In the name of

ALLAH

Most Gracious Most Merciful

Thanking

HIM

with a full heart and devoted tongue

DNA METHYLASE IN MOUSE CELL NUCLEI

By

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TO MY PARENTS

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ABBREVIATIONS.

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

Brd Urd	Bromodeoxyuridine
BSA	Bovine serum albumin
DTT	Dithiothreitol
L-929	Mouse fibroblast cells
mRNA	Messenger ribonucleic acid
PBS	Phosphate buffered saline
p.c.m.b.	p-chloro mercuribenzoate
PMSF	Phenyl methyl sulphonyl flouride
rDNA	Ribosomal deoxyribonucleic acid
SAM	S-adenosyl-L-methionine
SAH	S-adenosyl-L-homocysteine
SDS	Sodium dodecyl sulphate
TCA	Trichloroacetic acid

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Summary.

Extraction of isolated nuclei from Krebs II mouse ascites cells (or mouse L-929 cells) with low salt (0.2M-0.4M) is sufficient to solubilise the major proportion of DNA methylase. The properties of this enzyme were such as to suggest that it could perform a role in maintenance of DNA methylation pattern or catalyse the de novo methylation of unmethylated DNAs.

A brief search for soluble nuclear factors that might modify the actiivity of this enzyme was carried out but no spe-cific factors that would meaningfully modulate the activity of this enzyme were obvious.

On the other hand a search for further DNA methylase activity in these nuclei revealed activity which remained bound to nuclear structures even after extraction with 2M NaCl. The occurrence of this enzyme activity in such nuclear structures which resist to solubilise with 2M NaCl and the results from alkaline CsCl density gradient analysis are suggestive that this enzyme activity is a part of replication complex (see Pardoll et al., 1980) and is possibly involved in maintenance of the DNA methylation pattern after DNA replication.

The properties of bound DNA methylase have been compared to those of DNA methylase already solubilised at low salt concentration. Response to NaCl, pH and DNA substrates as well as sedimentation characteristic, indicate that bound DNA methylase is quite similar to soluble DNA methylase.

The inhibition of DNA synthesis with hydroxyurea released bound DNA methylase activity into the soluble form, suggests that occurrence in the bound form is dependent on DNA replication. In cells treated with 5-azadeoxycytidine, more than 80% of the DNA methylase was lost from the soluble form and further experiments support the notion that it may be bound to DNA nuclear matrix in an inactive form.

The solubilisation of bound DNA methylase has been achieved by using extremely large amount of micrococcal nuclease. Since this procedure was quite expensive an alternative method, the "abstraction" of DNA methylase from the bound form with denatured E. coli DNA was employed. However, despite this solubilisation the methylase remained bound to the 'abstracting' DNA.

By using 30% ammonium sulpahte, the solubilisation of bound DNA methylase from nuclear components, with high yield, has been carried out. Such study revealed that whereas 2M NaCl failed to dissociate enzyme from nuclear structure, the ammonium sulphate has proved to be an efficient reagent.

In view of the results obtained after the treatment of cells with 5-azadeoxycytidine and with DNA synthesis inhibitors, a model showing the biological relationship between these two forms of DNA methylases has been presented.

INTRODUCTION

1. Occurrence of methylated bases in DNA.

In addition to the four common bases, the presence of methylated bases in DNA is a feature of most groups of organisms (Shapiro, 1976). The presence of 5-methyl cytosine, as a constituent of nucleic acid in tubercle bacilli was first reported by Johnson and Coghill (1925). Thirty three years later Dunn and Smith (1958) isolated another methylated base in E. coli 15T, 6-methyl adenine. Since then several groups of workers (Doskocil and Sormova 1965a,b, Fujimoto et. al., 1965, Vanyushin et. al., 1968, 1970; Culp et. al., 1970) have established the presence of methylated bases in DNA of both prokaryotes and eukaryotes.

Higher eukaryotes contain 5-methyl cytosine as the only methylated base in their DNAs while certain insects and prokaryotes contain both 5-methyl cytosine and 6-methyl adenine.

1.1 Methylated bases in prokaryotes.

Bacterial DNA commonly contains small amounts of either 5-methyl cytosine or 6-methyladenine. These bases result from enzymatic modification of either cytosine or adenine in polymeric DNA.

The content of 6-methyl adenine in E. coli and Serratia marcesens is about 0.5 to 0.7 mole % and the mole % of 5-methyl cytosine is normally somewhat less than that of 6-methyl adenine lying in the range 0.02-0.2 mole % (Vanyushin, 1968). Both modifications may be present in the same strain of bacteria.

The phage ϕX174 has only one 5-methyl cytosine residue out of its 5375 nucleotide genome. It has been suggested that this base may play an important role in virus maturation (Razin et. al., 1975, Friedman and Razin 1976). In the DNA of λ-phage, the methylated base appears as 5-methyl cytosine (0.08 mole %) (Ledinko, 1964).

A phage which infects Shigella dysenteriae has shown to contain small amounts of the base 7-methyl guanine (Nikolskaya et. al., 1976) and a phage which infects Xanthomonas oryzae has all its cytosine residues replaced by 5-methyl cytosine (Ehrlich et. al., 1975, Kuo and Tu, 1976).

1.2 Methylated bases in eukaryotes.

Wyatt (1950) initially showed that 5-methyl cytosine was present in small amounts in several animal DNAs and wheat germ DNA. In eukaryote DNA, the amount of 5-methyl cytosine is generally small of the order between 2-7% of the total cytosine. For example, in vertebrates 5-methyl cytosine constitutes about 2 mole % of the total bases in DNA, in insects the quantity is very much lower (0.002 mole %). In Drosophila the level of 5-methyl cytosine is extremely low [i.e. an upper limit of only one methylated base in 300,000 bases (Dawid, 1974)].

The plant DNAs are more methylated than animal DNAs (up to 30% of the cytosine being methylated). For example, wheat germ DNA contains 27% of its cytosine residues as methyl cytosine (Chargaff and Davidson 1955, Shapiro and Chargaff 1960).

Vanyushin and Kirnos (1974, 1977) have reported that in several vertebrates and protozoa the mitochondrial DNA has a higher proportion of methyl cytosine than does the corresponding nuclear DNA. On the other hand earlier investegators have reported a very low level of mitochondrial DNA methylation (Shied et. al., 1968, Evans and Evans 1970., Nass, 1973). Dawid (1974) was even unable to detect 5-methyl cytosine in mitochondrial DNA from HeLa and Xenopus cells. Positive results have been criticised on the basis that mitochondrial DNA preparation used may have been contaminated with nuclear DNA.

Recently, Groot and Kroon (1979) also failed to detect 5-methyl cytosine in the CCGG sequence in a variety of mitochondrial DNAs.

Chloroplast DNA in Chlamydomonas in the vegetative stage has no methyl cytosine but in female gametes and in zygotes it is methylated (Royer and Sager 1979., Sano et. al., 1981., Sager et. al., 1981., Dyer, 1982). Chloroplast DNA in male gametes is also found unmethylated.

Another modified base which is found in eukaryotic DNA is 6-methyl adenine. This was one time thought to be present only in prokaryotes, its presence in unicellular eukaryotic DNA is established only a decade ago. Gorovsky et. al., (1973) demonstrated that 0.65-0.80 mole % of the adenine in the macronuclear DNA of the ciliate Tetrahymena pyriformis is methylated. Shortly after this, Cummings et. al., (1974) reported that macronuclear DNA of another ciliate Paramecium aurelia contains 2-2.5 mole % of the adenine as 6-methyl adenine, however they did not detect 5-methyl cytosine from Paramecium DNA.

Nuclear DNA of chloromycean algae Chlamydomonas reinhardi contains 6-methyl adenine to the extent of 0.5% of the total adenine and 5-methyl cytosine to 0.7% of the total cytosine. Chloroplast DNA from both mt^+ and mt^- Chlamydomonas reinhardi contains less than 0.05% methylated bases (Kenny and Hattman, 1977). Fungi are reported to have no methylated bases (Guseinov et. al., 1972).

1.3 Methylated bases in DNA of viruses.

The DNA of animal viruses polyoma and Simian virus-40 (SV-40) have no detectable methylated bases (Kaye and Winocour 1967, Fiers et. al., 1978, Soeda et. al., 1979), but their DNA copies integrated in transformed cell lines appear to be methylated in the 'late' region of the virus genome (Doerfler, 1981).

The DNA isolated from other animal viruses, for example herpes simplex virus and adenovirus is also normally reported unmethylated or methylated to a very limited extent (Low et al., 1969 Gunthert et. al., 1976, Sharma and Biswall 1977, Sutter et. al., 1978, Von-Acken et. al., 1979). When adenovirus and herpes virus integrate into the host cell genome to bring about transformation methylation of the viral DNA sequences occurs. Adenovirus (Ad-2, Ad-12) DNA was found extensively methylated when integrated in the DNA transformed and tumour cells (Sutter et. al., 1978, Sutter and Doerfler 1980, Vardimon et. al., 1980, 1981, Kuhlman and Doerfler 1982). In adenovirus transformed cells these regions of the integrated adenovirus DNA which are expressed (i.e. those coding for early genes) and not as highly methylated as regions which are not expressed (Sutter and Doerfler 1980).

In most cells, transformed with herpes virus, the integrated viral DNA is found heavily methylated e.g. in marmoset lymphoid cell line 1670, more than 80% of Hpa II (-CCGG-) sites are methylated (Desrosiers et. al., 1979). Similarly retrovirus DNA is methylated when integrated into the host cell genome (Cohen 1980, Stewart et. al., 1982, Jahner et. al., 1982, Gautsch and Wilson 1983).

2. DNA and Chromosome structure of eukaryotes.

2.1 Molecular organisation and function of the genome.

The genome of lower eykaryotes e.g. Drosophila contains approximately 50 times the amount of DNA found in E. coli (Laird, 1971), whilst the human genome contains roughly 1000 times the amount of DNA of E. coli (Sober 1970, Rees and Jones 1972). Whilst the prokaryotic genome has sufficient for approximately 3000 informational genes (McCarthy and Bolton 1964, Grouse et. al., 1972), eukaryotic genome has enough DNA to encode over 150,000 to 3 million such genes.

Renaturation experiments done on highly sheared DNAs have shown that eukaryotic nuclear genomes contain DNA sequences of various degrees of repetition. These sequences are specifically organised; repetitive and non-repetitive (unique) sequences either alternate in short periods i.e. short repeats flanked by non-repetitive sequences less than 2 kilobases long or in longer periods with repetitive and/or non-repetitive sequences extending for several kilobases (Schmidtke and Epplen 1980). In mammals repetitive sequences usually account for 30-40% of the genome and non-repetitive sequences for the remaining 60-70% (Davidson et. al., 1975).

Detailed studies of sea urchin and xenopus genomes (Davidson et. al., 1973, Graham et. al., 1974) revealed that unique sequences averaging 1000 base pairs alternating with repetitive sequences averaging 200-400 base pairs represent structural genes. This alternation of repetitive and unique sequences has also been termed "interspersion" (Davidson et. al., 1973). These authors showed that 80-100% of the messenger RNA molecules present in sea urchin embryos are transcribed from unique sequences in the genome. These unique sequences finely interspersed with short repetitive sequences represent 40% of the total genome (Davidson et. al., 1975). Similar findings have been made in the exploration of sequence representation in mouse RNA (Kuroiwa and Natori, 1979) In mouse liver and brain only about half of the nuclear RNA is transcribed from repeat contiguous unique sequences but cytoplasmic RNA is derived mainly (73-96%) from unique sequences adjacent to middle repetitive sequences.

2.2 Organisation of DNA in chromosomes of eukaryotes.

The DNA in mammalian chromosomes or chromatin (during interphase) is organised in repeating nucleoprotein subunits

"nucleosomes" (Olins and Olins 1974, Kornberg 1974) which on nuclease digestion, can be distinguished into two domains, a protected nucleosome core and exposed linker DNA segment. The core of each nucleosome consists of 145 base pairs DNA fragment arranged around the outside of an octomer of histones. The octomer contains two types of each H_2A,, H_2B (lysine rich histones) H_3 and H_4 (arginine rich histones). Histone H_1, which has been reported that it plays a fundamental role in stabilizing the nucleosomes and maintaining the higher order structure of the chromatin fibres (Thoma et. al., 1979, Butler and Thomas 1980, Thoma and Koller 1981), is associated with a further 20 base pair of DNA adjoining the core to complete full superhelical turn of 80 base pairs each around the histone octomer (Simpson 1978, Thoma et. al., 1979, Laskey and Earnshaw 1980) i.e. at the point where DNA enters and exits from the core particle. Each nucleosome is separated from the next one by a segment of linker DNA. The length of the linker varies from about 15 base pairs to 100 base pairs depending upon the organism and tissue of origin (Morris 1976, Thomas and Thompson 1977, Felsenfeld 1978). Even within a single cell type, the spacing is not homogenous (Lohr et. al., 1977). It has been proposed that shorter repeat lengths are correlated with higher levels of gene activity (Morris 1976, Thomas and Thompson 1977) although this proposal does not seem to be held for lower eukaryotes (Lohr and Ide 1979).

2.2.1 Chromosomal Scaffold.

In addition to the repeating structure (Nucleosomes), chromatin posses a complex range of local variations in the distribution of non-histone proteins which include DNA and RNA polymerases, nucleases, chromosomal structural proteins and possible

regulators of gene expression. These non-histone proteins have been shown responsible for higher order structure of eukaryotic chromosomes and these proteins are organised into structurally independent entity, the "chromosomal scaffold" (Paulson and Laemmli 1977). Since they are not sensitive to DNAase and RNAase and dissociate by mild trypsin or chymotrypsin treatment are generally known as non-histone scaffolding proteins (Paulson & Laemmli 1977, Howell and Hsu 1979). These proteins can be obtained by treating chromatin or metaphase chromosomes with 2M NaCl or polyanions (Dextran sulphate/heparin) (Adolph et. al., 1977a). The 2M NaCl removes all histones together with about 70% of the non-histone proteins. The remaining non-histone proteins are associated with DNA sequences at the base of each loop. Observations with electron microscopy further revealed that these loops attached to the scaffold are anchored (with both ends) at the same place in the scaffold with a 30-90 kilobases in length (Adolph et. al., 1977b, Paulson and Laemmli 1977).

Several reports support scaffold model. For example, sedimentation studies on metaphase chromosomes showed that DNA is compacted by nonhistone (Scaffolding) proteins (Adolph et. al., 1977a): Electron microscopy revealed a halo of DNA loops converging on the central skeletal structure, the scaffold, which retained the overall metaphase morphology of histone depleted chromosomes. More recent electron micrographs of transverse, thin sections through swollen, but unextracted chromosomes showed a star like arrangement of chromatin fibre, consistent with the proposed radial loop model (Marsden and Laemmli 1979., Adolph 1980a). A central longitudinal "core" or scaffold has also been observed in mitotic chromosomes by using silver staining and light microscopy (Howell and Hsu 1979, Satya-Parkash et. al., 1980). The question of whether the scaffold

is really present in the chromosome or merely represents an experimental artifact during preparation of dehistonised chromosomes has also been raised (Goyanes et. al., 1980, Okada and Comings 1980, Hadleczky et. al., 1981). Lewis and Laemmli (1982) have recently reported that scaffold requires specific metalloprotein interaction for stability. The histone depleted structures of chromsomes isolated in metal depleted form, were specifically and reversibly stabilised by Cu^{+2} and less specifically with Ca^{+2}. The pattern of protein structure in the scaffold that was reproducibly generated following treatment with Cu^{+2}, was found composed primarily of two high molecular weight proteins Sc_1 and Sc_2 (170,000 and 135,000 daltons). While the addition of metal chelating agent in the extraction buffer lead to dissociation of scaffolding and to complete unfolding of the DNA (Lewis and Laemmli 1982). More recently, the microscopic studies with metaphase chromosomes revealed that chromosomal scaffold is a defined structure, not a non-specific aggregate (Earnshaw & Laemmli 1983).

2.3 Organisation of DNA in interphase nuclei.

Several reports indicate that the chromatin of interphase nuclei is subject to similar topological constraints as the DNA of metaphase chromosomes. "Nucleoids", cells from which protein has been released by lysis in NaCl and detergent exhibit a biphasic alteration in sedimentation rate with exposure to increasing concentrations of ethidium bromide which suggests that DNA is constrained in closed loops (Cook and Brazell 1976, 1978., Benyajati and Worcel 1976, Vogelstein et. al., 1980). The presence of these DNA constraints has also been suggested by Igo-kemenes and Zachau (1978), who have shown that the maximum length of soluble chromatin

obtained by mild nuclease digestion is about 75 kilobases. The length of the proposed DNA domains has also been estimated to about 85 kilobases in Drosophila tissue culture cells (Benyajati and Worcel, 1976) and about 220 kilobases in HeLa cells (Cook and Brazell, 1978). These observations suggest that chromatin is attached to a framework inside the interphase nucleus and it has been proposed that nuclear matrix is involved in this organisation of DNA (Comings and Okada 1976, Vogelstein et. al., 1980).

2.3.1 Nuclear matrix.

The nuclear matrix is a residual structure, obtained from isolated nuclei following extraction with nucleases and high salt (Berezney and Coffey 1974, 1977, Mitchelson et. al., 1979). This structure accounts for about 10-20% of the total nuclear proteins depending on the tissue origin and has an overall composition: 98.2% protein, 0.1% DNA, 1.2% RNA, 0.5% phospholipid (Berezney and Coffey 1974).

Electron microscope sections of nuclear matrix preparation show a peripheral shell derived from the lamina or pore lamina elements of nuclear envelope (Aaronson and Blobel, 1975; Gerace and Blobel, 1980, Hancock 1982, Hancock & Hughes 1982), together with an internal "framework" or "scaffold" to which the DNA is attached (Adolph 1980b, Agutter and Birchall 1979, Agutter and Richardson (1980), Berezney 1980, Berezney and Buchholtz 1981, Bouvier et. al., 1982, Fisher et al., 1982). This framework is composed of non-histone proteins and retains some of the morphological features of nuclei (Shaper et. al., 1979, Lebkowsky and Laemmli, 1982a). DNA binding studies on histone depleted HeLa nuclei further revealed that among these proteins there are three prominent proteins of 60,000 to

70,000 Mr which are the major DNA binding proteins (Labkowsky and Laemmli, 1982b). Studies have shown that these proteins are the major components of peripheral lamina of the nucleus (Gerace et. al., 1978, Krohne et. al., 1978).

Metalloprotein interaction in nuclear matrix of histone depleted HeLa nuclei has also been characterised (Lebkowsky & Laemmli 1982a). It has been suggested that metals appear to play a role in the long range DNA compaction in both histone depleted metaphase chromosome and interphase nuclei.

2.3.2 DNA replication and the nuclear matrix.

In prokaryotes, replication complex is attached to the cell membrane (Ganesan and Lederberg 1965., Smith and Hanawalt 1967., Sueoka and Quinn 1968., Worcel and Burgi 1972). A similar mechanism would have an equal utility in eukaryotes, where an enormous amount of DNA must be ordered specially during replication. Hence many investigators have carried out studies analogous to those bacteria. Most of investigators have found no preferential association of replicating DNA with nuclear membrane (See for review Lewin 1974).

Recent work has shown, however, that other structural elements exist in the nucleus in addition to the nuclear membrane, for example the nuclear matrix (Berezney and Coffey 1974, Comings and Okada 1976, Keller and Riley 1976, Herman et. al., 1978, Miller et. al., 1978). Berezney and Coffey (1975) found that nuclear matrix is associated with newly synthesised DNA in regenerating rat liver. One minute after rats were injected with [^{3}H]-thymidine, more than 90% of the total tritium in the nuclear DNA was found associated with the matrix DNA (25% of the total nuclear DNA). Similar results have found with 3T3 cells (Vogelstein et. al., 1980), and with viral DNA in polyoma

infected 3T6 cells (Buckler-White et. al., (1980). While Nelkin et. al., (1980) showed a three-fold to seven-fold enrichment of SV40 DNA relative to total cellular DNA in the nuclear matrix DNA prepared from SV40 infected 3T3 cells.

