characterisation of DNA methylases from animal cells. DNA methylase activity was reported in nuclei from pea seedlings by Kalousek and Morris as far back as 1969 but no further purification has since been achieved with the pea enzyme. Two types of DNA methylase activities in plants had been postulated to explain the occurence of 5-methylcytosines in plant DNA in the sequences CG and CNG. Preliminary work in our laboratory (Adams, personal communication), have shown that the pea enzyme was active in similar reaction conditions to that for mouse ascites DNA methylase. The studies reported here, with purified pea DNA methylase indicate some similarity between this enzyme and the mammalian enzymes in its location and ability to perform both the maintenance and de novo methylation activity in vitro.

4.1.1. Cellular distribution.

The enzyme appears to be located entirely within the nuclear fraction, from pea seedling homogenate, there being no evidence of enzymic activity associated with chloroplasts or with plastids isolated from etiolated seedlings. The absence of enzymic activity from the chloroplasts (Table 2), is consistent with the finding that these organelles contain no methylcytosine (van Grisven and Kool, 1988; Kirk and Tilney-Bassett, 1978). There has been some controversy recently over whether or not plastid DNA is methylated in dark grown plants and in differentiated plastids (chromoplasts and amyloplasts).

The results in Table 2 show no enzyme activity in plastids from dark grown seedlings and therefore do not support the original observation made by Ngernprasirtsiri et al. (1988).

The nuclear localisation of DNA methylase appears to be universal. The first report on DNA methylase activity by Burdon and his group, in 1967 was made from a nuclear enriched preparation from mouse cells. The cellular distribution was confirmed by Sheid and his group who prepared the enzyme from chromatin from rat liver cells (Sheid et al., 1968). The same situation has been observed with other mammalian enzymes studied. Except for calf thymus cells, which have a greater proportion of the DNA methylase in the cytoplasm, the enzyme in all cases, is present in nuclear preparations (Kalousek and Morris, 1968; Sheid et al., 1968; Turnbull and Adams, 1976; Roy and Weissbach, 1983).

4.1.2. **Nuclear methylation**.

Pea DNA methylase is able to methylate endogenous DNA present in the isolated pea nuclei in the absence of exogenous DNA. The endogenous methylation of DNA present in the nuclear preparation from pea seedling, has also been observed for nuclear preparations from mammalian cells, though to a lesser extent (Kalousek and Morris, 1968; Burdon and Adams, 1969). In particular, it has been reported for nuclear DNA methylase from rat liver cells, rat spleen cells (Kalousek and Morris, 1969), and mouse ascites cells (Turnbull and Adams, 1976). Methylation in the absence of added DNA substrate is evidence for under methylated recognition sites and is evidence for a delayed methylation in pea nuclei, similar to that occuring in nuclear DNA from mammalian cells (Burdon and Adams, 1969).

The average DNA : protein ratio in the preparation of pea nuclei is similar to the values reported (Setlow, 1976). The protein assay by the dye binding method of Bradford (1955), is based on the shifting of the absorbance maximum of the dye, Coomassie brilliant blue G from 465nm to 595nm on binding to a protein. The method has the advantage of being simple and less susceptible to interference by other substances, than other methods of protein estimation, such as the Folin phenol method, a modified version of the method of Lowry et al. (1951).

4.1.3. Distribution of DNA methylase activity.

The tissue showing the greatest activity of DNA methylase is the rapidly growing shoot tip (Table 4). This is as expected for an activity whose presumed substrate (hemimethylated DNA) is formed largely in the S phase of the cell cycle (Adams and Hogarth, 1973).

The greater activity of DNA methylase from 5 day pea seedling shoot tips, is in agreement with the observed relative activity of this enzyme from various animal tissues at different stages of development. There is more enzyme activity in growing cells than in the corresponding non growing cells (Morris and Pih, 1971; Turnbull and Adams, 1976; Simon et al., 1978). The increase in enzyme activity has been associated with an increase in the synthesis of novel proteins and not as a result of cellular mobilisation of an inactive precursor protein. It has been shown that DNA methylase activity in actively growing cells could be inhibited by actinomycin D and p-fluoro-phenylalaline, suggesting that the increase in activity may be dependent on the synthesis of both RNA and new protein (Morris and Pih, 1971). The synthesis of proteins and DNA (a result of increase in cell number) in active growing seedlings has been shown to attain its peak on the 5th day post imbibition of growth.

If DNA methylase activity depends on de novo protein synthesis, then the incidence of peak activity from 5 day old actively growing shoot tips is to be expected.