Pulse Labelling experiments further showed that the fraction of the DNA remaining tightly attached to the matrix is highly enriched in newly synthesised DNA (Pardoll et. al., 1980, Hunt and Vogelstein 1981, McCready et. al., 1980, Aelen et. al., 1983). It has been suggested that the growing point of the DNA replication is attached to the nuclear matrix, a fixed site, in which DNA replication complexes are anchored to the nuclear matrix and the DNA is reeled through these complexes as it is replicated (Pardoll et. al., 1980, McCready et. al., 1980, Buongiorno-Nardelli et. al., 1982). These observations support the notion of a dynamic interaction of the matrix protein and DNA.

2.3.3 DNA sequences associated with nuclear matrix.

Several reports have recently appeared reporting on DNA sequences found to be associated with the nuclear matrix. Jeppesen and Bankier, (1979) and Razin et. al., (1979) reported that the scaffold DNA prepared from either Chinese hamster or mouse chromosomes was enriched in middle repetitive DNA sequences. Kuo (1982) has also found a slight enrichment of repetitive DNA sequences in the chicken chromosome scaffold DNA. The presence of significant amount of unique sequence has also been shown in the scaffold DNA.

Cook and Brazell (1980) reported that the human α-globin gene but not the β- and γ-globin genes is especially associated with the nuclear matrix which isolated by centrifuging intact HeLa cells through sucrose gradient containing 2M NaCl. Considerable amount of ovalbumin and conalbumin gene sequences have also been shown

associated with the nuclear matrix of hen oviduct cells but not with nuclear matrix of hen liver and brain cells (Robinson et. al., 1982, 1983).

2.3.4 Role of nuclear matrix in transcription.

Jackson et. al., (1981) have reported that DNA associated with the nuclear matrix from HeLa cells is highly enriched in transcribing sequences, as demonstrated by hybridizations of the total DNA and the matrix DNA to an excess of nuclear RNA. Using pulse labelling experiments, these authors also demonstrated that nascent RNA is synthesised at the nuclear matrix. Similarly, pulse labelling experiments done on SV40 infected cells also showed that viral RNA remaining in the nucleus is found in association with the nuclear matrix. (After 10 minute pulse with [5,6 ^{3}H]-uridine over 85% of the viral RNA was found associated with the nuclear matrix) (Ben-Ze'ev & Aloni, 1983).

3. Specific methylated sequences in eukaryotic DNA.

In eukaryotes, the methylated cytosine residues are distributed along the DNA molecule in a non-random fashion. The major portion of the methylated bases is associated with the dinucleotide -CG-. Early studies involving digestion of DNA with DNAase I showed that 5-methyl cytosine was recovered predominantly in the dinucleotide -m^{5}CG- (Sinsheimer 1955, Doskocil and Sorm 1962). Studies with developing sea urchin embryos showed that 90% of the 5-methyl cytosine exists in the sequence -CG- (Grippo et. al., 1968). However, the presence of 5-methyl cytosine is not limited to this sequence. The comparison of 5-methyl cytosine content of DNA from somatic bovine tissue and the frequency of -CG- occurrence in

those DNAs suggests that other dinucleotides must also contain 5-methyl cytosine residues. Sneider 1972, reported the existence of small amounts of 5-methyl cytosine residues in the sequence -CT- while other data indicates the occurrence of 5-methyl cytosine in the dinucleotide -CC- and possibly -CA- (Sneider 1980, Vander-Ploeg and Flavell 1980, Vander-Ploeg et. al., 1980, Simon et. al., 1980, Gruenbaum et. al., 1981b, Romanov and Vanyushin 1981). It is clear, however, that not all -CG- dinucleotides are methylated in eukaryotes. Even in bovine thymus at least 10% of the recoverable -CG- sequences are unmodified (Sinsheimer, 1955).

Several restriction endonucleases which have a -CG- sequence in their recognition sequence, have been used as probes for 5-methyl cytosine (Mann and Smith 1977, Gautier et. al., 1977, Bird and Southern 1978). A pair of endonucleases of identical sequence specificity (isoschizomers) but different sensitivity to methylation are Hpa II and Msp I. Hpa II cleaves -CCGG- but not -Cm^5CGG-, whereas Msp I cleaves both.

The use of such restriction endonucleases have confirmed very low level of methylation in insects like Drosophila (Rae and Steele, 1979) and extensive methylation in vertebrates like mouse in which more than 70% of the Hpa II sites were in methylated form, while in the sea urchin Echinus esculentus and other non-arthropod invertebrates around one third of the genome comprises heavily methylated sequence blocks, but the remaining two thirds are not detectably methylated (Bird and Taggart, 1980). The level of methylation of Hpa II sites in other vertebrates is also reported e.g. in rabbit DNA, these sites are 50% methylated (Singer et. al., 1979), in human DNA 60% (Vander-Ploeg and Flavell, 1980) and in calf thymus DNA 90% methylated (Cedar et. al., 1979).

3.1 Methylation of DNAs of different kinetic complexity.

3.1.1 Satellite DNA.

The DNA of eukaryotic organisms contain serially repeated sequences which vary in amount and complexity from the bulk DNA in G+C content and hence appear as satellites, when DNA is banded in CsCl density gradient.

Several satellite DNAs such as those of mouse, the fly Rhynchosciara and several species of Drosophila have been shown by RNA-DNA hybridisation to be located in the centromeric heterochromatin. The amount of 5-methyl cytsoine in DNA is related to some extent to the frequency of occurrence of dinucleotide -CG-. For example, highly repeated HS-β satellite DNA of Kangaroo rat has a basic 10 base pair repeat containing one methyl cytosine (Fry et. al., 1973) and the basic 34 base pair satellite DNA from the plant Scille sieberica has more methyl cytosine than cytosine (Deumling, 1981). Some of these methyl cytosines are in -mCT- and -mCA- dinucleotides. That both -AT- and -GC- rich satellite DNAs often have high levels of 5-methyl cytosine (Salomon et. al., 1969, Gautier et. al., 1977, Horz and Altenburger 1981) may point to a special function of the methylated base in satellite DNA.

Considerable tissue variation exists in the extent of methylation of some satellite DNAs. Thus in bovine sperm, satellites are not methylated (at Hpa II sites), whereas the same satellites are methylated in thymus, skin and thyroid (Kapat and Sneider 1979, Sturm and Taylor 1981)

Recently, Sano and Sager (1982) have determined the level of 5-methyl cytsoine residues in bovine satellite I DNA by sequence

analysis of native purified satellite I DNAs from three bovine tissues and from cloned DNA. They found that satellite I DNA from thymus contained a total of 5% methyl cytosine, whereas that from liver and brain contained 4.4% and 2.6% methyl cytosine respectively. It was found that there was specific extent of methylation of satellite I DNA in every tissue studied.

3.1.2 Repeated and unique sequences.

In addition to highly repeated sequences (satellite DNA) another class of repeated sequences called inverted repetitive sequences, hairpin on foldback DNA, has recently been characterised in eukaryotes. These sequences are formed by denaturing DNA in any of a number of a different ways (heat, alkali, formamide) and then reassociation conditions favouring the formation of intrastrand duplexes with very fast (first order) kinetics (Walker and McLaren 1965, Schmid et. al., 1975).

Studies with rapidly renaturing DNA from Chinese hamster ovary (CHO) cells showed that this DNA is enriched two to four fold in 5-methyl cytosine and this was particularly true for DNA made early in S-phase (Schneidermen and Billen 1973). It was further suggested that the origins of replicons may be enriched in such sequences. This enrichment of inverted repeat DNA in 5-methyl cytosine is not affected by the presence in the DNA of high levels of bromouracil (Singer et. al., 1977) or azacytosine (Adams et. al., 1982).

The methylation of inverted repeat sequences in cultured mouse cells (P815 mastocytoma cells) is about 50% higher than repetitive DNA which itself is about three fold higher than unique and middle repetitive sequences (Drahovsky et. al., 1979). The middle repetitive and unique sequences are usually either all highly

methylated (vertebrates) or unmethylated (arthropods).

3.2 Distribution of methylated bases with respect to chromosomal structure.

3.2.1 Distribution with respect to chromosomal proteins.

Razin and Cedar (1977) showed a preferential localisation of 5-methyl cytosine in micrococcal nuclease resistant DNA. They conclude that since at 20% digestion most of the DNA was found in core particles, a very high percentage of methyl groups were located preferentially in core regions. However, Adams et. al., 1977, could find no significant difference between 5-methyl cytosine content of nucleosomal core DNA and total DNA in Chinese hamster cells. What the effect of nucleosomal structure on DNA methylation *in vivo* is unknown.

3.2.2 Distribution with respect to chromosome ultrastructure.

The use of flourescent antibodies to 5-methyl cytosine has shown a concentration of this base in C-band regions of heterochromatin of mouse and human chromosomes where satellite DNA occurs (Miller et. al., 1974, Schreck et. al., 1977). This is consistent with the finding of high level of 5-methyl cytosine in certain satellite DNAs. For example, in the human, 5-methyl cytosine clusters in the C-banding region of chromosomes 1, 9, 16 and 15 corresponding to the sites where satellites II or IV and to a lesser extent, satellites I and III are concentrated. Whilst in the mouse, the methylated regions correspond to the locations of satellite DNA i.e. the C-band region of virtually every mouse chromosome (Miller et. al., 1974).

3.3 Species and tissue specificity of DNA methylation.

3.3.1 Level of 5-methyl cytosine in different species.

5-methyl cytosine has been reported to be present as an obligatory minor base component in all animal DNAs studied (Wyatt 1951, Vanyushin et. al., 1970, 1973). The level of 5-methyl cytosine in the total nucler DNA of higher eukaryotes varies from extremely low values found in insects where only one cytosine in 600 cytosine residues is methylated while in higher plants, one third of cytosine may be methylated. Vertebrates come in the middle of the range and have about one methyl cytsoine for every 30 cytosines in their DNA. Lower eukaryotes either lack 5-methyl cytosine or have less than 1 cytosine in 100 methylated.

3.3.2 Level of 5-methyl cytosine in different tissues.

Kappler (1971) has shown a tissue specificity in the levels of 5-methyl cytosine in the DNA of various cultured chick cells, and there are a number of reports on the effect of transformation on the level of 5-methyl cytosine in the DNA of cells in culture (Rubery and Newton 1973, Cato et. al., 1978). No significant changes have been found in the levels of 5-methyl cytosine during development of sea urchin (Baur and Kroger 1976) or mouse (Singer et. al., 1979) and during early rabbit development the DNA from the zygote after 9-10 divisions is found with the same level of methylation as in other tissues (Manes and Menzel 1981).

Minor changes have been reported in the level of 5-methyl cytosine during differentiation of teratocarcinoma cells (Singer et. al., 1979, Fabricant et. al., 1979) and the induction of globin synthesis in Friend erythroleukemia cells may be accompanied by small reduction in levels of DNA methylation (Christman et. al., 1977).

Considerable tissue specific variation in the extent of methylation of various Hha I and Hpa II sites in chicken DNA has recently been reported (Mandel and Chambon 1979). In the oviduct of laying hen, the genes coding for ovalbumin, conalbumin (ovatransferrin) and ovamucoid are expressed and are less methylated at a few sites relative to all other tissues studied (Sperm being the most highly methylated).

4. Possible functions of DNA methylation.

4.1 Restriction and modification.

Studies on bacteriophage infection have revealed that bacterial cells contain on the one hand restriction endonucleases which recognise specific set of nucleotides within DNA and make a double stranded cut, and on the other hand modification enzymes which recognise the same nucleotide sequence and modify them by specific base methylation to prevent from being cleaved (Arber and Dussoix 1962, Arber 1974) [two exceptional cases are R.DpNI and R.Apy I (Miller et. al., 1978, Razin et. al., 1980) which require methylation for cleavage activity].

The endonucleases are of two types. Class I restriction endonucleases are high molecular weight multisubunit enzymes which show DNA methylating and as well as endonuclease activity and require ATP and S-adenosyl-L-methionine for activity (Lautenberger and Linn 1972, Eskin and Linn 1972., Reiser and Yuan 1977, Roberts 1976). Class II restriction endonucleases have a single polypeptide chain of relatively low molecular weight and require neither ATP nor S-adenosyl-L-methionine for activity (Bingham and Atkinson 1978, Roberts 1980, 1983). It is believed that for each class II

endonuclease there is a corresponding but separate DNA methylase but this has been proved in only few cases (Haberman et. al., 1972, Dugaiczyk et. al., 1974, Mann and Smith 1977, Hattman et. al., 1978, Fuchs et. al., 1980, Van Heuverswyn and Fiers 1980).

Class I endonucleases recognise a more complicated site on the DNA (Ravetch et. al., 1978, Lautenberger et. al., 1978, Kan et. al., 1979), the site where methylated bases may present on both strands of the DNA. If both strands are methylated the enzyme fails to interact with the recognition site. If one strand is methylated the enzyme methylates the second strand. If neither strand is methylated the enzyme enters its restriction mode. Since Class I endonucleases give heterologous products they are of little value as genetic tools.

The class II endonucleases and methylases recognises the same symmetrical site on the DNA. The site usually consists of four or six bases which exists as short palindrome. Thus E.co R_1 endonuclease and methylase recognise

5' - G ↓ A * A T T C - 3'

3' - C T T A A G - 5'
* ↑

and the Hpa II endonuclease and methylase recognise

5 - C ↓* C G G - 3'

3' - G G C C - 5'
*↑

where the asterisk indicates the methylated base and if the sequence is not methylated the endonuclease will cleave the DNA as indicated by the arrows. If the DNA is methylated on one or both strands no nuclease action occurs.

After the discovery of "restriction modification" system is prokaryotes a number of observations has suggested that such mechanism might exist in eukaryotic cells. These range from selective (uniparental) inheritance of chloroplasts (Sager 1972, 1979, Sager and Lane 1972, Sager and Ramanis 1974) and mitochondrial DNAs (Bolotin et. al., 1971, Perlman and Birsky 1974), chromosomal elimination in interspecies somatic cell hybrids (Weiss and Epharussi 1966, Carlile 1972) to X-chromosome inactivation in placental mammals (Lyon 1972, Brown and Chandra 1973, Riggs 1975). However, no restriction like "site-specific" endonuclease has been reported in eukaryotes, although an S-adenosyl-L-methionine stimulated DNAase from nuclei of cultured hamster kidney (BHK) fibroblasts (Cato and Burdon 1979) has been reported. This enzyme does not require ATP or Mg^{+2} for activity and is inhibited by S-adenosyl-L-homocysteine. An endonuclease stimulated by ATP or S-adenosyl-L-methionine has also been found in green monkey cells (Brown et. al., 1978) and endonuclease activities isolated from Chlamydomonas (Burton et. al., 1977), Epstein-Barr virus infected cells (Clough, 1979) and from variety of mammalian sources (McKenna et. al., 1981) have also been reported that may be site specific.

4.2 Interplay between DNA replication and methylation.

In 1968, Billen showed that in E. coli, methylation of DNA occurs only on the nascent DNA close to the grown replication fork. The occurrence of methyl groups in the origin of replication of E. coli DNA indicate some interrelation between the process of DNA replication and methylation.

Several studies with bacteriophage ϕX174 suggest a function for the 5-methyl cytosine residues found in the viral DNA in

terminating a round of replication (Razin et. al., 1973, 1975). This single stranded DNA contains no GATC sequence but the sequence CCTGG at position 3501 , has always found methylated. The accumulation of abnormal replicative intermediates, when DNA methylation is inhibited by nicotinamide (Friedman and Razin, 1976) and when cells are infected with a virus, defective in gene A, and more recently the experiments on synchronised cultures of E. coli (Szyf et. al., 1982) suggest that methylation occur at the replication fork.

It has been established that DNA synthesis in E. coli is discontinuous, small DNA pieces (Okazaki fragments) are synthesised and are subsequently joined to yield high molecular weight DNA. The methylation of Okazaki fragments can occur before ligation (Marinus 1976), this can be contrasted to the situation in eukaryotes where newly synthesised DNA is not methylated (Adams 1974).

4.3 Gene regulation and differentiation.

4.3.1 Correlation of gene activity with undermethylation.

The active chromatin regions are known to be sensitive to DNAase I (Weintraub and Groudine 1976). Kuo et. al., (1979) have reported that undermethylated DNA in the region of chicken ovalbumin gene is also sensitive to DNAase I, but for chicken globin gene it was not found necessarily although the gene was undermethylated (McGhee and Ginder 1979) and sensitive to DNAase I (Weintraub and Groudine 1976).

Results of more general nature have been reported by Navah-Many and Cedar (1981), who used DNAase I treatment of chick erythrocyte chromatin to introduce nicks into transcriptionally active genes. Using Hpa II and Msp I restriction enzymes, they conclude

that only 43% of CCGG sites were methylated in active regions compared with 70% in total DNA.

With the help of these isoschizomers (Hpa II and Msp I) Waalwijk and Flavell (1978b) showed that a particular site in the large intron of the rabbit β-globin gene was methylated to different extents in different tissues. Sperm (100%) and brain (80%) DNA were most heavily methylated, while most somatic (erythroid and non-erythroid) tissue DNA was about 50% methylated. Shen and Maniatis (1980) considered 13 CCGG sites in a region of the rabbit genome covering 4 β-type globin genes. Again brain was found heavily methylated.

With chicken globin gene, McGhee and Ginder (1979) showed that certain specific methylation site (Hpa II sites) are less methylated in erythrocytes and reticulocytes than in oviduct tissue. Corresponding results have been obtained by Mandel and Chambon (1979), who studied the chicken ovalbumin gene activity. Similar conclusion has been reached from studies on rabbit and human globin genes and chicken α-globin genes (Shen and Maniatis 1980, Vander-Ploeg and Flavell 1980, Haigh et. al., 1982 respectively).

The DNA of adeno virus type 2 or 12 is not methylated in virions or when free or integrated in productively infected cells (Vardimon et. al., 1980, Sutter and Doerfler 1980). In contrast the majority of CCGG sequences in this DNA are methylated in transformed cells. A similar is emerging from herpes siamri virus DNA studies (Desrosiers et. al., 1979).

4.3.2 Ethionine and 5-azacytosine.

Ethionine, an inhibitor of DNA methylation, is an effective inducer of globin gene expression in Friend erythroleukemia cells.

In spite of the fact the ethionine is toxic and affects many cellular processes Christman et. al., (1977) suggested that induction of globin gene production is caused by undermethylation of DNA.

The incorporation of nucleoside analogues 5-azacytidine and 5-azadeoxycytidine (carbon atom at position 5 in the pyrimidine ring is replaced by nitrogen atom and thus cannot accept a methyl group) into DNA even in trace amounts reduces the level of DNA methylation probably by inhibiting DNA methylase activity (Jones and Taylor 1980, 1981, Adams et. al., 1982, Cruesot et. al., 1982). Cell growth is severely affected following incorporation of the drug. Adams et. al., 1982 have selected from such cultures, variants of mouse L-929 cells with only one third of amount of 5-methyl cytosine from the parental cell DNA.

Like ethionine, incorporation of 5-azacytosine brings about changes in differentiation of cells. Thus mouse embryo cells can be induced to differentiate into muscle cells (Jones and Taylor 1980) and 3T3 cells into muscle cells, chondrocytes and adipocytes (Taylor and Jones 1979). The differentiation is observed in about 1% of cells several days or weeks after treatment with the analogue, implying that cell division was obligatory for the expression of the new phenotypes.

Compare and Palmiter (1981) observed that only four hour exposure of 5-azacytidine on mouse thymoma cells is necessary for metallothionein gene to make it sensitive to induction by cadmium or glucocorticoids, although DNA synthesis is also required.

The treatment of the chick cells carrying an inactive retrovirus provirus (ev-1) with azacytidine lead to hypomethylation accompanied by activation of ev-1 and the acquisition of nuclease hypersensitive region within the ev-1 chromatin region (Groudine et. al., 1981). Similarly when Tk deficient derivatives of cell line 101

were grown in the presence of 5-azacytidine, a 6 to 23-fold increase in the number of Tk^+ derivative resulted (Christy and Scangos 1982).

4.3.3 Demethylation.

In early studies (Riggs 1975, Holliday and Pugh 1975) it was suggested that at some point, perhaps in the early embryos,DNA would be undermethylated or non-methylated, providing a clean slate upon which to laydown a methylation pattern during differentiation. DNA in mammalian sperm and early embryos is highly methylated (Waalwijk and Flavell 1978, Mandel and Chambon 1979, Singer et. al., 1979) and undermethylation is correlated with gene activity, there must be specific event like demethylation.

A model of demethylation is suggested (Singer et. al., 1979). In early undifferentiated state, the DNA is fully methylated and all sites are fully saturated. During development sequence specific proteins inhibits methylation of DNA during replication.

Whilst this model does not require the participation of a specific demethylation activity but relies on the limited inhibition of DNA methylation, a demethylation enzyme activity in the nucleus of murine erythroleukemia cells has recently been reported (Gjerset and Martin 1982).Nucleoplasm was prepared and used for enzyme activity which removes methyl groups from DNA methylated at the internal cytosine of the sequence '5-CCGG-3' (Hpa II sites). So far this result has not been reproduced. Indeed data of Adams et. al., (1974) suggest that such an activity is absent from L-929 cells.

4.4 Chromosome inactivation and methylation.

The Lyon (1961) hypothesis predicts that in each female mammalian cell, the genes of only one X-chromosome are active and the genes on

each additional X-chromosome are not. Inactive X-chromosome show characteristic common to all classes of heterochromatin i.e. they remain highly condensed throughout interphase, show minimum transcriptional activity and are late replicating.

Sager and Kitchen (1975) and Riggs (1975) independently proposed that DNA methylation may be limited with such inactivation of chromosomes or parts of chromosomes. They draw that conclusion from a study of chloroplast inheritance and chromosome elimination in cell hybrids and inactivation of one X-chromosome in female mammals.

Mohandas et. al., (1981) have obtained a mouse-human hybrid cell where the only one copy of hypoxanthine phosphoribosyl transferase (HPRT) gene is on an inactive human X-chromosome. When these hybrid cells were treated with 5-azacytidine, the rate of appearance of the variants was increased to 1000 fold.

Using specifically purified antibodies to 5-methyl cytosine, Eastman et. al., (1980) have shown an increased level of 5-methyl cytosine in the polytene chromosome of dipterin salivary glands, which are transcriptionally inert. Similarly methylation is correlated with reduced activity in amplified RNA genes. In Xenopus laevis the highly active amplified ribosomal RNA genes in oocytes are unmethylated while less active unamplified ribosomal RNA genes in somatic cells are methylated (Dawid et. al., 1970, Bird and Southern 1978). Studies on inactive amplified ribosomal RNA genes from vertebrates where high level of methylation was observed (Tantravahi et. al., 1981, Miller et.al., 1981) also show that there may be a link with the proposed function of methylation in gene expression.

Inactivation of DNA may involve a methyl cytosine induced condensation of chromatin, but the action may be more direct. Thus

the presence of 5-methyl cytosine in DNA increases both the melting temperature and the formation of Z-DNA (a left handed helical form (Behe and Felsenfield 1981). However, as methyl cytosines are seldom clustered (mostly they are very widely spaced) such effect on physical structure is unlikely to be important.

In Chlamydomonas the function of methylation in chloroplast DNA seems much clearer (Royer and Sager 1979, Sano et. al., 1981, Sager et. al., 1981). The level of methylation in chloroplast DNA is very low or zero in vegetative cells and male (mt^-) gametes. The chloroplast DNA in female (mt^+) gametes is heavily methylated. This methylation is brought about by a site specific DNA methylase (Sano and Sager 1980, Sager et. al., 1981) present only in female gamates. In Chlamydomonas, the chloroplast genes are maternally inherited. They are transmitted from the female parent to all progeny, while corresponding genes from the male parent are lost.

4.5 Other possible functions of DNA methylation.

4.5.1 Mismatch repair.

When mismatched nucleotides are incorporated into DNA and not excised before polymerisation continues, a potential for mutation arises. However, in bacteria, efficient methods exist for replacing a mismatched base pair created by incorporation of the wrong residues by DNA polymerase during DNA synthesis (Radding 1978, Glickman and Radman 1980). An important feature of this repair is its control by methyl groups in the DNA. The question arises, how the incorrect base is recognised and replaced. E. coli DNA contains a small amount of 5-methyl cytosine and 6-methyl adenine to protect itself from its own restriction enzymes. Immediately after replication the newly

synthesised DNA strands will be temporarily unmethylated. Therefore if a mismatch occurs in newly synthesised DNA, the correct base will be the one in the methylated parental strand and the incorrect base will be the one in the unmethylated daughter strand.

Several papers have been published to report that strand recognition for mismatch repair is methyl directed (Marinus and Morris 1974, Glickman et. al., 1978, Herman and Modrich 1981). In 1980, Meselson and his associates found that when heteroduplex lambda DNA was constructed in which only one strand was methylated, mismatch repair occurs and the progeny obtained when this DNA was transfected into E coli was strongly inclined towards the genetic information contained in the methylated strand. If both strands are methylated, little if any repair occurs; if neither strand is methylated, repair occurs but without strand discrimination. (Wagner and Meselson 1976).

These results are consistent with the conclusion that DNA methylase levels are controlled in vivo to allow transient undermethylation of the newly replicated DNA (Marinus 1976). This permits selective recognition and repair of the unpaired base in the newly replicated DNA which is more likely to be incorrect.

As some evidence exists for the occurrence of such a mechanism in bacteria, Radman's group are looking for parallel evidence in eukaryotes (Radman et. al., 1978, Glickman & Radman 1980, Radman 1981).

4.5.2 Mutation.

5-methyl cytosine residues may be sites for spontaneous mutation (Salsar 1977). Cytosine residue in the DNA, when subject to deamination gives rise to uracil, which can be enzymically removed (Coulondre et. al., 1978, Lindhal 1979). But deamination of 5-methyl

cytosine produces thymine, which must be lead to a heritable change by transition from-GC-to-AT-(Scarano 1971). In eukaryotic DNA, 5-methyl cytosine is preferentially present in-CG-sequencies. This sequence is also under represented (methylated plus non-methylated forms) to various extents in vertebrates (Russel et. al., 1976). The observed deficiency of the-CG-dinucleotide was suggested to result from high mutability of 5-methyl cytosine (Salser 1977). In recent study of the extent of methylation of-CG-sequences in the DNAs of large variety of eukaryotic organisms, a reciprocal correlation was observed between the content of 5-methyl cytosine and the frequency of-CG-dinucleotides (Bird 1980). This observation could support the idea that methylation of-CG-renders it hypermutable.

5. Patterns of DNA methylation: its maintenance and alteration.

Having described the patterns of DNA methylation a key question arises how these patterns are maintained following cell division and how they are altered during development.

5-methyl cytosine residues are largely found in the symmetrical dinucleotide $-m^5CG-$. Thus immediately following DNA replication an unmethylated -CG- in the nascent DNA strand is paired with a $-m^5CG-$ in the parental strand forming hemimethylated DNA (Turnbull and Adams 1976). Such hemimethylated DNA is believe to be a natural substrate for DNA methylases (or DNA methyl transferases) and turns out to be an efficient acceptor of methyl groups *in vitro* (Compare and Palmiter 1981, Jones and Taylor 1981, Gruenbaum et. al., 1982).

Considerable evidence has been obtained from transfection studies demonstrating that methylation patterns can be copied over

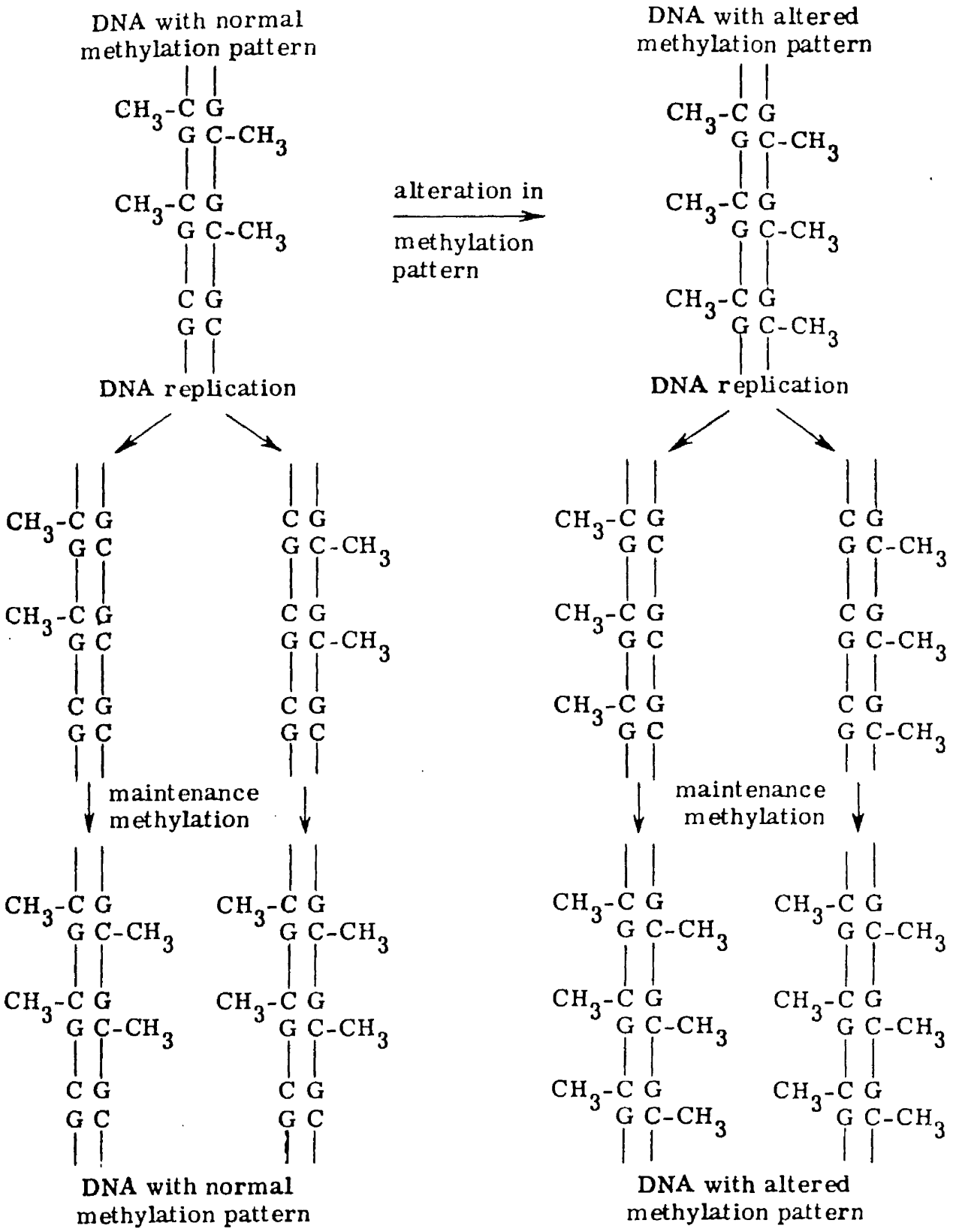


Fig.1 Maintenance and alteration of DNA methylation patterns.

many divisions in eukaryotic cells (Wigler et. al., 1981, Harland 1982). For example, when bacteriophage ϕX174 DNA and cloned chicken thymidine kinase gene methylated in vitro with Hpa II methylase were introduced into tk^- cultured mouse cells by DNA mediated gene transfer technique, the inheritance of DNA methylation was observed over 25 cell generations (Wigler et. al., 1981). This support the hypothesis that cells contain methylase activities capable of efficient modification of hemimethylated sites and it has been shown in several cases (Riggs 1975, Holliday and Pugh, Bird 1978, Cedar et. al., 1979, Wigler 1981, Wigler et. al., 1981).

In order to alter the pattern of methylation, methyl groups must either be added to or removed from DNA. Loss of methyl groups can result from replication in the absence of methylation (Fig. 1) or possible by the action of specific demethylases if they exist. While the addition of methyl groups would have to be brought about by a methylating enzyme which would not require to recognise a hemimethylated site.

6. DNA methylases.

The methylation of DNA bases is brought about by DNA methylases. These enzymes have been isolated and purified from a variety of eukaryotic and prokaryotic organisms, and use S-adenosyl-L-methionine as a methyl group donor, which in turn is derived from L-methionine and ATP.

DNA + S-adenosyl-L-methionine $\xrightarrow{\text{DNA methylase}}$ methylated DNA + S-adenosyl-L-homocysteine.

(See Figure 2).

6.1 DNA methylases from prokaryotes.

Prokaryotic DNA methylases isolated so far, recognise a

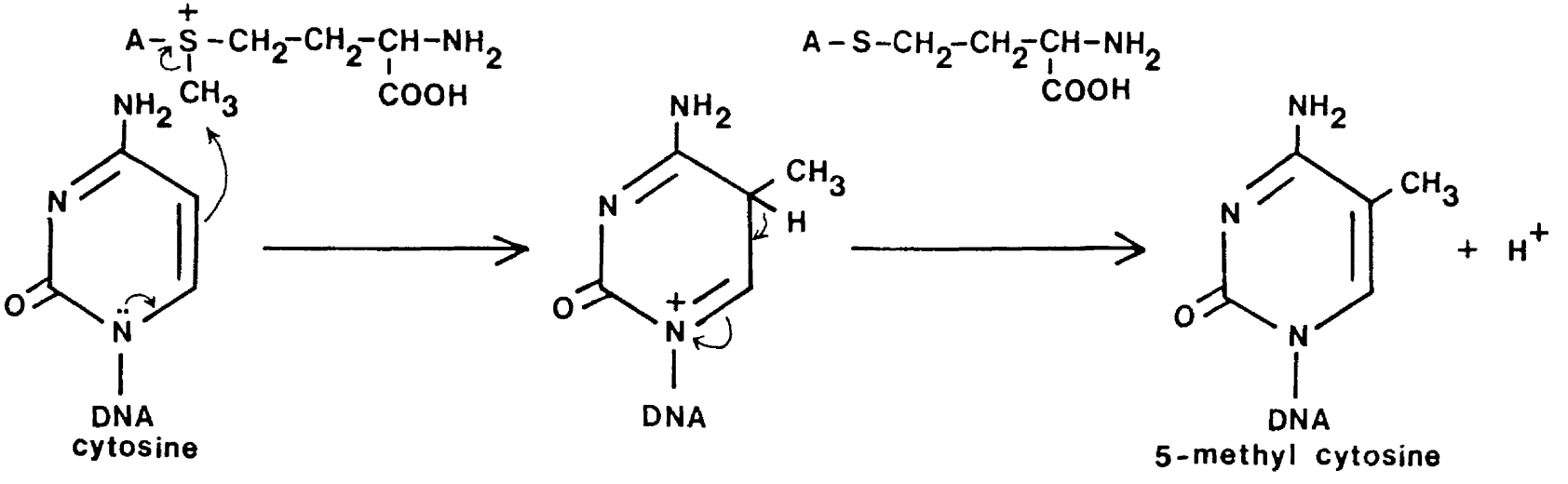


Fig.2 DNA cytosine methylation.
The reaction catalyzed by DNA cytosine methylase.
A=adenosine

specific nucleotide sequence since few of the many possible cytosines and adenines are methylated. The methylation of newly synthesised DNA occurs near the replication point (Billen 1968, Lark 1968) resulting in a very low *in vivo* concentration of unmethylated DNA. This is confirmed by the findings of Lautenberger and Linn (1972). These enzymes have shown incapable of *in vitro* of methylating (homologous) DNA from the same strain whilst methylating DNA from heterologous source more efficiently (Gold et. al., 1963, Gold and Hurwitz 1964a, b, Fujimoto et. al., 1965). These early findings are now clearly understood in terms of their being a wide variety of sequence specific DNA methylases existing in prokaryotes.

6.1.1 *The dam and dcm methylases*.

In *E. coli* strains DNA methylation in fact is not associated with restriction modification. This methylation is carried out by at least two different methylases coded by the dam and mec genes (Marinus and Morris 1973). The physical separation of these two enzymes has recently been achieved (Nestrenko et. al., 1979, Hattmen 1977, Urieli et. al., 1979, Nikolskaya et. al., 1979). The dam product methylates adenine residues within the sequence GATC and the mec product (dcm methylase) methylates cytosine residues at the $CC^{A}_{T}GG$ sequence (Pollock et. al., 1980). In *E. coli* DNA, these sites seem to be 100% methylated on both strands as are those sequences in extrachromosomal DNA propagated in the cell like plasmid or phage DNA (Pollock et. al., 1980). From the calculated number of GATC sites in *E. coli* DNA and the number of detected 6-methyl adenine residues, it appears that the methylatable sites can account for all 6-methyl adenine residues observed.

In addition to these methylating activities, the strains E. coli B and E. coli K12 are known to contain host specific modification enzymes (Haberman et. al., 1972, Kuhnlein et. al., 1969). Two methylases analogous to those coded by the dam and mec genes in E. coli have been found in Salmonella typhi and Salmonella typhimurium (Gomez-Eichelmann 1979) but not in Bacillus subtilis or Staphlococcus aureus (Dresiseikelmann and Wackernagel 1981).

The isolation of dam and dcm methylases from E. coli C has recently been reported (Urieli-shoval et. al., 1983). The substrate and sequence specificity has been studied and found that these methylases are different from the mouse DNA methylase (see below) which methylates hemimethylated DNA at a rate of 100-fold higher than that for unmethylated duplex DNA (Gruenbaum et. al., 1982).

6.1.2 DNA methylases in bacteriophage infected cells.

Hausmann and Gold (1966) reported a 100-fold increase in the capacity of extracts of E. coli B cells to methylate adenine residues [these cells have no methylated cytosine residues and they are devoid of DNA cytosine methylases (Fujimoto et. al., 1965)] 6 minutes after infection by T2 bacteriophage. de novo protein synthesis was necessary for this increase to occur. The properties of the DNA methylase of phage-T2 infected bacteria suggest that it is a new DNA methylase synthesised after infection. T_4 and T_1-phage infected E. coli B cells also showed host DNA methylase activity but the increase was smaller compared to T_2-phage infected cells. T_7 and λ-phages had no effect on the host DNA methylases (Hausmann and Gold, 1966).

When Xp12 bacteriophage infects Xanthomonas oryzae cells to induce the activity of an enzyme that is responsible for the unusual ocurrence of methylated bases in Xp12 phage DNA (Kuo and Tu 1976).

In this phage DNA, cytosine is completely replaced by 5-methyl cytosine residues (Erhlich et. al., 1975, Kuo and Tu 1976). However unlike DNA methylases, this enzyme methylates cytosine residues at the nucleotide level before polymerisation of the DNA. In this enzyme reaction methyl group of 5-methyl cytosine residues is derived from the 3.carbon of serine rather than from the thio methyl carbon of methionine (Kuo and Tu 1976).

6.2 DNA methylases from mammalian sources.

6.2.1 Solubilisation and purification of DNA methylases.

6.2.1.1 Solubilisation of DNA methylases.

Burdon et. al., (1967) were the first to detect DNA methylase in mammalian cell nuclei. They also reported that the enzyme in the cell is firmly bound to the chromatin fraction. Similarly when Sheid et. al., (1968), reported the existence of DNA methylase from rat tissues, the enzyme remained associated with the nuclear insoluble fraction. Studies with rat liver (Kalousek and Morris 1968), however, have shown that DNA methylase activity can be solubilised from chromatin but under only stringent extraction conditions e.g. homogenisation alternatively in sucrose containing $MgCl_2$, and Tris-HCl (pH 7.8) buffer.