4.1.4. Assay for DNA methylase.

The usual assay mixtures for DNA methylases are incubated for 1 or 2 hours at the appropriate temperatures of optimum activity. The assay for eukaryotic DNA methylase activity does not include the use of cofactors such as Mg^{2+} and ATP. This has been discussed in section **1.4.3.** S-adenosyl-L-methionine can serve as a methyl donor in enzymic transmethylation reactions not only to DNA but also RNA, carbohydrates and proteins and small molecules. The inclusion of phenol, ethanol precipitation and alkali treatment steps in the assay for DNA methylase activity was found sufficient to remove acid precipitable products other than DNA (Kalousek and Morris,1969). This takes care of the ability of of the pea DNA methylase preparations, like that of the mammalian enzymes, to transfer methyl groups from S-adenosyl-L-methionine to RNA and protein, present in the enzyme preparations (Paik et al.,1968; Kalousek and Morris, 1968).

In plants, deproteinisation has the additional effect of removing some green material that might be present in the nuclear preparations in particular, from interfering with scintillation counting of the tritiated DNA product. The concentration of AdoMet (3.3µM) used in the assay is less than the Km value obtained for the mouse and wheat enzymes (Adams and Hogarth, 1973; Theiss et al., 1987). This was for economic reasons. The presence of glycerol in the buffer helps to increase the stability of the enzyme. For efficiency of recovery, a carrier DNA is incorporated. The effect of dithiothreitol is to prevent

oxidation of the enzyme and cross linking of SH groups. The specific activity of DNA methylase depends on the DNA substrate used and the value for the mammalian enzyme falls into the range of 600-70,000 units/mg, depending on the DNA substrate, being highest for hemimethylated DNA. This argument is developed further elsewhere (section **4.4.2**).

4.1.5. Identification of methylated base in the DNA product.

The relative amount of the five bases in pea DNA was determined by separation of the formic acid hydrolysate by high performance liquid chromatography (HPLC; Figure 6). A 26% methylcytosine content was obtained. This result is close to the value (23%) obtained by Wagner and Capesius (1980) and Gruenbaum et al. (1981). Among the methods used for the determination of methylcytosine content in DNA, such as paper and thin layer chromatography, HPLC in an ion exchange column, (Aminex A6) gives the better result because of the higher capacity of the column, better separation of the bases and easier quantification. It is faster and more accurate and only small amounts of DNA (5-20µg) are required for accurate estimation of the molar proportion of minor bases such as methylcytosine. Formic acid hydrolysis of pea DNA did not degrade methylcytosine to thymine and the only peak with tritiated methyl group incorporation was 5-methylcytosine.

4.1.6. Stability of enzyme activity with storage.

Isolated and purified pea DNA methylase is highly unstable in terms of the polypeptide composition, depending on the protein concentration, even in 50% glycerol containing buffer at -20^{0}C. After a week of storage at -20^{0}C the soluble enzyme looses 50% of its activity and after 3 weeks over 90% of the activity is lost (Figure 8).

The enzyme activity of the bound form (pea nuclei), is more stable under the same storage conditions. Over 60% of the enzyme activity is present after 3 weeks of storage. Nevertheless the stability of the pea enzyme seems to be greater than the stability of the mouse ascites DNA methylase (Adams et al., 1986a).

4.2. Extraction of pea DNA methylase.

In 1971, Morris and Pih described a general method for extracting soluble DNA methylase from nuclear enriched cellular preparations with NaCl containing buffers. The interaction between most nuclear proteins and DNA is thought to be electrovalent, hence the presence of ions would help dissociate these proteins. This seems to be the case with DNA methylases in animal and plant cells. Some of the pea enzyme was soluble in 0.2M NaCl solution in buffer M^+ (Figure 7). However much of the pea shoot DNA methylase is firmly bound within the cell nucleus and only a small fraction can be extracted with 0.2M NaCl containing buffers. The proportion of enzyme readily soluble in 0.2M NaCl containing buffer is much smaller in pea nuclei than in nuclear preparations obtained from animal cells such as mouse ascites cells (Tables 5.1 and 5.2). A large proportion of the enzyme is present within the chromatin in a salt resistant complex. Only following brief digestion with nuclease could the bulk of the enzyme be extracted. This could mean that most of the enzyme activity is present in the linker region of chromatin but some activity is left even in the matrix bound or nucleosome core fraction left after 0.2M NaCl extraction of nuclease digested nuclei (Tables 5.1 and 5.2). Higher salt concentrations did not improve the yield.

In vertebrates most of the DNA methylase is readily solubilised with only a small fraction remaining tightly bound.

This difference may reflect the rate of growth of the cells used, or the higher proportion of cytosines methylated in plant DNA. Thus, the methylation of 30% of the cytosines in pea DNA may require a greater fraction of the enzyme to be associated with the replicating DNA than is the case when only about 5% of the cytosines are to be methylated in animal cells DNA.

The presence of a salt resistant, matrix bound enzyme complex has also been reported for mouse ascites DNA methylase (Qureshi et al., 1982). This has been attributed to an association of the DNA methylase with the replication complex of DNA, RNA and protein, involved in the maintenance methylation (Adams and Burdon, 1983).

I am confident of the absence of pea DNA from the 0.2M NaCl nuclear extracts since DNA methylase activity in the extracts depends entirely on the addition of exogenous DNA substrate. The absence of DNA from the soluble enzyme preparation is important as such DNA might interfere with the subsequent assays.

4.3. Purification of pea DNA methylase.

DNA methylase has been purified from a number of mammalian cells to give preparations with high specific activity but one that varies depending on the DNA substrate used. Turnbull and Adams (1976), extracted DNA methylase from Krebs II mouse ascites tumour cell nuclei using 0.4M NaCl. They purified the extract using a 30-60% ammonium sulphate cut, gel filtration, phosphocellulose and DEAE cellulose chromatographic steps. Later improvement (Adams et al., 1986a) gave an enzyme preparation of a specific activity of 11,500. Similar chromatographic steps were tried in the purification of pea DNA methylase.

Losses with ammonium sulphate fractionation and irreversible binding to phosphocellulose led to the adoption of the present steps (Table 6). The use of Heparin Sepharose and an FPLC Mono Q columns for the purification of pea DNA methylase (section **2B.4.1.**) gave the best purification in terms of enzyme activity and the disappearance of other protein bands. At each step in the purification, only one peak of enzyme activity was obtained and so far only one enzyme activity has been obtained in the purification of mammalian DNA methylases. The purification relative to the activity in the whole cell homogenate is 19,000. The specific activity of the nuclear extract is higher than the comparable value for the vertebrate enzyme (Adams et al., 1986a), yet only a modest purification relative to nuclear extract was obtained and the final specific activity obtained was low compared to the purified mammalian enzymes (11,500 units/mg protein, for mouse ascites DNA methylase and only 1738 units/mg for pea DNA methylase). This might be responsible for the low in vitro rate of de novo methylation observed for plasmid and phage DNA substrates (Figure 13).

The use of ammonium sulphate fractionation was abandoned because the protein undergoes denaturation on stirring which is an essential step required to dissolve the ammonium sulphate crystals.

4.4. Characteristics of pea DNA methylase.

4.4.1. Molecular weight.

The final preparation showed one major band of 160,000 daltons on a silver-stained SDS polyacrylamide gels (Figures 10.1 and 10.2). This corresponded to the native molecular mass of the enzyme as estimated by gel filtration on a FPLC Superose 6 column.

Fractionation by FPLC uses the same principle as standard gel filtration chromatography but one advantage of this system is the speed of operation (because of the high pressure of the column). The FPLC Superose 6 column also gives better reproducibility and resolution as a result of the fine resins and control systems employed. This provides strong evidence that the native molecular mass of pea DNA methylase is about 160,000. The enzyme is quite unstable even in the presence of PMSF and breaks down on storage to lower molecular weight polypeptides which still retain activity. This may explain the molecular mass of 50,000 obtained for the enzyme from wheat germ (Theiss et al., 1987). This assumption may not be entirely valid as a second explanation of the anomalous molecular weight, could be due to the unusual migration of certain amino acid sequences on SDS-polyacrylamide (Kaufman et al., 1984; Kleinschmidt et al., 1986). However DNA methylases purified from mammalian cells are also known to be highly susceptible to proteolytic degradation (Pfeifer and Drahovsky, 1986; Adams et al., 1986a), so this might be a more plausible explanation for the variations in molecular mass of the plant enzymes. The similarity between the molecular mass of the wheat germ DNA methylase to that reported for the enzyme from the green alga Chlamydomonas reinhardii (Sano and Sager, 1980), was thought to depict a significant difference between DNA methylases from mammalian and plant cells (Theiss et al., 1987). However Sano et al. (1981) have also reported the presence of a second high molecular mass (200,000) DNA methylase from the same organism and proposed that the earlier small molecular mass enzyme reported, could well be a subunit of the larger enzyme. DNA methylase purified from pea seedlings is of high molecular weight.

Since it has also been shown that this protein is highly unstable in very dilute solutions (0.05mg/ml) of the enzyme, this molecular mass of the pea enzyme must be the correct value.