6.2.1.2 Purification of DNA methylases.

The purification of DNA methylases after solubilisation have been reported from number of sources ranging from rat spleen and rat liver (Kalousek and Morris 1969, Morris and Pih 1971), Krebs II ascites tumour cells (Turnbull and Adams 1976), Novikoff rat hepatoma cells (Sneider et. al., 1975), HeLa cells (Roy and Weisbach (1975) and

BHK-21 cells (Cato et. al., 1978). The methods applied for the purification are similar. Most involve ammonium sulphate precipitation, gel filtration, DEAE-cellulose, phosphocellulose and hydroxylapatite chromatography.

Simon et. al., (1978) purified DNA methylase from regenerating liver cells to 660-fold. Although this enzyme was not purified to apparent homogeneity its degree of activity was at least 7-fold higher than reported for other DNA methylases from mammalian cells.

Recently, Adams et. al., (1979), have reported an improved method (involve initial treatment of nuclear extract with phosphocellulose and taking the 30 to 60% saturated ammonium sulphate insoluble fraction through gel filtration) for the purification of DNA methylase from Krebs II ascites tumour cells. The enzyme sedimented at 8.3S on glycerol gradients and a major band on SDS-polyacrylamide gel electrophoresis had a molecular weight of 184,000 daltons.

6.3 Substrate specificity of DNA methylases.

Two types of DNA methylase activities have been postulated (Riggs 1975, Holliday and Pugh 1975, Drahovsky and Boehm 1980, Razin and Friedman 1981) which may exist in higher eukaryotes i.e. maintaining activity, able to complete a half methylated methylation site and initiating or alteration activity, methylating previously unmethylated sites (sometimes also called *de novo* methylation).

DNA methylase isolated from mammalian sources (Drahovsky and Morris 1971 a, b., Roy and Weisbach 1975, Sneider et. al., 1975, Turnbull and Adams 1976, Simon et. al., 1978) *in vitro* seem to express initiating rather than maintaining properties as they methylate a number of bacterial DNAs and synthetic polymers which do not contain

5-methyl cytosine. On the other hand observations with DNA mediated gene transfer and bromodeoxy-uridine experiments indicate the presence of maintenance methylase in eukaryotes (Pollock et. al., 1980, Jones and Taylor 1981, Stein et. al., 1982,). This raises the question of whether present procedures for the isolation and study of mammalian DNA methylases are adequate. Where are the maintenance activities? Can the DNA methylase presently studied be modulated in such a way as to act in the maintenance or initiation of methylation patterns?

6.4 Sequence specificity of DNA methylases.

When hemimethylated ØX174 DNA was inserted into mouse L-cells by the technique used for DNA mediated gene transfer, only methylation at -CG- residues was inherited from generation to generation (Stein et. al., 1982). This selectivity was presumably determined by the enzymic specificity of DNA methylase. Greunbaum et. al., (1982) tested that hypothesis by methylating hemimethylated ØX174 DNA by ascites tumour cell methylase *in vitro* and analysed the sequence specificity of the reaction. Modified nearest-neighbour analysis of *in vitro* methylated ØX174 DNA indicated that 95% of the -CG- residues were modified. Other sequences like -GCGC- -CCGG- and -ACGT- that share in common -CG- dinucleotide are also reported to be partially methylated (Razin and Riggs 1980). This raises the question; is the methylation of these sequences a characteristic of one specific DNA methylase or alternatively do several highly specific methylases exist in the cell each methylating one specific sequence. Can further purification separate these activities?

6.5 Interaction between DNA methylase and DNA.

Early studies concerning interaction between DNA methylase and

DNA are performed with a partially purified DNA methylase from rat liver (Drahovsky and Morris 1971a). The results showed that the enzyme forms two types of complex with DNA in the absence of adenosyl methionine (a) weak complex (dissociates in 0.2M NaCl at $0^{o}c$) and (b) tight complex (formed at high temperatures and stable in 0.2M NaCl). The tight complex appeared to be for methylation to occur, it was not known whether formation of this tight complex involves recognition of specific binding sites. It was later found (Drahovsky and Morris 1971b) that the enzyme can form a tight complex at $0^{o}c$ with denatured DNA not with native DNA.

It was also shown that liver DNA methylase functioned processively i.e. it continues to walk along the DNA transferring methyl groups at appropriate sites without detaching (Drahovsky and Morris 1971a). Later Simon et. al., (1978) using more purified preparation of rat liver DNA methylase confirmed the temperature dependent formation of salt resistant tight complex. Similar results have been obtained by Taylor and Jones (1982) with mouse spleen DNA methylase and hemimethylated DNA containing high concentration of 5.azacytosine. On the other hand, highly purified DNA methylase from Krebs II ascites tumour cells has been shown to bind loosely and reversibly to DNA perhaps at random and methylation occurs only when enzyme binds to methylatable sites (its tight binding is depending upon the source of DNA (Turnbull and Adams 1976).

AIMS

From the above introduction it is clear that our knowledge concerning substrate and sequence specificity of eukaryotic DNA methylases is limited compared with that of DNA methylases from prokaryotes. Occurrence of 5-methyl cytosine in different sequences and preferenece of certain DNA methylase preparations for one type of DNA substrate and not the other indicate that there may be more than one methylase activity working in eukaryotes. The aim of this study was to determine, whether there may be multiple methylase activities, or additional factors which might modulate the activity and specificity of DNA methylase in mouse tumour cells.

For instance are there DNA methylases (a) capable of methylating cytosines in different sequences (b) capable of, either maintaining or altering DNA methylation patterns? Or are there factors which might modulate the activity of a single DNA methylase to modify different types of sequence and to both maintain and alter DNA methylation patterns?

MATERIALS AND METHODS

A. MATERIALS.

1. Chemicals.

Most of the general chemicals used were products of British Drug House Chemicals Limited, Poole, England and were Analar grade whenever possible except for the following.

Dithiothreitol	Koch-light Laboratories Limited, Colnbrock, England.
2,5 diphenyl oxazole (PPO) (Scintillation grade)	"
Trichloro acetic acid	"
Toluene (AR) grade	"
Tween-80	"
Hyamine hydroxide (1M Solution in methanol)	Fison Scientific Apparatus. Loughborough, England.
Triton X-100	Rohm and Haas Limited U.K.

2. Fine Chemicals.

The fine chemicals were obtained as specified below.

Actinomycin-D	Calbiochem Limited Hereford, England.
Streptomycin sulphate	Sigma (London) Chemicals Co., Limited U.K.
Cycloheximide	"
Hydroxyurea	"
Phenyl methyl sulphonyl fluoride (PMSF)	"
Trypsin inhibitor	"
E. coli DNA	"
Calf thymus DNA	"
Salmon testes DNA	"

M. luteus DNA	"
Histone type IIA (from calf thymus)	"
Bromodeoxyuridine	"
Nicotinamide adenine dinucleotide	"
5-azacytidine	"
5-azadeoxycytidine	(a generous gift from Dr. Roger Adams).
A. albopictus DNA	"
Adenosine triphosphate	Boehringer Manheim Corporation, London.
Bovine serum albumin	Armour Pharmaceutical Co., Limited, Eastbourne, England.

3. Radiochemicals.

S-adenosyl-l-[methyl-^{3}H]-methionine	500 mCi/mmole Radiochemical Centre, Amersham, England.
[U-^{14}C]-deoxycytidine	478 mCi/mmole Radiochemical Centre, Amerhsam, England.
[6-^{3}H]-thymidine	23 Ci/mmole Radiochemical Centre, Amersham, England.
[4,5-^{3}H]-leucine	50 Ci/mmole Radiochemical Centre, Amersham, England.

4. Enzymes.

Deoxyribonuclease-1	Sigma Chemicals Co., Limited.
Ribonuclease-A	"
Yeast alcohol dehydrogenase	"
Trypsin	"
Micrococcal nuclease	Boehringer Manheim Corporation, London.

Pronase — Calbiochem, San Diego, California, U.S.A.

5. Chromatographic materials.

The following Whatman products were purchased from H. Reeve Angle Limited, London.

3MM filter discs (dia 2.5 cm)

cellulose phosphate P11.

B. METHODS.

1. Standard solutions.

1.1 Buffer M.

This is the standard buffer used for routine storage, assay and isolation of the DNA methylase. Its composition is as follows.

50mM	Tris-HCl (pH 7.8)
1mM	EDTA
1mM	DTT
10% (v/v)	glycerol and
60µg/ml	PMSF.

1.2 Phosphate buffered saline.

This was stored as three separate components. Solution A, B and C which were mixed immediately before use in the ratio 8:1:1 by volume. These solutions had the following compositions.

Solution A.

NaCl	10 g
KCl	0.25 g
Na_2HPO_4	1.44 g and
KH_2PO_4	0.25 g per litre.

Solution B.

$CaCl_2 . 2H_2O$ 1.0 g per litre.

Solution C.

$MgCl_2 . 6H_2O$ 1.0 g per litre.

1.3 Tris-EDTA buffer (TE) pH 7.5.

It had the following composition.

10mM Tris-HCl

10mM EDTA.

2. Stopper solutions.

2.1 Stopper solution-A.

This solution was used for the termination of enzyme activity in standard DNA methylase assay. It had the following composition.

2% (w/v) SDS

4mM EDTA

6% (w/v) 4-amino salicylic acid

10% (v/v) n-butanol

0.5M NaCl and

2mg/ml salmon testes DNA.

The salmon testes DNA served as a carrier DNA in the subsequent precipitation step.

2.2 Stopper solution-B.

This solution was also used for the termination of enzyme activity, but no carrier DNA was used. It had the following composition.

1% (w/v) SDS

2 M NaCl

2mM EDTA

heated to dissolve and kept at $37^{o}C$.

3. Phenol solutions.

3.1 Phenol- m-cresol solution.

This solution had the following composition.

88% phenol

12% m-cresol

0.1% 8-hydroxy quinoline.

3.2 Buffer saturated phenol solution.

To 500 g of phenol melted at 60°C overnight was added 0.1 g of 8-hydroxy quinoline and 500 ml of T.E. buffer, shaken well and supernatant was then decanted off. The solution was again saturated with equal volume of T.E. buffer and the supernatant was removed. The solution was stored in a cold dark place.

4. Tumour propagation.

4.1 Krebs II ascites tumour cells.

These cells were propagated and maintained as described by Martin et. al (1961) by serial intra peritoneal transplantation in mice (Porton strain) of the departmental colony, and were harvested routinely after 7-8 days of growth. Cells were washed twice by suspension in ice cold PBS (phosphate buffered saline) followed by centrifugation at 1000 g at 4°C for 10 minutes.

5. Cell culture techniques.

5.1 Medium.

5.1.1 A modification of Eagle's minimum essential medium was used, henceforth referred to as the Glasgow's modification of Eagle's minimum essential medium. It consists of:

Calf serum (Difco) 10% (v/v)

1F solution (see below)	10% "
$NaHCO_3$ Solution (see below)	5% "
Penicillin	(100 units/ml)
Streptomycin	(100 µg/ml)

5.1.2 1F solution.

Glasgow modification of Eagle's medium with glutamine without sodium bicarbonate with 3x arginine- HCl had the following components.

D-glucose	4500 mg/ml
Magnesium sulphate-$7H_2O$	200 "
Potassium chloride	400 "
Sodium chloride	6400 "
Sodium hydrogen orthophosphate-$2H_2O$	140 "
Calcium chloride $2H_2O$	264.9 "
L-arginine HCl	126.4 "
L-cysteine disodium	28.42"
L-glutamine	584.6 "
L-histidine hydrochloride-H_2O	21.0 "
L-isoleucine	52.46"
L-leucine	52.46"
L-lysine - HCl	73.03"
L-methionine	14.92"
L-phenylalanine	33.02"
L-threonine	47.64"
L-tryptophane	8.16"
L-tyrosine	36.22"
L-valine	46.86"
D-calcium pantothenate	2.0 "

Choline chloride	2.0 "
Folic acid	2.0 "
Nicotinamide	4.0 "
Pyrodoxal hydrochloride	2.0 "
Riboflavin	0.2 "
Ferric nitrate $9H_2O$	2.0 "
Thiamine-Hcl	0.1 "
Phenol red	17.0 "

This solution was stored X10 concentration in 50 ml and 100 ml amounts.

5.1.3 Sodium bicarbonate solution.

Sodium bicarbonate	5.6% (w/v)
Phenol red	0.0015% (w/v)

Sterilized by millipore filtration using 0.22 micron membrane.

5.2 Propagation of L-929 cells.

L-929 cells (mouse fibroblast cells) were grown as monolayers in rotating 80 oz Winchester bottles or 20 oz flat bottles (Roux bottles). The growth medium consisted of Glasgow's modification of Eagle's essential medium supplemented with 10% (v/v) calf serum as described above. Generally cells were seeded at $20x10^6$ per 80 oz glass bottle (100 ml medium) or $4x10^6$ per Roux bottle (30 ml medium) and these allowed to grow to stationary phase (about 8-10 days by changing medium at 2 day intervals). The cells always grown at 37^oC in an atmosphere of containing 5% CO_2.

5.2.1 Subculturing of L-929 cells.

Cell line was maintained by subculturing from confluent

monolayers. The medium was replaced by about 10 ml of a solution made up of four volumes "Versene" solution (0.6 mM EDTA, 0.17M NaCl, 3.4 mM KCl, 10mM Na_2HPO_4, 2.4mM KH_2PO_4) and one volume trypsin (0.25% (w/v) trypsin, 10.5mM NaCl, 1.0mM sodium citrate, 0.002% (w/v) phenol red pH 7.8) at 37°C. The monolayer was then treated with a further 10 ml of trypsin/versene solution until opaque, at which time the solution was poured off, leaving approximately 1 ml of a solution on a monolayer. As soon as the cell layer began to peel off the glass surface, 15 ml of growth medium was added, and the cells were shaken into suspension. The cell density of the suspension was measured using a haemocytometer.

5.2.2 Contamination checks.

All media and passaged cells, were checked regularly for bacterial, fungal or PPLO infection as follows.

a. Bacterial contamination: aliquots were added to blood agar plates and brain-heart infusion broth at 37°C. Results were considered to be a negative if no growth is seen after 7 days.

b. Fungal contamination: aliquots were added to Saboraud's medium at 32°C. No growth after 7 days assumed to indicate the absence of fungal contamination.

c. PPLO infection: agar plates were seeded with passaged cells by piercing the agar surface with a charged pasteur pipette. The plates were grown in an atmosphere of 5% CO_2 in N_2 at 37°C. Infected cells resulted in the occurrence of the characteristic "fried egg" appearance of PPLO colonies on examination of the plates under the microscope, contaminated cultures are discarded.

5.3 Harvesting of cells.

The culture was decanted and the monolayer of cells was washed

twice with ice cold PBS solution. The cells were scraped off the glass in PBS using a rubber wiper and collected by centrifugation at 1000 g at $4^{o}C$ for 10 minutes.

6.0 DNA methylase assay.

The DNA methylase assay was done according to the procedure of Adams et. al (1979). The assay involves measuring of incorporation of radioactivity from S-adenosyl-L(methyl-^{3}H)-methionine into DNA. Steps were incorporated to remove RNA and proteins which may also be likely to be methylated by other methylating activities.

The standard assay mixture (70 μl) contained either 10 μl of denatured calf thymus DNA (20 μg) or 10 μl of denatured E. coli DNA (20 μg), 10 μl S-adenosyl-L-(methyl-^{3}H) methionine (1.6 μCi, obtaining by mixing different S-adenosyl-L-(methyl-^{3}H)-methionine of specific activities 500 mCi/mmole and 10 Ci/mmole respectively), 20 μl of enzyme fraction and 30 μl of buffer containing NaCl to give final concentration of 0.1M.

After incubation at $37^{o}C$ for an hour, the reaction was terminated by adding 1.5 ml of stopper solution-A and the protein was removed from the mixture by extraction with a solution of phenol-m-cresol. After centrifugation at 1000 g for 20 minutes at $25^{o}C$, the upper aqueous layer (containing DNA) was removed leaving protein in the interphase and phenol layer.

DNA was precipitated by mixing upper aqueous layer with 2 volumes of absolute alcohol. After centrifugation at 1000 g for 20 minutes at $-10^{o}C$, the precipitate was dried and dissolved in 50 μl of 0.5M NaOH. The solution was incibated for 30-45 minutes at $37^{o}C$ to hydrolyse any RNA in the mixture.

The solution containing ^{3}H-methyl DNA was then transferred to

Whatman 3MM filter paper discs and washed 5 times in ice cold 5% TCA (about 10 ml per filter disc), twice with methylated spirit and once in ether. The filter was dried in air and heated in 0.3 ml of hyamine hydroxide at 60^{o}C for 20 minutes. 3 ml of toluene-PPO was added to each vial and radioactivity counted using a scintillation spectrophotometer.

A unit of enzyme activity is defined as that amount which catalyses the incorporation of 1 p.mole of methyl groups into DNA in one hour.

7. Preparation of DNA methylase.

7.1 Preparation of nuclei.

Krebs II ascites tumour cells or L-929 cells, harvested as described in sec.5.3, were washed twice in PBS following centrifugation at 800 g for 10 minutes. The cells were then allowed to swell in 10 volumes of ice cold water for 10-15 minutes. Afterwards the cells were resuspended in 1% Tween-80 in water (Burdon, 1971) and homogenised using a tightly fitting teflon glass homogeniser (15-20 strokes). The preparation was examined by phase contrast microscopy for integrity of nuclei and absence of whole cells, after which the nuclei were recovered by centrifugation at 800 g for 10 minutes.

The nuclei were washed in buffer M and used for either DNA extraction or the preparation of DNA methylase (see below).

7.2 Nuclear soluble fraction.

Nuclei obtained from either Krebs II ascites tumour cells or L-929 cells, were resuspended in buffer M and made 0.2M with respect to NaCl (nuclei first resuspended in 5 volumes of buffer M and then equal volume of buffer M containing 0.4M NaCl was added). This was stirred

gently for 15-20 minutes and centrifuged at 12,000 g for 20 minutes to yield a preparation termed nuclear soluble fraction (NSF).

7.3 Nuclear insoluble fraction.

The pellet obtained after centrifugation at 12000 g was resuspended in buffer M and 0.4M Nacl as described in sec.7.2, and thus two more extractions were made to remove any remaining soluble DNA methylase from the pellet. The pellet was then resuspended in buffer M containing 2M NaCl for 20 minutes with constant stirring. The suspension was then centrifuged at 190,000 g in Beckman SW 50.1 rotor for 40 hours. The pellet was washed in 10 volumes of buffer M (twice) following centrifugation at 12,000 g for 20 minutes and homogenised in buffer M. This preparation is termed nuclear insoluble fraction (NIF).

All steps were carried out at 0-4^{o}C.

7.4. SCHEME FOR ISOLATION OF DNA METHYLASES.

Nuclei

(Suspended for 15 minutes in buffer M + 0.2M NaCl)

Centrifuged at 12,000 g for 20 minutes.

Pellet	Supernatant
resuspended in buffer M + 0.2M NaCl	Nuclear soluble fraction (NSF)
(This extraction was repeated three times to remove remaining soluble DNA methylase, if any)	contains Soluble form of DNA methylase.

Pellet	Supernatant
resuspended in buffer M + 2M NaCl for 15 minutes	
Centrifuged at 190,000 g for 40 hours	

Pellet	Supernatant
rinsed with buffer M (twice)	includes histones
Centrifuged at 12,000 g for 20 min.	

Pellet	Supernatant
homogenised in buffer M.	
Nucler insoluble fraction (NIF) contains	
Bound form of DNA methylase and nonhistine proteins.	

Note: All steps were carried out at 0-4^{o}C.

7.5 Other preparation.

7.5.1 Phosphocellulose chromatography of NSF.

7.5.1.1 Preparation of phosphocellulose.

20 g of cellulose phosphate P11 was resuspended in 300 ml of water, several cycles of phosphocellulose suspension was carried out to remove fine particles. It was then collected on Buchner funnel and resuspended in 1 litre distilled water, with constant stirring 100 ml of 0.5M NaOH was added and stirring was continued for further 5 minutes. The suspension was washed with one litre water, to it 100 ml of 0.5M HCl was then added with constant stirring and stirring was continued for further 5 minutes. The suspension was washed with water until all the aid has been removed.