4.4.2. Substrate specificity.

Pea DNA methylase was assayed with eukaryotic, prokaryotic, plasmid and phage DNA from various sources. The enzyme is active on all DNA types, whether double or single stranded (Figure 14). The ability to methylate single stranded DNA substrate is similar to the observation made for the animal enzymes. The difference between the pea enzyme and the mammalian enzymes is the finding that native homologous DNA is the best natural substrate found for the pea enzyme. The preference for double stranded pea DNA, differentiates the pea enzyme from the mammalian DNA methylases. This is also the case with the enzyme from wheat embryo (Theiss et al., 1987). Native wheat DNA was the best substrate for the wheat enzyme. Native mouse DNA is a poor substrate for the mouse DNA methylase, but its effectiveness is improved when the DNA is isolated from rapidly dividing cells or cells temporarilly deprived of methionine (Turnbull and Adams, 1976). Microbial DNAs, are better methyl acceptors than vertebrate DNA or homologous DNA. This has been attributed to the high CG content (72%) of M.luteus DNA. It was suggested that the different rates observed with different DNA substrates were proportional to the total available sites of methylation, that is to the proportion of CG dinucleotide sites in the DNA (Gruenbaum et al., 1981). The inability of homologous DNAs to be good substrates for the vertebrate enzyme may imply a saturation of available sites. A similar explanation may account for the difference in methylase activity of homologous DNA in nuclear preparations from 5 to 11 day old pea seedling shoot tips (Table 8).

The deficiency in methylase activity in older nuclei may not be due solely to a deficiency of the enzyme, since the purified enzyme also showed greater methyl group incorporation with DNA from 5 day old pea shoots than with DNA from older seedlings. The purified vertebrate enzymes prefer single stranded DNA as substrate regardless of its source (Drahovsky and Morris, 1971; Sano et al., 1983; Roy and Weissbach, 1975; Turnbull and Adams, 1976). Heat denatured DNAs are better methyl acceptors than native DNAs even under assay conditions containing low salt concentrations (Adams et al., 1986a) and are much better in the presence of 100mM NaCl. The preferred substrates however are hememethylated DNAs (Roy and Weissbach, 1975; Pfeifer et al. 1983; Spiess et al.,1988) and the same is true for the pea enzyme. Pea DNA which was shown to be the preferred substrate for the pea enzyme, is believed to be rich in hemi- methylated sites.

Although hemimethylated 5-methylcytosine rich DNAs (pea DNA) is a much better substrate than 5-methylcytosine deficient DNAs (M.luteus DNA) the pea enzyme is able to bind to M.luteus DNA even in the presence of pea DNA. This shows that the enzyme binds to DNA irrespective of its suitability as a substrate, which is similar to the situation observed for the mammalian enzyme (Wang et al., 1984).

4.5. Mechanism of action of pea DNA methylase.

4.5.1. General.

Apart from the difference in the molecular mass of the purified wheat embryo DNA methylase, other aspects of the in vitro methylation activity are closely comparable to those of the mammalian DNA methylases.

This include specificity for AdoMet as methyl donor, lack of cofactor or metal ion requirements and the capacity for methylation over a long period of time (Sano et al., 1983; Pfeifer, 1983; Roy and Weissbach, 1975; Turnbull and Adams, 1976). The product is 5-methylcytosine (Figure 3).

4.5.2. Salt inhibition studies.

Although the initial association of the pea enzyme with DNA is sensitive to inhibition by low levels of NaCl, a salt resistant complex quickly forms at 30^0C and is highly stable, in terms of resistance to dissociation by salt (Figure 15). This may be analogous to the tightly bound nuclear enzyme which remains behind even in the partially digested chromatin. Salt is thought to exert a stabilising effect upon the helical configuration of DNA. Drahovsky and Morris (1971), suggested that some form of transition from the helical to a single-stranded-like structure, was a prerequisite for the methylation of helical DNA and salt by stabilising the helix, could prevent this transition from occurring which would result in inhibition of binding of methylase to double helical DNA. The strong binding on preincubation at 30^0C may be a result of temperature enhanced uncoiling of double stranded DNA. Drahovsky and Morris (1971) proposed a temperature dependent binding of DNA methylase to DNA, after which the enzyme moves along the double strand during its catalytic action. The results with the mouse ascites enzyme did not agree with this mechanism (Turnbull and Adams, 1976). The results with pea DNA methylase seem to conform with this hypothesis; the binding of the enzyme to double stranded DNA is inhibited by NaCl, whereas once the binding has taken place in the absence of salt, methylation can proceed even in the presence of salt, though at a reduced rate (Figure 11a).

This complex formation appears to involve the binding of more than one enzyme molecule to one molecule of substrate DNA as deduced from the non linear enzyme concentration curve (Figure11d). Thus with excess DNA the enzyme shows co-operative kinetics, but this is not obvious when the DNA concentration is limiting, when a linear response to enzyme concentration is observed. The complex once formed, is long lived and does not dissociate during a 4 hour incubation. NaCl, as well as interfering with the initial binding of the enzyme to DNA, also has an effect on the methylation reaction, either by slowing the rate of movement of the enzyme along the DNA or by interfering with the actual transfer of the methyl group.

The different effect of salt upon the methylation of single and double stranded DNA substrate observed for mammalian enzymes (Drahovsky and Morris, 1971; Simon et al., 1978; Turnbull and Adams, 1976; Adams et al., 1986a) was not observed with pea DNA methylase. For the mammalian enzyme, methylation of double stranded DNA substrate is inhibited by low salt concentrations and the same salt concentrations stimulated methylation of single stranded DNA substrates. The optimal salt concentration is the same for both double and single stranded DNA substrate with the pea enzyme (Figure 16). This difference between the pea enzyme and the mammalian enzyme may also explain why single stranded DNAs are poorer substrates for the pea enzyme in spite of the easier binding facilities they possess (Drahovsky and Morris, 1971). Drahovsky and Morris (1971) have shown that this initial unwinding of helical DNA was only transient since they were able to recover double stranded methylated DNA product. The inability of single stranded DNA to reanneal in the reaction, may affect cooperative binding which might be a prerequisite for stabilising the process of processive methylation seen with double stranded DNA.

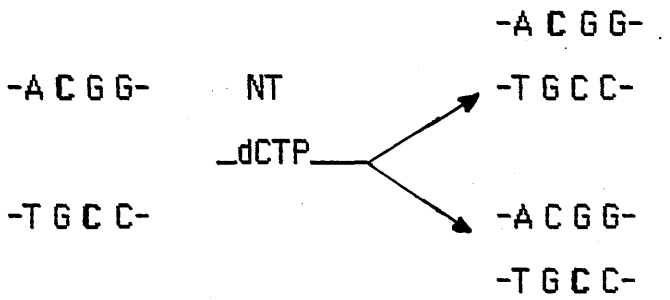
This would explain the fall in rate and amount of actual incorporation when denatured DNA is used as substrate for pea DNA methylase.

4.5.3. Optimum assay condition for pea DNA methylation.

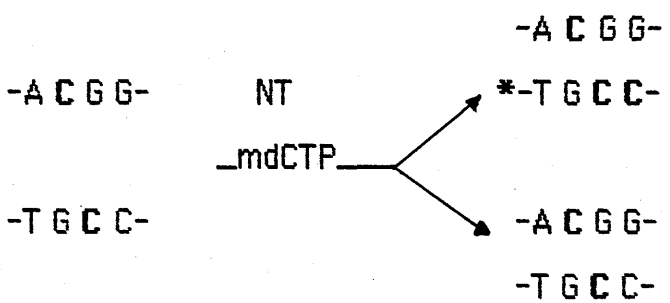
The standard assay reaction using crude enzyme preparations is linear for 4 hours (Figure 11a) and then continues for an additional one hour or more at a reduced rate. The purified enzyme, even at limiting DNA concentrations, is capable of methylation for many hours, the reaction remaining linear for as long as 12 hours, and continuing for several hours more, at a slowly declining rate. This is dependent on the enzyme concentration, and is probably a reflection of the stability of the enzyme (Figure 13). Thus cessation of enzyme activity has been attributed to loss of enzyme activity rather than saturation of available sites on the DNA substrate.

The mammalian enzyme displays an initial rate depending on the DNA substrate used which determines the total amount of available sites. The pea enzyme also displays a reaction rate depending on the DNA substrate (Figure 14a). The enzyme activity is dependent on the availability of hemimethylated sites in the DNA substrate as shown in the preferential methylation of hemimethylated M.luteus DNA and calf thymus DNA (Figure 14b). With calf thymus DNA the only difference is the slight increase in the number of hemimethylated sites present by the replacement of cytosine with methylcytosine from mdCTP. This conclusion is also supported by the result obtained for calf thymus DNA nick translated with dCTP. This reduces the proportion of 5-methylcytosine present in the DNA as mCNG (A) but increases the amount of hemimethylated CG sites as shown in page150. 5-methylated cytosine is shown in bold type.

A.



B.



The results for the nearest neighbour analysis (Table 13) show that pea DNA methylase has a strong preference for CNG sequence methylation hence reduction in the availability of these sites, makes the DNA a poor substrate. Nick translation with mdCTP, however increases the number of mCNG (B*) and these would likely be present as hemimethylated sites and thus act as good substrate for pea DNA methylase.

Although pea DNA methylase prefers hemimethylated DNA substrates, the enzyme is able to bind any available DNA substrate even in the absence of methylatable sites (e.g M.luteus DNA). This has also been observed for the mammalian DNA methylases.

They exhibited non preferential binding to any available DNA substrate as shown by the inhibition of enzyme activity in the presence of competing concentrations of a poor DNA substrate (Wang et al., 1983).