Phosphocellulose was then resuspended in buffer M containing 0.2M NaCl in a 500 ml beaker, pH was adjusted to 7.8 with washings of buffer M containing 0.2 M NaCl. Nonspecific binding sites of phosphocellulose were saturated with bovine serum albumin (100 µg/ml). Suspension was then washed with buffer M containing 0.5M NaCl to remove all excess of BSA. Afterwards suspension was washed with buffer M containing 0.2M NaCl and the optical density at 280 nm was measured until zero.

7.5.1.2 Chromatographic procedure.

To a nuclear soluble fraction (NSF) phosphocellulose suspension (see sec 7.5.1.1.) was added and stirred for 30 minutes at $0^{o}C$. After centrifugation at 12,000 g for 20 minutes at $4^{o}C$, the pellet was re-suspended in buffer M containing 0.2M NaCl and was pumped into the column (dia 1 cm), washed with buffer M containing 0.2M NaCl at a flow rate of 30 ml per hour until the optical density at 280 nm fell to zero. To elute the enzyme the column was then washed with buffer M containing 0.5M NaCl and 1 ml fractions were collected until the 280 nm

fell to zero. The column was again washed with buffer M containing 1M NaCl and 1 ml fractions were collected. The fractions with peak optical densities (of 0.5M and 1M NaCl elutes) were pooled and dialysed against two changes of buffer M (100 volumes) overnight at 4°C.

7.5.2 Ammonium sulphate precipitation of NIF.

Solid ammonium sulphate was slowly added with stirring to the nuclear insoluble fraction, until the latter was 30% saturated with respect to ammonium sulphate. Stirring was continued for 2 hours and the sample was centrifuged at 190,000 g overnight at 4°C in Beckman SW 50.1 rotor. The precipitate was discarded and the supernatant made to 60% saturation with ammonium sulphate. The sample was stirred again for 2 hours at 4°C and centrifugation was carried out as above for 5 hours. The precipitate was then dissolved in minimum volume of buffer M.

7.5.3 Glycerol density gradient centrifugation of NIF.

It was done as described by Turnbull (1976). Linear 10-25% glycerol gradients were prepared by layering successively 25%, 20%, 15% and 10% (v/v) buffered glycerol containing 0.2M NaCl in centrifuge tubes. The tubes were kept standing in the refrigerator for 8 hour to allow the density gradient to form by diffusion. 0.2 ml of the enzyme sample along with marker enzyme was layered on to the gradient and then centrifuged at 134,000 g for 16 hours at 4°C in Beckman SW 50.1 rotor. Gradients were harvested by upward displacement with 50% glycerol using MSE gradient harvester, 8 drop fractions being collected.

7.5.3.1 Alcohol dehydrogenase assay.

The method is based on the absorption of NADH at 340 nm. With ethanol in excess the rate of NAD reduction is proportional to the enzyme

concentration.

$$CH_3CH_2OH + NAD^+ \longrightarrow CH_3CHO + NADH + H^+$$

In 1 ml cuvette at room temperature following solution was added.

0.032M tetrasodium pyrophosphate (pH8.8)	= 0.45 ml
25 mM Nicotinamide adenine dinucleotide(NAD)	= 0.30 ml
2M absolute alcohol	= 0.15 ml

The optical density at 340 nm was adjusted to zero and then to it 50 μl of the enzyme sample was added and the optical density was measured against a blank solution at 15 second intervals.

8. Isolation of DNA.

8.1 Extraction of DNA.

DNA was prepared according to the procedure of Flint et. al., (1976) with slight modification.

The nuclear pellet was suspended in 20 volumes of buffer M. Sodium dodecyl sulphate and pronase (self digested for 3 hours at 37°C, 5 ml/ml distilled water (w/v)), was added to final concentrations of 0.5% (w/v) and 0.1% (w/v) respectively. After digestion at 37°C for 3 hours, the mixture was extracted twice with buffer saturated phenol and once with chloroform-isoamyl alcohol (24:1 v/v). The aqueous phase adjusted to 0.2M NaCl and nucleic acids were precipitatd overnight at -20°C after the addition of 2 volumes of ethanol. The precipitate was collected and dissolved in buffer M and incubated for 1 hour with 50μ g/ml H_2O ribonuclease-A (previously boiled for 10 minutes to remove any DNAase activity) at 37°C. Predigested pronase was then added to 50μ g/ml and incubation continued for 3 hours. The mixture was again extracted with buffer saturated phenol and chloroform-isoamyl alcohol and DNA was precipitated with 2 volumes of ethanol and 0.2M NaCl to the final concentration. The DNA was lyophilised and dissolved in 50mM KCl.

8.2 Preparation of denatured DNAs.

Denatured DNAs of E. coli, calf thymus and M. luteus were prepared by heating their solutions (dissolved in 50mM KCl) at 100°C for 10 minutes followed by rapid cooling in ice.

8.3 Preparation of heavy and light DNAs.

Heavy and light DNA from L-929 cells, was prepared with minor modification, according to the procedure of Adams (1971).

L-929 cells grown to stationary phase were transferred into Eagle's minimum essential medium containing 0.25 μCi ^{14}C-deoxycytidine, 2 μM aminopterin, 100 μM glycine, 60 μM hypoxanthine, 10 μM bromodeoxyuridine (Brd Urd), and 2 μM thymidine. After 20 hours incubation at 37°C, the cells were harvested in ice cold PBS buffer and the nuclei were made (see sec 7.1), Nuclear soluble and insoluble fractions were prepared (see sec 7.2 and 7.3 respectively) from such nuclei.

9. Analysis of DNA.

9.1 Analysis of the product of DNA methylase activity.

Nuclear insoluble fraction and S-adenosyl-L-(methyl-^{3}H)-methionine were incubated for 3 hours at 37°C without substrate DNA. The reaction was terminated with stopper solution-B. The normal DNA methylase assay was carried out to 5% TCA precipitation in a centrifuge tube. The DNA was then dissolved into 0.2 ml of formic acid and transferred into pyrolysis tube. The tube was sealed and the DNA was hydrolysed to the bases, by heating for 2 hours at 170°C. The formic acid was then evaporated and the dried sample was dissolved in 100 μl of 20mM ammonium carbonate buffer (pH 10.0). Enough sucrose was added to make the sample denser than running buffer. The bases were

separated on a column of Aminex-A6 by high performance liquid chromatography.

9.2 Analysis of in vitro methylation of nascent and/or parental DNA.

9.2.1 Preparation of DNA.

Nuclear insoluble fraction and S-adenosyl-L-(methyl-^{3}H)-methionine were incubated for 1 hour at 37^{o}C without substrate DNA. The reaction was terminated with stopper solution-B. The normal DNA methylase assay was carried out to TCA precipitation. The DNA was then dried under vacuum.

9.2.2 Alkaline CsCl density gradient centrifugation.

It was done according to the procedure of Adams (1971). The DNA obtained after lyophilisation was dissolved in alkaline CsCl solution (1.5 g CsCl/ml of 0.1M NaOH, density 1.79 g/c^{3}). The solution was transferred into polyalomer tube layered with paraffin oil and centrifuged at 83,000 g for 40 hours in Beckman SW 50.1 rotor at 20^{o}C. Two drop fractions were collected by piercing the tube in the bottom. Triton-toluene was added to each (5ml/vial) fraction and radioactivity was measured using scintillation spectrophotometery.

10. Determination of radioactivity.

10.1 Solutions required for assay of radioactivity by scintillation counting.

a. Toluene-PPO scintillation solution was prepared by dissolving 2,5-diphenyloxazol (PPO) in toluene at a concentration of 5 g per litre (0.5% w/v).

b. Triton-toluene scintillation solution consisted of 5 g PPO plus 0.5 g of p-bis(O-methyl-steryl)-benzene(Bis MSB), dissolved in 350 ml of triton-X100 and 650 ml of toluene.

11. Determination of protein and DNA.

Protein was estimated by the method of either Lowry et al., (1951) or Bradford (1976) using bovine serum albumin as standard.

DNA was estimated by the method of Burton (1956), using native calf thymus DNA as standard.

RESULTS

1. Search for separate DNA methylase activities in mouse cell nuclei.

Sections 5 and 6 of the introduction outlined the case for involvement of DNA methylases in initiation (or alteration) and maintenance of DNA methylation patterns, but no evidence is yet available that these patterns are performed by different DNA methylase activities. DNA methylases from various mammalian cell nuclei have been isolated by extraction with low salt concentration and have been further purified (Roy and Weissbach 1975, Sneider et al., 1975, Turnbull and Adams 1976, Cato et al., 1978, Simon et al., 1978). These enzymes have shown almost similar substrate specificity. However, the possibility cannot be excluded that nuclei contain additional methylase activity and that this is lost during the purification process or remains associated with the insoluble nuclear material. To examine this possibility the nuclei from Krebs II ascites tumour cells are re examined for further methylase activities or additional factors which might modulate the activity and specificity of the already extractable DNA methylase mentioned above.

1.1 Normal extraction of DNA methylase at low salt concentration.

Treatment of nuclei with buffer M containing 0.2M NaCl was followed by centrifugation at 12000 g for 20 minutes at 4^{o}C. This extracts about 86% of the total methylase activity into the supernatant. This is in close agreement with the results of Turnbull and Adams (1976), using buffer M containing 0.4M NaCl. This fraction which will be referred to as the nuclear soluble fraction (NSF) (Table 1).

1.2 Ion exchange chromatography on phosphocellulose.

The NSF fraction was then subject to phosphocellulose chromatography as described by Adams et al., 1979. As found by these

Table 1. Extraction of DNA methylase from Krebs II ascites tumour cell nuclei at low salt concentration and behaviour on phosphocellulose column.

Fraction	Volume (ml)	Total protein (mg)	Sp. activity (p.mole CH_3 incorporated $hr^{-1}mg^{-1}$)
Nuclear soluble fraction	276	303.	9.9
0.5M NaCl Phospho-cellulose eluate	5.5	7.97	63.0
1M NaCl phospho-cellulose eluate	1.6	0.25	0

The assay mixture (70 µl) consisted of 20 µl of each fraction, 10 µl of denatured calf thymus DNA (2mg/ml), 10 µl of S-adenosyl-L-(methyl-^{3}H)-methionine (1.6µCi) and buffer M containing 0.1M NaCl at the final concentration.

authors the NSF was applied in 0.2M NaCl and was completely retained. DNA methylase activity was found to elute with 0.5M NaCl. To determine whether any further DNA methylase activity remain bound to the column, The column was first washed with 0.5M NaCl until no further DNA methylase eluted and then 1M NaCl was applied and DNA methylase activity assayed in the eluate (table 1).

1.3 Removal of soluble DNA methylase by repeated extraction at 0.2M NaCl.

The nuclear pellet remained after first extraction of soluble DNA methylase was further extracted with buffer M containing 0.2M NaCl. Table 2 shows that whilst some further activity is extracted on the second occasion. Further extractions did not yield any more DNA methylase activity.

1.4 Extraction of remaining nuclear material with 2M NaCl.

There is accumulating evidence that chromatin after treatment with 2M NaCl yields a chromosomal scaffold structure consists of residual nonhistone proteins which are associated with DNA sequences at the base of each loop (Adolph et al., 1977 a,b., Paulson and Laemmli 1977, Benyajati and Worcel 1976, Cook and Brazell 1976, 1978). These proteins include DNA and RNA polymerases, nucleases, chromosomal proteins assumed to be responsible for the control of gene expression in eukaryotes.

In order to find out whether further methylase activity is present in such nuclear structure. The material remaining in the nuclear pellet was further treated with 2M NaCl in the extraction buffer. Since 2M NaCl has been used to remove all histone proteins along with about 70% of the nonhistone proteins (Oudet et al., 1975, Paulson and Laemmli 1977, Razin et al., 1978). However, Table 2 shows

Table 2. Soluble and bound DNA methylase activites from mouse cell nuclei.

Nuclear fraction	Total volume (ml)	Total protein (mg)	Specific activity (units+/mg)
Ist 0.2M NaCl extract	77.0	66.0	17.1
2nd " "	32.0	11.0	13.8
3rd " "	35.0	3.2	0.1
4th " "	37.0	1.2	0.1
1st 2M NaCl extract	22.0	8.8	0
Nuclear insoluble fraction *	30.0	36.0	5.1

* For assay this fraction is suspended in buffer M.

\+ A unit of enzyme activity catalyses the incorporation of 1 pmole of methyl groups into DNA in one hour. Single stranded calf thymus DNA was present in all incubations.

surprisingly, no further methylase activity is solubilised with 2M NaCl. On the other hand material remaining insoluble with 2M Nacl does show the enzyme activity which under the condition of the assay of the soluble activity is about 15% of the total enzyme activity in cell nuclei.

2. Various soluble nuclear extracts that modulate DNA methylase activity from soluble and insoluble fractions.

2.1 Effect of 1M NaCl phosphcellulose eluate on partially purified DNA methylase.

While 84% of the enzyme activity can be recovered in NSF and then partially purified when eluted from phosphocellulose column at 0.5M NaCl (Turnbull, 1976), there is no evidence for DNA methylase activity in the fraction eluted at higher concentration of salt (Table 1). However, to determine whether proteins eluted at 1M NaCl have any effect on the activity of DNA methylase, equal volumes of 1M NaCl phophocellulose eluate and 0.5M NaCl phosphocellulose eluate were mixed before assay of DNA methylase activity, Table 3 shows that the activity of partially purified DNA methylase from the 0.5M NaCl phosphocellulose eluate in the presence of 1M NaCl phosphocellulose eluate was inhibited about 44% (the protein ratio between 1M and 0.5M NaCl phosphocellulose eluates was 1:7).

The inhibition of DNA methylase activity can be postulated to operate in the following ways (a) either inhibition is caused by protection of methylatable sites in DNA possibly by DNA binding proteins, (b) the inhibitor was removing methyl groups from certain sites previously methylated by DNA methylase i.e. a DNA demethylase activity, (c) the inhibitor is removing potential unmodified sites in the DNA i.e. nuclease activity or (d) protease activity.

Several experiments were done to examine all these possibilities (Results not shown). It was found that the proteins eluting at 1M NaCl from phosphocellulose column was a mixture of different proteins.

Table 3. The effect of 1M NaCl phosphocellulose eluate on partially purified DNA methylase activity.

Fraction	Addition to incubation	Methylase activity (dpm incorporated hr^{-1})	% Control
0.5M NaCl phospho-cellulose eluate	None	6000	100
"	1M NaCl phospho-cellulose eluate.	3700	57

The assay mixture (70 µl) consisted of 22 µg protein of 0.5M NaCl phosphocellulose eluate and 3 µg protein of 1M NaCl phosphocellulose eluate, 20 µg of heat denatured calf thymus DNA, 1.6 µCi of S-adenosyl-L-(methyl-^{3}H)-methionine in buffer M containing 0.1M NaCl at the final concentration.

Whilst these included some DNA binding proteins, and the fraction also contained considerable nuclease activity which made further analysis difficult.

2.2 Effect of 2M NaCl extract on soluble DNA methylase activity.

To determine, whether proteins extracted with 2M NaCl have any effect on the activity of DNA methylase in nuclear soluble fraction (NSF). Table 4 shows that when 2M NaCl extract was added to nuclear soluble fraction before incubation, the enzyme activity of soluble DNA methylase is inhibited more than 50%. This inhibition of course could simply because of histones in the 2M NaCl extract. To examine this position the effect of histones and 2M NaCl extract on the enzyme activities in nuclear soluble and insoluble fractions was studied.

2.3 Comparison between the effect of 2M NaCl extract and histones on the enzyme activity in nuclear soluble and insoluble fractions.

Table 5 shows that histones present in the 2M NaCl extract may be a likely explanation of inhibition of soluble DNA methylase activity. Histones may simply bind more specifically to the substrate DNA and block methylation by the soluble enzyme. However, neither histones nor 2M NaCl extract had an effect on the bound activity. It may be that the bound activity is part of a complex which included DNA and other components. That is why it is inaccessible to histones and is unaffected.

3. Studies on DNA methylase remaining insoluble after 2M NaCl extraction of nuclei.

3.1 Product of insoluble DNA methylase.

Before an enzyme can be unequivocally characterised as a

Table 4. The effect of 2M NaCl extract on the enzyme activity from nuclear soluble and insoluble fractions.

Fraction	addition to incubation	Methylase activity (dpm incorporated hr^{-1})	% control
Nuclear soluble fraction	None	1016	100
"	2M NaCl extract (10μg protein)	412	41
Nuclear insoluble fraction	None	492	100
"	2M NaCl extract (10μg protein)	460	94

The assay mixture (70μl) consisted of 20μg protein of each soluble and insoluble fraction, 10μg protein of 2M NaCl extract, 20μg of heat denatured calf thymus DNA and 1.6μCi of S-adenosyl-L-(methyl-^{3}H)-methionine and buffer M containing 0.1M NaCl at the final concentration.

Table 5. The effect of histones and 2M NaCl extract on the enzyme activity in nuclear soluble and insoluble fractions.

Fraction	addition to incubation	DNA substrate	methylase activity (dpm incorporated hr^{-1})	% control
Nuclear soluble fraction	None	+	1016	100
Nuclear soluble fraction	2M NaCl extract	+	412	41
Nuclear soluble fraction	histones	+	390	37
Nuclear insoluble fraction	None	+	492	100
Nuclear insoluble fraction	2M NaCl extract	+	460	94
Nuclear insoluble fraction	histones	+	490	100
Nuclear insoluble fraction	None	-	480	100
Nuclear insoluble fraction	2M NaCl extract	-	440	92
Nuclear insoluble fraction	histones	-	444	93
-	2M NaCl extract	+	0	0
-	histones	+	0	0

Assay mixture (70µl) contained 20µg protein of each nuclear soluble and insoluble fractions, 20µg of histone proteins, 10µg of 2M NaCl extract, 20µg of single stranded DNA of calf thymus and 1.6µCi of S-adenosyl-L-(methyl-^{3}H)-methionine in buffer M containing 0.1M NaCl at the final concentration.

eukaryotic DNA methylase, one must be certain that only product is 5-methyl cytosine. Nuclear insoluble fraction was used as the source of enzyme and also for DNA substrate. The isolated methylated DNA was digested with formic acid and the bases were separated on Aminex A6 column by high performance liquid chromatography, a most sensitive method used for the analysis of 5-methyl cytosine. The only base methylated by the enzyme in nuclear insoluble fraction was cytosine. Figure 3 shows the distribution of radioactive label in endogenous DNA methylated by the enzyme bound to insoluble nuclear components.

3.2 Accessibility of the methyl product to DNAase-I.

Results given in table 6 show that when nuclear insoluble fraction incubated in the presence of S-adenosyl-L-(methyl-^{3}H)-methionine for 6 hours was digested with DNAase-I, about 80% of the radioactivity was found in acid insoluble precipitatable material. This low figure of digestion may show that DNA in nuclear insoluble fraction is closely associated with other proteins in such a way that it is inaccessible to DNAase-I. Similarly when thymidine labelled nuclear insoluble fraction was digested with DNAase-I for 1 hour at 37^{o}C, the percent digestion of DNA of thymidine labelled nuclear insoluble fraction was found lower than that of isolated thymidine labelled DNA, it further supports the idea that DNA in the nuclear insoluble fraction is closely associated with other proteins.