4.5.4. Stimulatory effect of a glycerol soluble fraction.

The duplex DNA in chromatin on which the pea DNA methylase will act in vivo, is complexed with histones and other proteins. These proteins may affect methylation in a specific or non specific way and might thus limit or stimulate the rate of in vivo methylation. The glycerol soluble fraction is the supernatant obtained from isolated pea nuclei stored at $-20^{0}C$, in 50% glycerol containing buffer M^{+}. The stimulatory effect of the glycerol soluble fraction, on pea DNA methylase activity (in both the extracted nuclei fraction II, and the soluble extract with pea DNA substrate) may be an illustration of such an effector protein. The stimulation of enzyme activity in stored extracted nuclei is much higher than the stimulatory effect on the enzyme activity, with purified DNA substrate (Tables 11.1 and 11.2). This could be supporting evidence for the existence of such an effector protein.

It has been shown that histone proteins are inhibitory to DNA methylation (Davis et al., 1986; Caiafa et al., 1989). Most of the methylation activity of purified mammalian enzyme were in the internucleosomal DNA (Davis et al., 1986). A non histone protein which is stimulatory to DNA methylation has been isolated (Caiafa et al., 1989). The possibility exists that accessory proteins which would not survive the process of purification may be bound to DNA methylase. Miller et al. (1985) suggested that the assay conditions for DNA methylase may not be optimal, since even the slight stimulatory effects of trace metal ions in the enzyme preparations may be masked by the presence of dithiothreitol and EDTA in the buffers used.

Further experimental evidence on the identity of the glycerol soluble fraction would contribute to the design of improved reaction conditions which would give the enzyme a higher specific activity, comparable to the *in vivo* methylation rate.

4.6. Sequence specificity of pea DNA methylase.

4.6.1. Nearest neighbour analysis.

It has been reported that plant DNA is methylated at CG dinucleotides and CNG trinucleotides (Gruenbaum *et al.*, 1981) and this could mean the existence of one or more DNA methylases. I have only been able to find evidence of one enzymic activity in all enzyme preparations. Although much study has gone into the distribution of methyl groups in DNA, *in vivo*, little has appeared to show whether a similar distribution occurs *in vitro*. It has been demonstrated for the animal cell enzyme that the pattern produced in vitro is similar to the *in vivo* pattern, that is the methylation of CG dinucleotide sequences (Browne *et al.*, 1977).

To investigate the specificity of pea DNA methylase, pea DNA was nick translated with labelled deoxyribonucleotides (Josse *et al.*, 1960). It was then used as a substrate for the enzyme and the methylated product was hydrolysed to 3' monophosphate for nearest neighbour analysis.

The purified pea enzyme exhibited a broad range of sequence specificity, methylating cytosine residues in CpA, CpC, CpT and CpG dinucleotide sequences (Table 13). Until confirmation is obtained that the first three dinucleotide sequences are components of the trinucleotide CNG, it is difficult to confirm *in vitro* methylation of the trinucleotide, CNG by the purified pea enzyme but this is the likely explanation.

The process of purification may be responsible for the broad sequence specificity as observed in some mammalian enzymes (Simon et al., 1978; Sneider, 1980; Woodcock et al., 1987) where methyl groups were detected in CpT and others in addition to CpG. Indeed vertebrate and plant DNA methylases might be similar but physiological conditions might be responsible for the high rate of methylation of CNG sequences in plants compared with the rate in animal cells.

In pea DNA (as determined from the results of the estimation of the nearest neighbour) the mCpG dinucleotides account for only 13% of the total in vitro methylated cytosines. 50% of the methylcytosines are in the dinucleotide sequence mCpT, 8.5% are in mCpC, and 33% in mCpA dinucleotide sequences. This underrepresentation of CG dinucleotide sequences could be accounted for partly by the phenomenon of CG deficiency in eukaryotic DNA (Adams et al., 1987). However the proportion even when this is taken into account is rather low and it seems the enzyme at least in vitro, is exhibiting preferential site specific methylation of the complementary CTG and CAG sequences.

This in vitro sequence specificity was not observed in a second experiment with another batch of enzyme. This showed a higher proportion of CpG dinucleotide methylation (as would be expected from the ratio of cytosines in CpG and CpNpG sequences in pea DNA) and this might imply the presence of two enzymes (or two forms of the enzyme) present in different proportions in the two preparations.

4.6.2. Restriction enzyme studies.

Restriction endonucleases whose activities are blocked when their recognition sites are methylated have been used to detect modified CG sites in the genome of animal cells (Bird and Southern, 1978).

They have been used to define the methylation status of specific sites along the DNA by cleaving with restriction enzymes and interpreting the products of their digestion in terms of the presence or absence of methylated cytosine. However restriction sites represent only a small proportion of potential methylation sites.