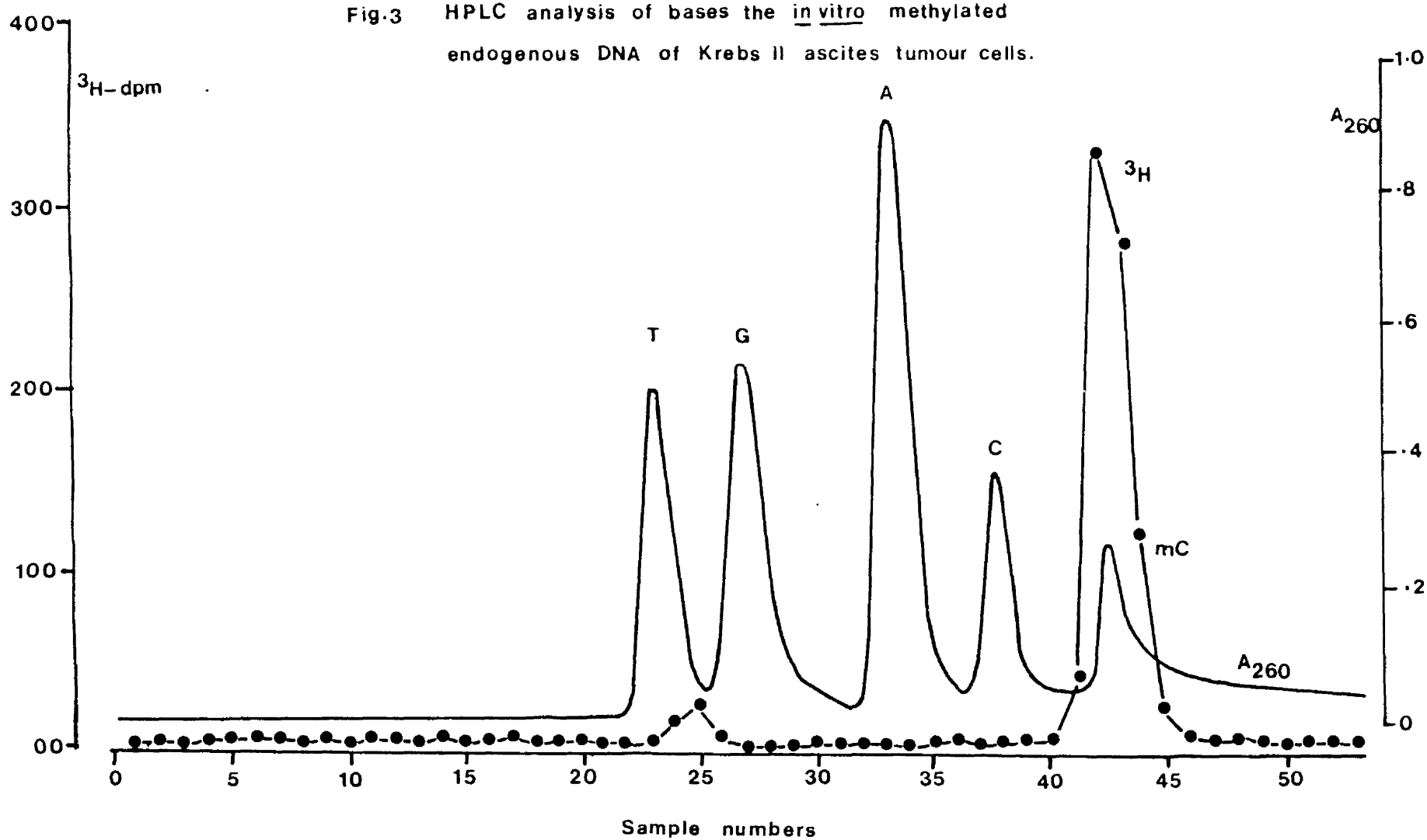
3.3 Independence of enzyme activity in nuclear insoluble fraction of added calf thymus DNA substrate.

Previous experiments showed that there is some proportion of enzyme activity bound to nuclear material. While examining the activity in the presence of exogenous DNA (calf thymus), it is revealed that whereas soluble DNA methylase requires the addition of exogenous DNA for

Figure 3. HPLC analysis of bases the in vitro methylated endogenous DNA of Krebs II ascites tumour cells.

The assay mixture (280µl) consisted of 160µl of nuclear insoluble fraction (NIF), 40µl of S-adenosyl-L-(methyl-^{3}H)-methionine (6.5µCi) and 80µl of buffer M was incubated for 3 hours at 37^{o}C. The reaction was terminated with stopper solution-B. The proteins were removed and the DNA was precipitated. The DNA was then hydrolysed in 100 l of 0.5M NaOH at 37^{o}C for 1 hour. The dissolved DNA was then precipitated with 20µl of 50% TCA and washed three times with ice cold 5% TCA with subsequent centrifugations at 800 g for 5 minutes at 4^{o}C. The DNA was then extracted with ethanol. After centrifugation at -10^{o}C for 20 minutes at 1000 g, the DNA was dried and hydrolysed in 0.3 ml of formic acid at 170^{o}C for 2 hours. The formic acid was then evaportated and the dried sample was dissolved in 200µl of 20mM ammonium carbonate buffer (pH 10.0) and was loaded on to the Aminex-A6 column. 1ml fraction was collected and the radioactivity was measured under scintillation counter.

Fig.3 HPLC analysis of bases the in vitro methylated endogenous DNA of Krebs II ascites tumour cells.



Labelling of Krebs II ascites tumour cells in vivo

^{3}H-thymidine was injected into the intraperitoneal cavity of tumour bearing mice (4 mice were injected, in each 50µCi [6-^{3}H]-thymidine (21 Ci/mmole)). After 15 minutes time from injection the ascites cells were harvested. Nuclei prepared from such cells were divided into the equal parts. One was used for DNA preparation and from other part nuclear soluble and insoluble fractions were made.

(3) ^{3}H-thymidine labelled NIF: It was incubated with DNAase-1 (1mg/ml) for 1 hour at 37^{o}C. The reaction was terminated with 1% SDS solution.

(4) ^{3}H-thymidine labelled DNA: The DNA prepared according to the procedure of Flint et al., (1976), was digested with DNAase-I as above.

Table 6. Deoxyribo-nuclease-1 digestion of nuclear insoluble fraction from Krebs II ascites tumour cell nuclei.

	Sample	% digestion *
(1)	Nuclear insoluble fraction incubated in the presence of S-adenosyl-L-(methyl)-^{3}H)-methionine was further digested with DNAase-1.	20
(2)	DNA isolated from nuclear insoluble fraction incubated with S-adenosyl-L-(methyl-^{3}H)methionine.	82
(3)	Nuclear insoluble fraction from cells labelled *in vivo* with ^{3}H-thymidine.	65
(4)	DNA isolated from cells, labelled with ^{3}H-thymidine *in vivo*.	85

* % digestion: The radioactivity remained in the acid soluble precipitates in comparison to control samples.

(1) Nuclear insoluble fraction (80µl) was incubated in the presence of 6.5µCi of S-adenosyl-L-(methyl-^{3}H)-methionine for 6 hours at 37°C (total incubation mixture: 280µl). Before termination of the reaction the mixture was digested with DNAase-1 (1 mg/ml) for further one hour at 37°C, and then usual assay was carried out.

(2) Nuclear insoluble fraction (160µl) was incubated in the presence of S-adenosyl-L-(methyl^{3}H)-methionine (13.0µCi) for 6 hours at 37°C the reaction was terminated with stopper solution-B (without carrier DNA) and the DNA was isolated according to the procedure of Flint et al., (1976). The DNA was then dissolved in buffer M and digested with DNase-I as above.

activity (table 7), the bound DNA methylase did not respond to added calf thymus DNA but appears to methylate the endogenous DNA with which it is associated, either directly or in a complex with nonhistone proteins. The activity of bound DNA methylase with other exogenous DNA substrates has been shown elsewhere (see sec.4.2.4).

3.4 Effect of divalent cations on DNA methylase activity in nuclear insoluble fraction.

When Bryan and her associates analysed the nuclei from calf thymus for metal content, after repeated washings in tris-buffer (pH7.4) about 0.1μg Zn^{+2}/mg DNA and 0.025μg Cu^{+2}/mg DNA was found associated with chromatin. This level of metal was essentially unchanged with subsequent washings (Bryan et al., 1981). Similarly scaffolding structure contains Cu^{+2} (or possibly Ca^{+2}) which stabilising this structure against dissociation by histone extraction buffer (Lewis and Laemmli 1982) Chelating of the metal leads to dissociation of the scaffolding structure and complete unfolding of the DNA.

In order to find out whether DNA methylase bound to nuclear components require any divalent cation for its activity. The nuclear insoluble fraction was assayed for DNA methylase activity in the presence of various concentrations of copper and magnesium. The results displayed in table 8 show that DNA methylase bound to nuclear components does not require divalent cations for its activity. Indeed a considerable inhibition of activity was observed with Mg^{+2}. With various concentrations of manganese and zinc, no real stimulation or inhibition was observed (results not shown).

3.5 The effect of sulphydryl group blocking reagent on enzyme activity in nuclear soluble and insoluble fractions.

Like DNA polymerases (Aposhian and Kornberg 1962, Handerson

Table 7. Independence of enzyme activity in nuclear insoluble fraction of added DNA substrate.

Fraction	DNA substrate added (20μg)	Activity p.mole CH_3-incorporated $hr^{-1}mg^{-1}$ protein
Nuclear soluble fraction	None	0
Nuclear soluble fraction	calf thymus	17.2
Nuclear insoluble fraction	None	5.8
Nuclear insoluble fraction	calf thymus	5.0

The assay mixture (70μl) consisted of 20μl of each fraction, 1.6μCi of S-adenosyl-L-(methyl-^{3}H)-methionine and buffer M containing 0.1M NaCl. In the incubation with nuclear insoluble fraction without substrate DNA, the protein DNA ratio was 1:1.3.

Table 8. The effect of divalent cations by DNA methylase activity in nuclear insoluble fraction.

Fraction	addition to incubation	dpm incorporated hr^{-1}
Nuclear insoluble fraction	None	188
" " "	5mM $MgCl_2$	112
" " "	10mM "	52
" " "	15mM "	68
" " "	20mM "	28
" " "	25mM "	28
Nuclear insoluble fraction	None	100
" " "	1mM $CuCl_2$	46
" " "	2mM "	72
" " "	3mM "	100
" " "	4mM "	100

Assay mixture (70μl) contained 20μl of nuclear insoluble fraction, 1.6μCi S-adenosyl-L-(methyl-^{3}H)-methionine, various amounts of $MgCl_2$ or $CuCl_2$ solution and buffer M.

1972), DNA methylases have shown sulphydryl reagent requirement for their activity (Roy and Weissbach 1975, Turnbull and Adams 1976, Simon et al., 1978). In order to find out whether DNA methylase bound to nuclear components require sulphydryl group for its activity. The results presented in table 9 demonstrate that when DNA methylases from nuclear soluble and insoluble fractions were incubated in the presence of 1mM p-chloromercuribenzoate (Sulphydryl-group blocking agent) the enzyme activity of both fractions was severely affected. 2mM concentration of p-chloromercuribenzoate (p.c.m.b.) completely inhibit the activity of both enzyme preparations.

3.6 Effect of actinomycin-D on DNA methylase activity in nuclear insoluble fraction.

Actinomycin-D is a cyclic polypeptide antitumour drug which intercalates preferentially between GpC in double helical DNA (Sobel 1973). Deoxyguanosine residues are essential for binding reaction (Wells and Larson 1970, Sobel 1972). It has been reported that when native calf thymus DNA incubated with 10μM actinomycin-D, 55% inhibition of soluble DNA methylase activity occurred while little effect was noted when denatured E. coli DNA was used as methyl acceptor (Adams and Burdon 1982b). Table 10 shows that when nuclear insoluble fraction which of course contain endogenous DNA was incubated with actinomycin-D the methylation of the endogenous DNA by the bound DNA methylase was also inhibited (about 53%).

3.7 Analysis of parental and nascent DNA for product of in vitro methylation.

In 1975, Holliday and Pugh, and Riggs independently proposed an attractive model suggesting that DNA modification may be involved in

p-chloromercuribenzoate reagent.

p-chloromercuribenzoate solution was prepared according to the procedure of Handerson (1972). Weighed amounts of well dried preparation of p-chloromercuribenzoate (p.c.m.b.) were dissolved in a slight excess of dilute NaOH and diluted with 50mM tris-Hcl pH 7.8.

Table 9. The effect of sulphydryl group blocking reagent on DNA methylase activity in nuclear soluble and insoluble fractions.

Fraction	addition to incubation	% control
Nuclear soluble fraction	None	100
Nuclear soluble fraction	1mM p-chloromercuri-benzoate	10
Nuclear soluble fraction	2mM p-chloromercuri-benzoate	0
Nuclear insoluble fraction	None	100
Nuclear insoluble fraction	1mM p-chloromercuri-benzoate	30
Nuclear insoluble fraction	2mM p-chlromercuri-benzoate	0

The incubation mixture (140µl) consisted of 16µg protein of nuclear soluble fraction and 24µg protein of nuclear insoluble fraction, 40µg of calf thymus DNA (denatured) and 1.6µCi of S-adenosyl-(methyl $-^{3}H$)-methionine in buffer M containing NaCl at the final concentration of 0.1M.

(100% enzyme activity of nuclear soluble and insoluble fractions correspond to 1280 and 484 dpm per assay respectively).

Table 10. Effect of actinomycin-D on DNA methylase activity of nuclear insoluble fraction.

Fraction	addition	p.mole CH_3 incorporated $hr^{-1}mg^{-1}$ protein
Nuclear insoluble fraction	None	3.84
Nuclear insoluble fraction	actinomycin-D (2μg)	1.79

The assay mixture (140μl) consisted of 25μg protein and 22μg endogenous DNA of nuclear insoluble fraction prepared as described in materials and methods sec. 7.3) 3.2μCi of S-adenosyl-L-(methyl-^3H)-methionine and buffer M.

differentiation and development. Vertebrate DNAs contain 5-methyl cytosine as a modified base and modification is introduced specifically into progeny strand following DNA replication (Bird 1978). Furthermore, the methylation pattern present in the parental strand is semiconservatively copied after replication so that the pattern is passed to the daughter cells after division.

In order to find out which strand is methylated in vitro by the DNA methylase bound to nuclear components, a nuclear insoluble fraction containing heavy and light strands of endogenous DNA was prepared from mouse L-929 cells, by growth of the cells in the presence of [U-^{14}C]-deoxycytidine and bromodeoxyuridine (Brd Urd) for 20 hours at 37^{o}C (see materials and methods sec.8.3). The nuclear insoluble fraction was then prepared from these cells and incubated in vitro with S-adenosyl-L-(methyl-^{3}H)methionine. The methylated DNA of the L-cell nuclear insoluble fraction was then isolated and was dissolved in alkaline CsCl solution (1.79g/C^{3}) and separated into heavy and light strands by buoyant density gradient centrifugation. Figure 4 shows an incorporation of ^{3}H-methyl counts from S-adenosyl-L-(methyl-^{3}H)-methionine into the heavy strand, which suggest that DNA methylase bound to the L-cell nuclear insoluble fraction is methylating cytosine residues of the newly synthesised strand of the duplex DNA associated with that fraction.

4. Possible significance of "soluble" and "bound" forms of DNA methylase.

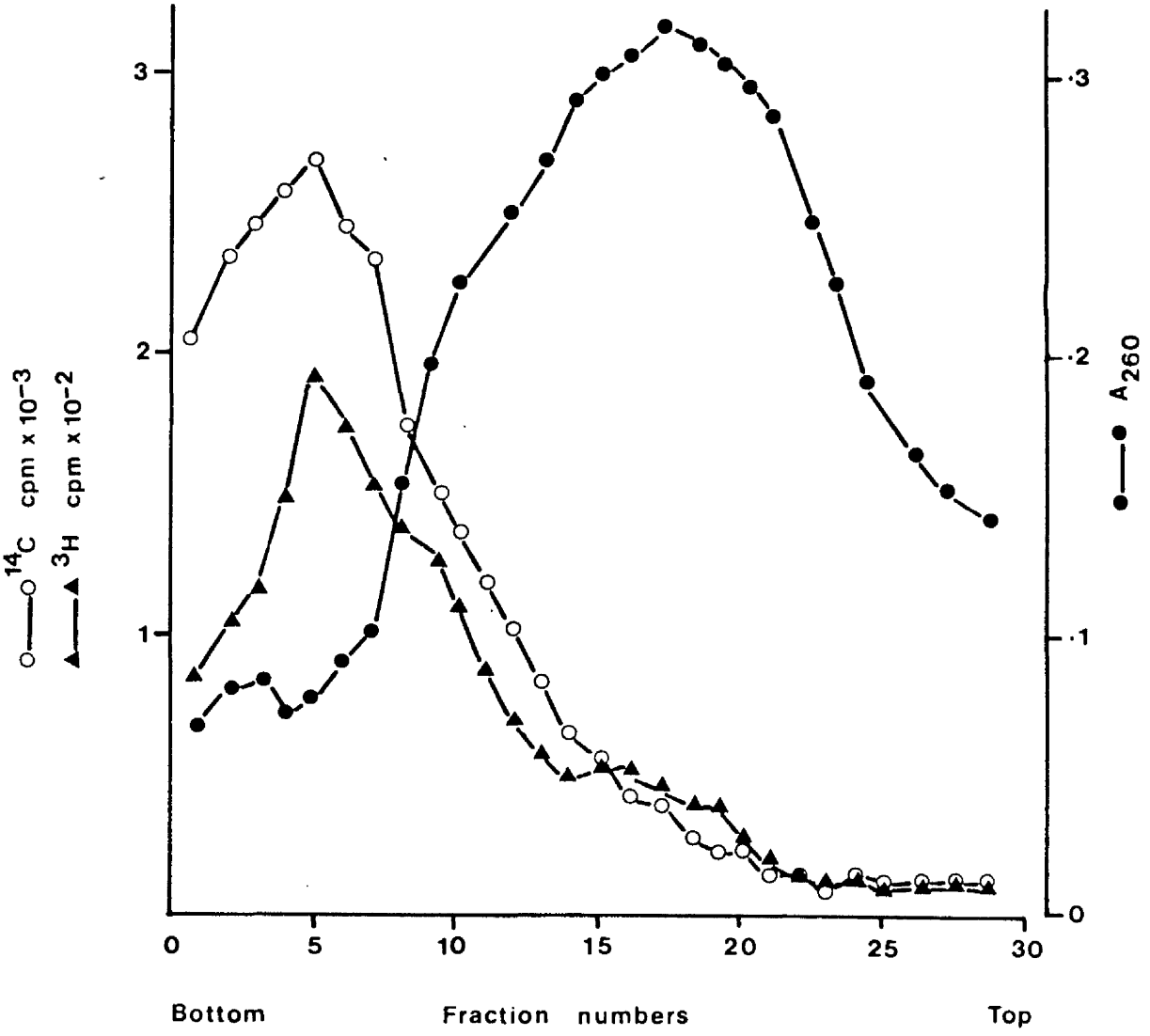
4.1 Examination of in vivo properties.

4.1.1 Methylation of endogenous DNA of rapidly growing and stationary mouse L-929 cells by "bound" DNA methylase.

In 1970, Kappler showed a linear rate of incorporation of

Figure 4. Alkaline CsCl equilibrium density gradients of in vitro methylation reaction product.

The DNA methylated by nuclear insoluble fraction was dissolved in alkaline CsCl solution (See materials and methods Sec. 9.2). The solution was centrifuged at 83,000 g for 40 hours in the Beckman SW50.1 rotor at 20°C. 2-drop fractions were collected starting from bottom of the gradient.


^{14}C cpm x 10^{-3}
^{3}H cpm x 10^{-2}
A_{260}
3
2
1
·3
·2
·1
0
0
5
10
15
20
25
30
Bottom
Fraction numbers
Top

radioactivity into cytosine and methyl cytosine of DNA, when ^{14}C-labelled deoxycytidine was administrated into rapidly growing cultured mouse cells, while other workers have shown a considerable delay between the time of synthesis of DNA and its eventually complete methylation (Burdon and Adams 1969, Adams 1974). In certain instances it appears that methylation continues well beyond the end of S-phase (Evans et al., 1973, Drahovsky and Walker 1975) but in general it is supposed that methylation would be complete prior to initiation of a new round of DNA synthesis.

In addition it has been shown that in eukaryotes, the DNA from homologous source can accept methyl groups *in vitro* indicating that DNA isolated from rapidly growing cells is probably not fully methylated *in vivo*. DNA isolated from nongrowing cells is on the other hand, in general, a poor acceptor of methyl groups probably due to methylation of most of the potential sites *in vivo* during the round of DNA synthesis. When nuclear insoluble fractions and their associated DNAs were isolated from rapidly growing and nongrowing mouse L-cells and incubated *in vitro* with S-adenosyl-L-(methyl-^{3}H)-methionine over a period of 12 hours (Figure 5), the methylation of the endogenous DNA of the nuclear insoluble fraction from rapidly growing cells is 4-fold higher than that prepared from nongrowing cells. This supports the view that the bound enzyme is probably methylating the newly synthesised strand of the duplex DNA which remained partly unmethylated in the preparations from the growing cells.

4.1.2 Effect of hydroxyurea on "soluble" and "bound" forms of DNA methylase from L-929 cells.

To investigate the relationship between these two forms of DNA methylases *in vivo*, mouse L-929 cells were preincubated with

Figure 5. Assay mixture (140µl) consisted of 36µg protein and 56µg DNA of nuclear insoluble fraction of L-929 log cells (–O—O–) and 36µg protein and 66µg DNA of nuclear insoluble fraction of L-929 stationary cells (–●—●–), 3.2µCi of S-adenosyl-L-(methyl-^{3}H)-methionine in buffer M.

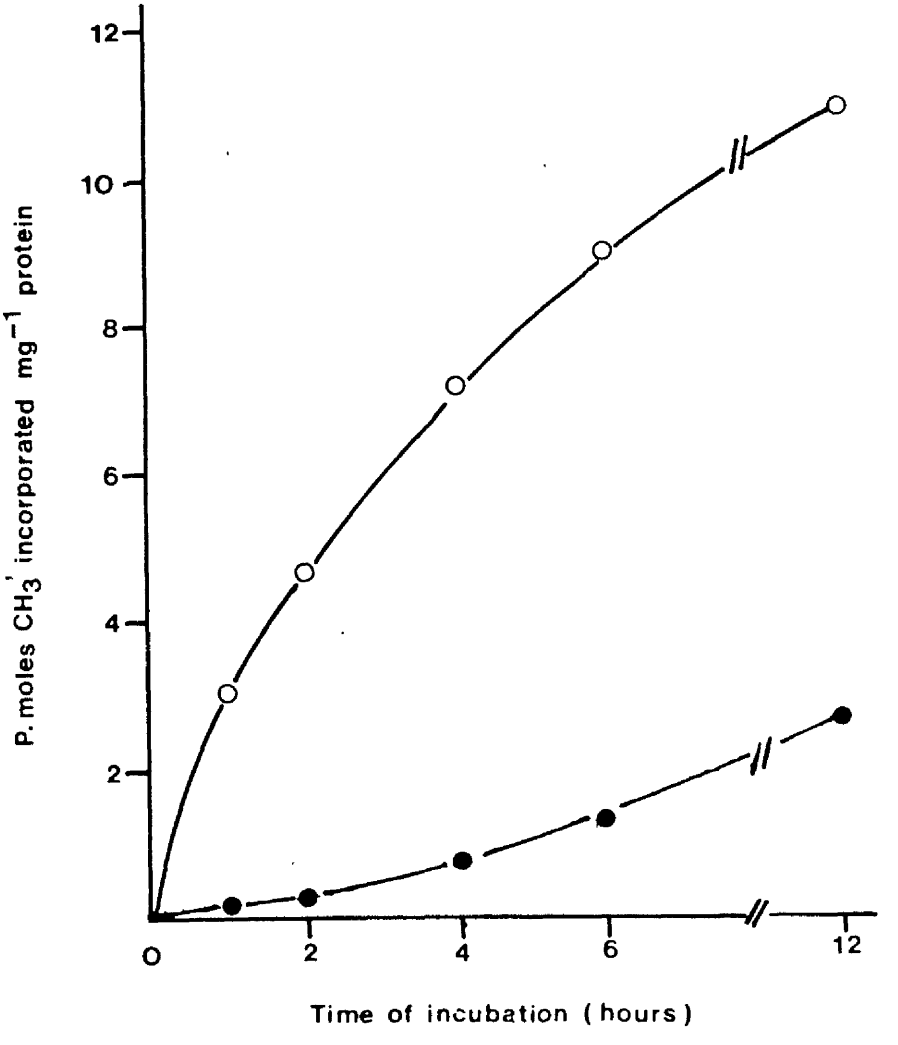


Figure 5. DNA methylase activity in nuclear insoluble fractions from L-929 cells harvested at stationary and log phase of growth.

hydroxyurea. This compound inhibits the activity of ribonucleoside diphosphate reductase (Krakoff et al., 1968, Theiss and Fisher 1976, Berglund and Sjoberg 1979, Engstron et al., 1979) causing an intracellular level of dATP and dGTP to become depleted (Skoag and Nordens-Kjold 1971, Skoag and Bjursell 1974, Theiss and Fisher 1976), with a consequent inhibition of DNA synthesis. Figure 6 shows that prior incubation of mouse L-929 cells with the drug for 6 hours leads to reduction of DNA methylase activity in the bound state and an increase in the soluble form. Thus, the association of DNA methylase with insoluble nuclear components in bound form probably requires continuing DNA replication.

4.1.3 Effect of cycloheximide on "soluble" and "bound" forms of DNA methylase from L-929 cells.

When L-929 cells were incubated for 8 hours with the potent protein synthesis inhibitor, cycloheximide, there was an increase in the activity of soluble form and a reduction in the activity of DNA methylase of bound form (Figure 7). The drug cycloheximide not merely inhibits the synthesis of protein but also inhibits the synthesis of DNA. From table II it can be seen that when L-929 cells were incubated in the presence of cycloheximide for 8 hours and labelled for further one hour with ^{3}H-leucine and ^{3}H-thymidine to examine protein and DNA synthesis, the tritium label incorporated into protein and DNA showed a reduction in the synthesis of both protein and DNA (2% and 5% respectively). This data supports the findings reported by Creusot et al., (1982) where synthesis of DNA in Friend erythroleukemia cells have shown inhibited by cycloheximide. Thus the association of DNA methylase with insoluble nuclear components in a bound form probably requires continuing DNA replication may be also protein synthesis.

Figure 6. Assay mixture (140µl) consisted of 20µg protein of each fraction (soluble and bound DNA methylase), 40µg denatured calf thymus DNA, 3.2µCi S-adenosyl-L-(methyl-^{3}H)-methionine and buffer M containing 0.1M NaCl. No substrate DNA used in the incubations with bound DNA methylase, as the preparation already contained DNA.

—O——O— DNA methylase activity from control cells.

—●——●— DNA methylase activity from cells treated with 2mM hydroxyurea for 6 hours.

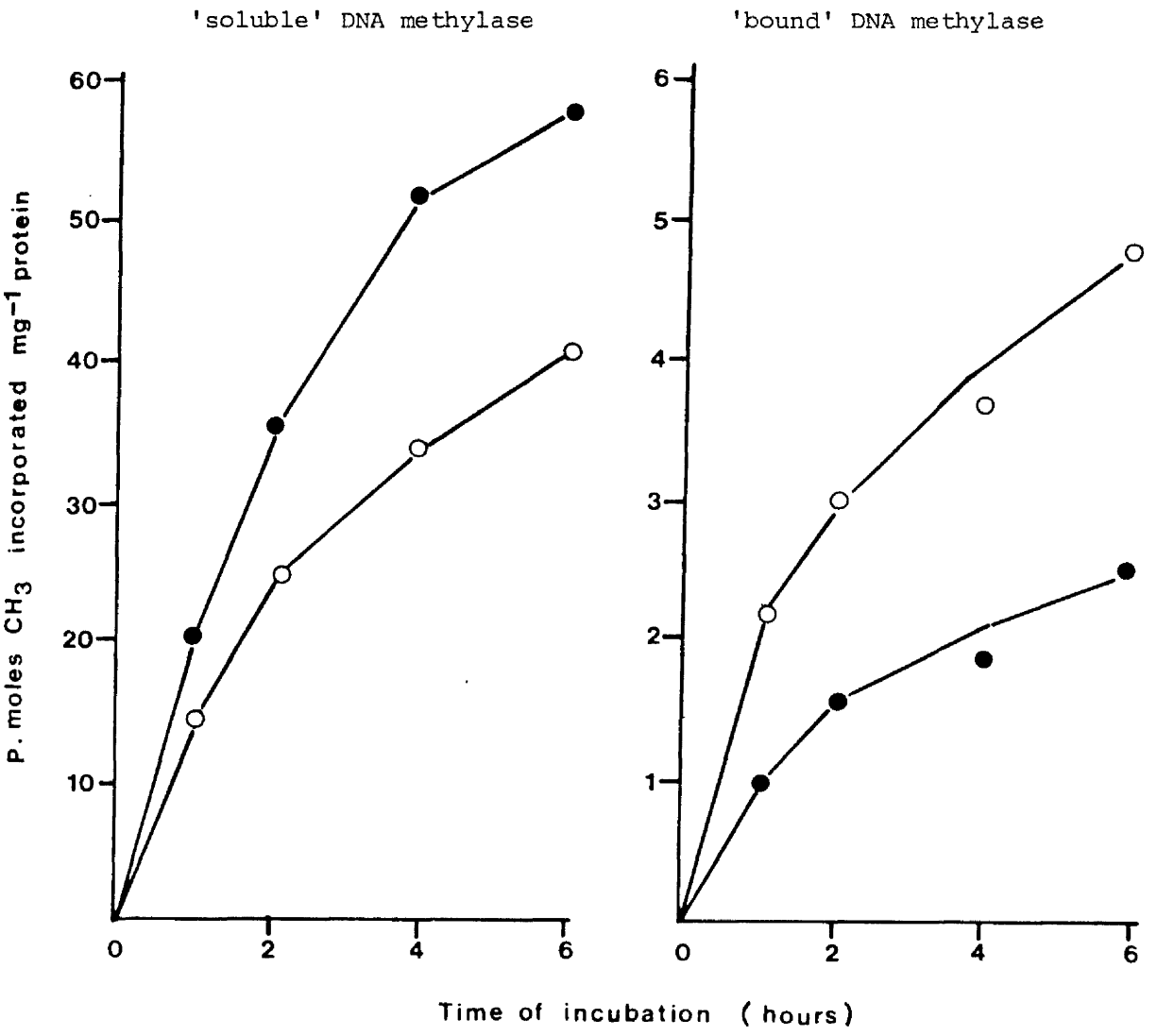


Figure 6. Effect of 2mM hydroxyurea on DNA methylase activities in mouse L-929 cells.

Figure 7. a. Assay mixture (140µl) consisted of 20µg protein of nuclear soluble fraction (soluble DNA methylase), 40µg denatured calf thymus DNA, 3.2µCi S-adenosyl-L-(methyl-^{3}H)-methionine and buffer M containing 0.1M NaCl.

b. Assay mixture (140µl) consisted of 40µg protein of nuclear insoluble fraction (bound DNA methylase), 3.2µCi S-adenosyl-L-(methyl-^{3}H)-methionine and buffer M containing 0.1M NaCl. No substrate DNA used as the preparation already contained DNA.

—O——O— DNA methylase activity from control cells.

—●——●— DNA methylase activity from cells treated with cyclo heximide (25µg/ml) for 8 hours.

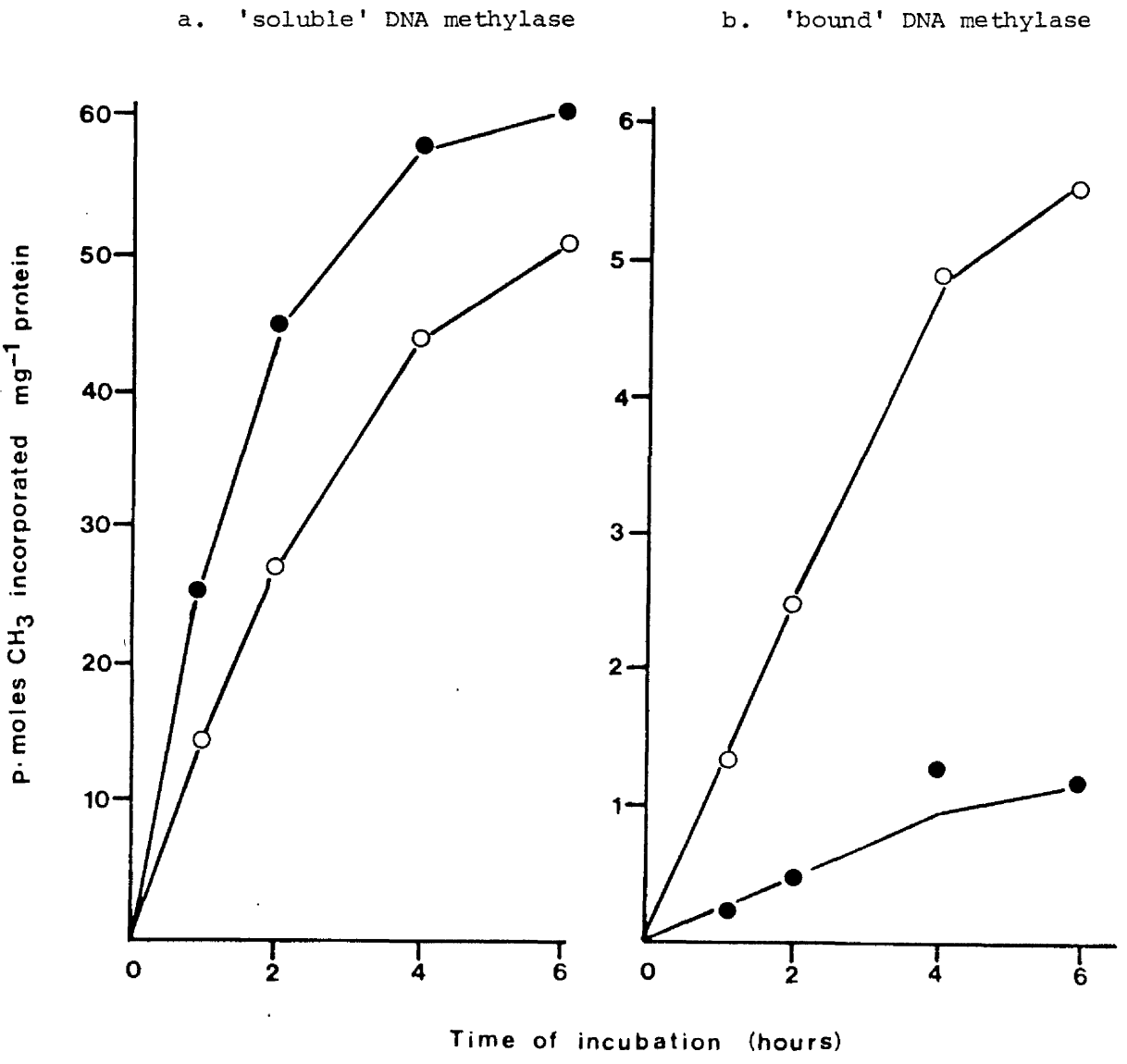


Figure 7. Effect of Cycloheximide on DNA methylase activities in mouse L-929 cells.

Table 11. The effect of cycloheximide and hydroxyurea on the synthesis of protein and DNA of L929 cells.

L-929 cells ($8x10^4$) were incubated into 5 cm dishes in the Glasgow's modification of Eagle's essential medium supplemented with 10% (v/v) calf serum as described in 'materials and methods'. Cells were allowed to grow for 23 hours and then the drugs cycloheximide (25μg/ml) and 2mM hydroxyurea were added and the incubation was continued for further 8 hours. Cells were labelled with ^{3}H-thymidine (2μ Ci/ml, specific activity 23 Ci/m.mole) and ^{3}H-leucine (1μ Ci/ml, specific activity 50 Ci/m.mole) for further one hour. After harvesting the cells in PBS buffer, were washed twice with PBS buffer and five times with 5% TCA following centrifugation at 800 g for 5 minutes at 4°. Afterwards the cells were washed in absolute alcohol and then incubated in 1ml of 0.3M NaOH for 30 minutes at 37°C. 0.5 ml of each aliquote was transferred in plastic vial with the addition of 5 ml of Triton-toluene and the radioactivity was measured under scintillation counter. Average incorporation of radiolabel into acid precipitatable materials by untreated cells was 117526 cpm/ 10^6 cells/hour for ^{3}H-thymidine, and 7049 cpm/10^6 cells/hour for ^{3}H-leucine. All values represent the average of two determinations on extracts from the same culture.

Table 11. The effect of cycloheximide and hydroxyurea on the synthesis of protein and DNA of L-929 cells.

	Rate of Synthesis	
Addition to medium	DNA	Protein
	% control	
cycloheximide (25µg/ml)	5	2
Hydroxyurea (2mM)	0.1	63

4.1.4 Effect of 5-azadeoxycytidine on DNA methylase activity in mouse (L-929) cell nuclei.

The antileukemic drug 5-azacytidine is capable of inhibiting a wide variety of critical cellular processes including synthesis of proteins, RNA and DNA (Cihak 1974). One of its effects, however is the inhibition of the DNA methylation process. Recently Creusot et al., (1982) have found that 5-azacytidine or 5-azadeoxycytidine causes a concentrations and time dependent loss of soluble DNA methylase activity from Friend erythroleukemia cells. They conclude that primary cause of inhibiton of DNA methylation may be the loss of DNA methylase activity rather than replacement of potentially methylatable cytosine residues by an analogue which cannot be methylated i.e. 5-azadeoxy cytidine. The treatment of Friend erythroleukemia cells for 10 hours with 1μM 5-azadeoxycytidine caused about 90% inhibition of DNA methylation.

In order to determine the effect of 5-azadeoxycytidine on both soluble and bound DNA methylase activities, mouse L-929 cells were incubated for 10 hours in the presence of 1μM 5-azadeoxycytidine. Figure 8 shows that the DNA methylase activity in the nuclear soluble fraction (soluble DNA methylase) is considerably reduced, which is in close agreement with the results of Creusot et al., (1982) as well as with the results of Adams et al., (1982) where level of DNA methylation was reduced to 20%, when L-929 cells were exposed to 1μM 5-azadeoxy cytidine for 2-3 days. On the other hand there is an increase in the activity of the bound enzyme. Precise quantitative comparison of these changes is difficult because the soluble enzyme is measured with exogenous DNA (E. coli) whereas the bound enzyme is methylating endogenous DNA.

4.1.5 Effect of cycloheximide on DNA methylase activities of 5-azadeoxycytidine treated L-929 cells.

Figure 8. Assay mixture (140µl) contained 20µg protein of each fraction, 40µg denatured E. coli DNA, 3.2µCi S-adenosyl-L-(methyl-^{3}H)-methionine and buffer M containing 0.1M NaCl. No DNA substrate used in the incubations with bound DNA methylase (NIF) as it already contained DNA.

—○——○— DNA methylase activity from control cells.

—●——●— DNA methylase activity from cells treated with 1µM 5-azadeoxycytidine for 10 hours.