The methylation of CpG and CpNpG in plant DNA can be detected similarly by the effect of methyl groups on digestion by restriction endonucleases which contain CpG and CpNpG in their recognition sites (McClelland, 1981; Kessler et al., 1985; Nelson and McClelland, 1987). In higher plants only a portion of the 5-methylcytosine content is found in the CpG dinucleotide sequence (Gruenbaum et al., 1981; Vanyushin, 1984). Much of the 5-methylcytosine exists in the trinucleotide sequence CpNpG such as CAG and CTG (Gruenbaum et al., 1981). This trinucleotide is found in the recognition sequence of SstII, PstI, and EcoRII. The CpG dinucleotide methylation is present along with CCG and CGG trinucleotide methylation in the recognition sequence CCGG, of HpaII and MspI. HpaII does not cleave this site if the internal cytosine is methylated, wherees MspI is insensitive to methylation at this site. MspI will not cleave however if the 5' cytosine is methylated. Figure 19.1a shows no difference between pea DNA cleaved with HpaII and MspI, suggesting extensive in vitro methylation of both sequences.

To further investigate the specificity of pea DNA methylase, the purified enzyme was incubated with supercoiled viral DNA and plasmid DNA and the results are discussed below. Such incubation introduces no more nicks than in enzyme free controls which were used to show that the restriction digestion with a specific endonuclease was not the result of cleavage by the pea DNA methylase preparations.

4.6.2.1. Study with plasmid DNA, pVHCI.

Cytosine methylation within the CCGG sequence in the plasmid pVHCI, was studied using the restriction enzymes HpaII and MspI. There are 14 of these sites present in the plasmid DNA (Bryans, 1989). On digestion of 1µg samples of both methylated and unmethylated pVHCI DNA with 10 units each of these enzymes 9 bands of DNA fragments, were obtained (Figure 19.1a). Of the 14 DNA fragments possible, two of the bands (0.54 kb) would run in the same position and the smaller base pair fragments (0.034-0.11kb), would be too faint to be seen hence the 9 visible bands. The lack of any difference in the digestion pattern of methylated and unmethylated DNA samples, obtained with either restriction enzyme, indicates that the low in vitro methylation rate obtained in this experiment was not sufficient. The fluorograph of the gel however indicates the presence of incorporated tritium in the high molecular weight band (1.06 kb). The starting plasmid DNA contains two forms of the plasmid, the slower migrating band is the relaxed form and the faster migrating band is the supercoiled species. The latter is the major component.

4.6.2.2. Study with plasmid DNA, ColEI.

ColEI DNA also contains relaxed and supercoiled DNA species. The supercoiled DNA was converted partly to the relaxed form during the process of methylation. There are nine HpaII/MspI sites in the 6.4 kilo base, plasmid DNA, and 3 prominent bands of DNA fragments are seen in the restriction digest with HpaII/MspI (Figure 19.2a). Two faint bands are also visible but the other bands are too small and faint to be seen. There appears to be two restriction sites for PstI in the plasmid DNA which gives two DNA fragments, one of about 1.3 and another of 4.2kb.

There is only one site for SstII and restriction with this enzyme produces the linear form of the plasmid DNA. The loss of visible DNA fragments in the restriction digest of methylated ColEI, is due to poor recovery after methylation with pea DNA methylase (Figure 19.2b). This could be as a result of a strong complex of DNA methylase with the DNA substrate, resulting in some loss during phenolisation as a protein complex. The faint fragments visible are however similar in size to those obtained with unmethylated ColEI DNA. The presence of methylated DNA fragments in the enzyme digests, was not detected by fluorography, thus indicating a low *in vitro* activity of pea DNA methylase. Since the recovery of plasmid DNA after methylation are very low, the presence of high molecular weight bands would be faint and would have gone undetected.

4.6.2.3. Study with phage DNA, ϕX174 RF DNA.

Unmethylated uncleaved ϕX174 RF DNA, showing two forms of the phage DNA is shown in Lane 3 (Figure 19.3). They are the faster migrating supercoiled species and the slower migrating relaxed form. Lane 4 is methylated uncleaved ϕX174 RF DNA. During incubation with pea DNA methylase in an AdoMet free reaction mixture, the supercoiled form was nicked to give the linearised species of the phage DNA. The presence of smaller DNA fragments indicates the presence of exonuclease and endonuclease activities in the enzyme preparation.

ϕX174 RF DNA has 5 sites for HpaII/MspI. Complete cleavage with these enzymes would give 5 DNA bands (Sanger *et al.*, 1977). Adams *et al.* (1984) have shown that on methylation, methyl groups are added to the various DNA segments in proportion to the number of CpG dinucleotides present in each DNA fragment.

Two large DNA fragments and three faint fragments were obtained from HpaII/MspI digestion of ϕX174 RF DNA. No subset of fully methylated HpaII resistant DNA molecules is produced on methylating ϕX174 RF DNA with pea DNA methylase. Essentially the same pattern of restriction fragments was obtained for the methylated and unmethylated phage DNA. This is despite the fact that methylation to 12% of the cytosines was obtained, which compares favorably to the 26% of *in vivo* cytosine methylation of pea DNA. Furthermore, Drahovsky and Morris (1971) calculated a rate of 50bp/s which means that an enzyme molecule would travers a cyclic molecule of ϕX 174 RF DNA (5375bp), in 108 seconds and the present incubation was for 48 hours. The fluorograph in this case failed to show any incorporation of tritium into the methylated DNA bands despite the 12% methylation calculated from the methyl group incorporation. It is possible this was caused by the observed degradation of DNA which generated diffuse rather than sharp bands.

There are two EcoRII/BstNI sites in ϕX174 RF DNA, but some resistance to the enzyme is shown in both forms, (methylated and unmethylated), of the DNA. The absence of prominent DNA fragments in the lanes for BstNI enzyme digest, must be due to insufficient loading of DNA in the control and/or degradation in the methylated sample. Razin and other workers (1970) have shown that the commercially available ϕX174 RF DNA, is usually methylated in the cytosine residues present in CNG trinucleotide sequence, as a result of *dcm* methylation in the prokaryotic host. All EcoRII sites would be expected to be methylated.I did observe some cleavage, however, but the pattern of DNA fragments for the methylated and unmethylated DNAs were indistinguishable.

In vitro methylation activity of pea DNA methylase, could not be used to identify specific site methylation of CG and CNG DNA sequences in plant DNA from the experimental results obtained. This would be better studied with a more active enzyme preparation which would ensure methylation of all sites in the DNA substrate as obtained for the mouse ascites DNA methylase (Turnbull and Adams, 1976).

4.7. Identity of protein bands in purified fractions.

4.7.1. Cross reactivity with mouse methylase antiserum.

It has been shown in mammalian cells, that different cell types with different methylation patterns contain very similar or identical species of DNA methylase (Bestor et al., 1988). It has also been shown that monoclonal antibodies against human placenta DNA methylase cross reacted with DNA methylase from mouse cells. It would be interesting to know the extent of the conservation of this cellular protein amongst organisms of distant evolutionary relationship, such as plant and mammals, which utilise the process of post replicative DNA methylation for control of gene expression. The only plant enzyme purified (from wheat embryo), has been reported to be different not only in size but in specificity and immunological properties (Theiss et al., 1987). There was no cross reactivity reported between the wheat enzyme and antibody raised against any vertebrate DNA methylase.

The observed cross reactivity of pea DNA methylase, (fraction III), with mouse ascites DNA methylase antiserum (no. 401) (see Table 12.1) may reflect a similarity in the protein structure of the two enzymes. The interaction appears greater with fragments produced (by limited proteolysis) from the high molecular mass polypeptide, (160,000) with conformations recognised by the antiserum.

4.7.2. In situ DNA methylase activity.

The use of an in situ methylation assay to identify the protein bands in the fractions of pea DNA methylase has not been successful. Mouse ascites DNA methylase, showed strong evidence for the incorporation of tritiated methyl groups into the DNA substrate. No incorporation of tritiated methyl groups into the DNA in the polyacrylamide gel, was seen with the pea enzyme (Figure 20b). The lack of a positive response however does not rule out the existence of a protein(s) with DNA methylase activity in the purified fractions of pea DNA methlase.

The mouse ascites DNA methylase has a much higher specific activity than the most active pea DNA methylase fraction (11,500 units/mg as compared to 1738 units/mg). Also the mouse enzyme is much more concentrated. To prevent binding to the DNA incorporated in the gel, and to enhance electrophoretic migration at the low temperature of 4^0C, 0.4M NaCl was added to the electrophoretic buffer, (buffer E). Salt is strongly inhibitory to the pea enzyme and it has been shown to affect its binding to a DNA substrate. It is possible that the presence of salt may have caused an irreversible change in the protein conformation of pea DNA methylase which renders it inactive even after several washes with buffer to get rid of the salt or it could just be due to too little enzyme present in the pea DNA methylase fractions.

4.8. Conclusion and future work.

It is necessary to improve on the protocol designed for the purification of pea DNA methylase. This would improve the yield and give enzyme preparations with specific activities, comparable to those for the animal cells DNA methylase. The more active enzyme preparations would form the basis for future in vitro methylation studies. This would provide some knowledge into the mechanism of in vivo methylation of CG and CNG sites in plant DNA.

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