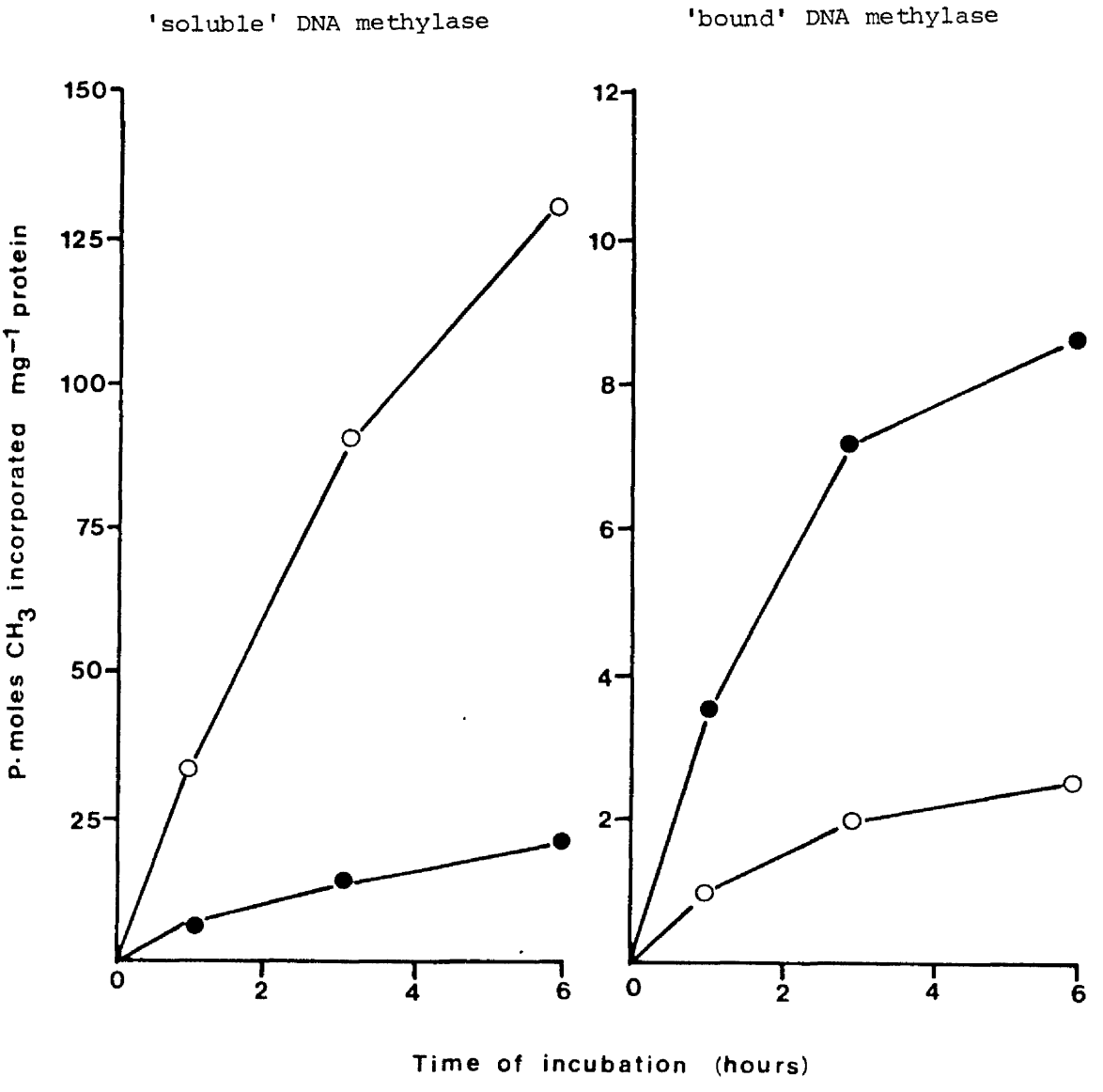


Figure 8. Effect of 5-azadeoxycytidine on DNA methylase activities in mouse L-929 cells.

When L-929 cells were exposed to 1μM 5-azadeoxycytidine for 10 hours, the activity of the soluble form of DNA methylase was reduced to about 14%. It has been suggested that the reduction of DNA methylation may be due to either arrest of DNA methylase which has been proposed to "walk" down the helix (Drahovsky and Morris 1971a) or irreversible binding of the enzyme closely to potentially modifiable sites containing 5-azacytosine residues (Jones and Taylor 1981). On the other hand from Figure 5 it can be seen that when protein synthesis was blocked in the presence of cycloheximide there was an increase in the activity of soluble form. In order to examine whether cyclo heximide can release any DNA methylase that becomes bound in presence of 5-azacytosine, cells treated with 5-azadeoxycytidine were subsequently treated with cycloheximide for 6 hours. Results given in table 12 shows that no significant amount of enzyme activity that becomes bound in presence of 5-azacytosine is released in cells treated with cyclo heximide.

4.1.6 Micrococcal nuclease digestion of nuclear insoluble fraction of 5-azadeoxycytidine treated L-929 cells.

Table 12 showed that cycloheximide treatment failed to release any significant amount of DNA methylase into a soluble form from the nuclear components of cells previously treated with 5-azadeoxycytidine. In order to find out more about the nature of binding of DNA methylase to nuclear components (including DNA), nuclear insoluble fraction from 5-azadeoxycytidine treated and untreated cells were incubated with high levels of micrococcal nuclease (2500 units/100μg chromatin DNA) for 45 minutes at 37°C. Figure 9 shows that while enzyme activity from untreated cells can be released from chromatin and be made dependent on exogenous substrate DNA, the bound DNA methylase activity from 5-azadeoxycytidine treated cells has not been so affected. It may be

Table 12. Effect of cycloheximide on the DNA methylase activity of 5-azadeoxycytidine treated L-929 cells.

L-929 cells grown to stationary phase in Glasgow's moeifications of Eagle's essential medium were split into four 80 oz rotating bottles (equal amount of cells in each bottle), which are treated as follows:

(1) L-929 cells grown for 20 hours in fresh Eagle's minimum essential medium and then harvested in PBS buffer.

(2) Cells were treated with 1μM 5-azadeoxycytidine at the 10th hour of growth and harvested at the 20th hour of growth. Referred to as L-aza cells.

(3) Cells treated as (2), at the 20th hour the medium was changed and left for further 6 hours and then harvested.

(4) Cells treated as (2), at the 20th hour the medium was changed with the fresh medium containing 25μg/ml cycloheximide and left for further 6 hours and then harvested.

Table 12. Effect of cycloheximide on the DNA methylase activity of 5-azadeoxycytidine treated L-929 cells.

Source	DNA methylase	total activity p.mole incorporated hr^{-1}	% control
1. L-929 cells	'soluble' DNA methylase	34.2	100
2. L-aza cells	"	6.2	18
3. L-aza + chase	"	7.9	23
4. L-aza + cycloheximide chase	"	11.2	33
1. L-929 cells	'bound' DNA methylase	7.5	100
2. L-aza cells	"	4.3	58
3. L-aza + chase	"	11.2	150
4. L-aza + cycloheximide chase	"	9.2	123

In 140µl incubation mixture, 20µg protein of each fraction, 20µg native M. leutus DNA, 3.2µCi S-adenosyl-L-(methyl-^{3}H)-methionine and buffer M containing NaCl at the final concentration of 0.1M.

Figure 9. Micrococcal nuclease digestion of nuclear insoluble fraction of mouse L-929 cells treated with 5-azadeoxycytidine.

Nuclear insoluble fractions (NIFs) of 5-azadeoxycytidine treated and untreated mouse L-cells were digested with micrococcal nuclease (2500 units per 100μg chromatin DNA) for 45 minutes at 37°C in a nucleasebuffer (Buffer M without EDTA, 1.2mM $CaCl_2$). The reaction was terminated with 1.6mM EDTA (pH7.5). The mixture was chilled in ice and then centrifuged at 12000 g for 30 minutes at 4°C. The supernatants were used for DNA methylase assay.

Assay mixture (210μl) consisted of 10μg protein of each fraction, 40μg denatured E. coli DNA, 3.2 μCi S-adenosyl-L-(methyl-^{3}H)-methionine and buffer M containing 0.1M Nacl.

▲, ■, ● DNA methylase activity from control cells.

△, □, ○ DNA methylase activity from cells treated with 5-azadeoxycytidine (1μM) for 10 hours.

▲——△ Undigested nuclear insoluble fraction with den. E. coli DNA.

■——□ Supernatant of digested nuclear insoluble fraction with den. E. coli DNA.

●——○ Supernatant of digested nuclear insoluble fraction without substrate DNA.

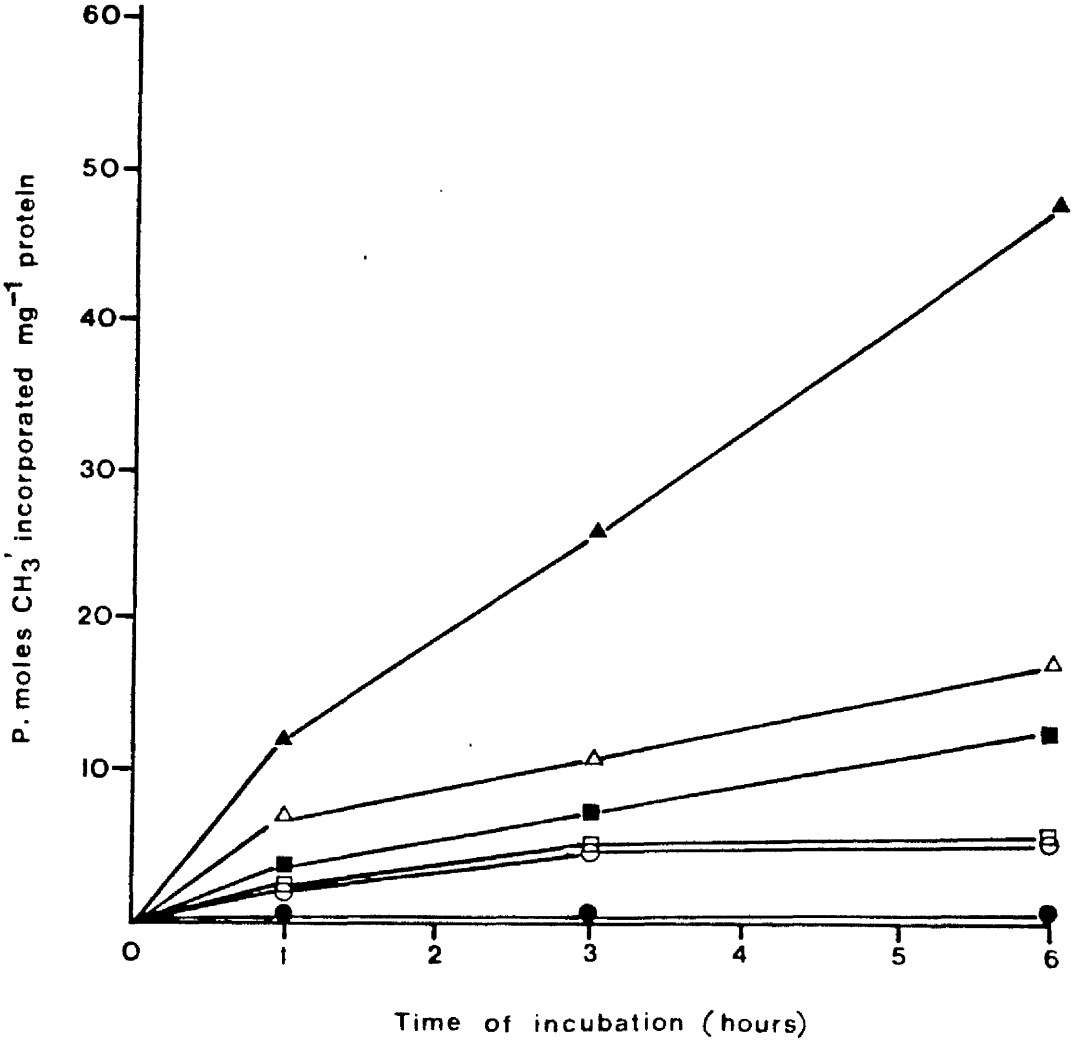


Figure 9. Micrococcal nuclease digestion of nuclear insoluble fraction of mouse L-929 cells treated with 5-azadeoxycytidine.

that the DNA methylase formed a covalent complex (Santi et al., 1983) with endogenous hemimethylated duplex DNA containing 5-azacytosine residues and this complex was not dissociated with micrococcal nuclease digestion in such a way as to release DNA methylase activity that would interact with exogenous substrate DNA.

4.1.7 Conclusion

The results presented in this section have shown that cells treated with hydroxyurea to block DNA replication have lesser amount of DNA methylase in the nuclear matrix fraction. Cycloheximide, which blocks protein synthesis and blocks DNA synthesis as well (table 11, Creusot et al., 1982) has shown similar effect on DNA methylase activity bound to nuclear matrix, It indicates that actual association of DNA methylase with such nuclear matrix is certainly closely linked to DNA replication.

The treatment of cells with 5-azadeoxycytidine, on the other hand, resulted in the loss of DNA methylase activity from the soluble fraction and an increase in the activity somestimes of bound form. The failure of cycloleximide to release DNA methylase from the bound state of 5-azadeoxycytidine treated cells supports the results of Creusot et al., (1982), Taylor and Jones (1982), that enzyme is irreversibly bound to 5-azacytosine molecules of the hemimethylated duplex DNA and was not affected by the blockade of DNA synthesis. Furthermore, when such nuclear matrix was extensively digested with large amounts of micrococcal nuclease, the concentration at which the bound enzyme was released from matrix of untreated cells, the DNA methylase from 5-azadeoxycytidine treated cells remained associated in a form that would not interact with exogenous substrate DNA. An other evidence that supports the irreversible binding of DNA methylase is perhaps the

formation of covalent complex between 5-azacytosine molecules and DNA methylase (Santi et al., 1983).

4.2 Comparison of enzymic properties in vitro.

4.2.1 Effect of pH on DNA methylase activity.

Holliday and Pugh (1975) hypothesis predicts that in a given organism different DNA methylases with different substrate sequence specifities are involved in the control of differentiation. Since then several reports have been published postulating that there may be at least two DNA methylase activities in higher eukaryotes (Riggs 1975, Drahovsky and Boehm 1980, Razin and Friedman 1981) and these activities (initiating and maintenance) might be performed at different conditions such as pH and ionic strength.

In early studies, it has been shown that DNA methylase isolated and purified from different mammalian sources are optimal at different pH. For example, DNA methylase from HeLa cells showed pH optimum at 6.5 (Roy and Weissbach 1975) while Sneider et al., (1975) reported that their purified but still heterogenous enzyme from Novikoff hepatoma cells gave a broad peak with native DNA but two distinct optima with denatured DNA (pH7.0 and 7.5). In view of this observation they suggested that there may be more than one species of DNA methylase in Novikoff hepatoma cells. Turnbull and Adams (1976) were only able to show a single pH optimum. This raises the question of whether bound DNA methylase activity exhibited the same or a different pH optimum. In Figure 10, the soluble and bound forms of DNA methylases have shown one sharp peak of maximum activity at a pH 7.8.

4.2.2 Effect of increasing salt concentration on DNA methylation.

From previous section it can be seen that soluble and bound DNA

Figure 10. Effect of pH on DNA methylase activities.

Buffers used were as follows:

(i) 0.05M sodium hydrogen maleate (pH 5.2-6.0).

This was prepared by mixing 50ml of sodium hydrogen maleate (0.2M) with 7.2 ml (for pH 5.2) and 33.0 ml (for pH 6.0) of 0.2M NaOH, and diluting to 200 ml with water. DTT, EDTA and glycerol were added at the same concentrations as in buffer M, before checking the pH and adjusting as necessary.
(Sodium hydrogen meleate is prepared by dissolving 23.2 g maleic acid and 8 g NaOH in water at a total volume of 1:1.

(ii) Tris-HCl buffers, pH 7.1, 7.5, 7.8, 8.3 & 8.8

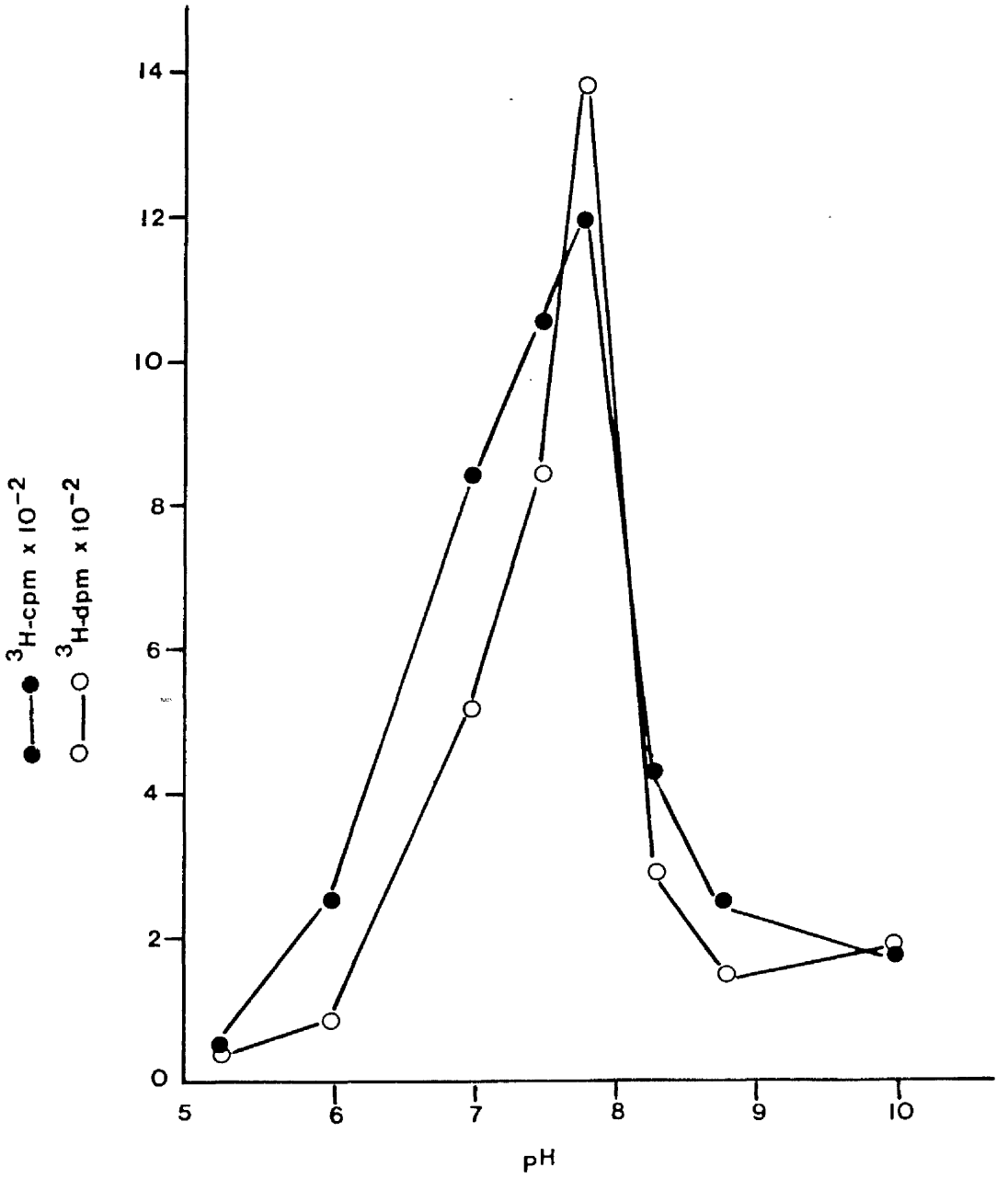
This consisted simply of buffer M containing 50mM tris-HCl at the appropriate pH.

(iii) 0.05M glycine-NaOH, pH10.0.

This was made by mixing 0.2M glycine (50ml) and 0.2M NaOH (30ml), adding DTT, EDTA and glycerol as in buffer M and making up to 200 ml with water after checking the pH.

-●——●-'soluble' DNA methylase activity

-O——O-'bound' DNA methylase activity.


14
12
10
8
6
4
2
0
5
6
7
8
9
10
pH
^{3}H-cpm x 10^{-2}
^{3}H-dpm x 10^{-2}

methylases did not show any difference at pH level. In order to examine the salt effect on the activities of both forms of DNA methylases, nuclear soluble and insoluble fractions were incubated with single stranded E. coli DNA at different salt concentrations for one hour at 37°C. Figure 11 shows that incubation of single stranded E. coli DNA with soluble and bound DNA methylases in the presence of Nacl results stimulation of enzyme activities above zero salt concentration to 0.1M Nacl. Whilst minor differences exist, both enzyme activities show similar responses to increasing salt concentration.

4.2.3 The effect of brief trypsin digestion on "soluble" and "bound" DNA methylase activities.

Recently, the structure and function of myosin subfragment-1 has been studied by limited tryptic digestion (Hozumi 1983). It has been suggested that large conformational changes were induced in the myosin subfragment-1 when it was trypsinised in the presence of actin. More recently, Adams et al., (1983), have shown that prior incubation of soluble DNA methylase from mouse ascites cells with trypsin over a period of 10-20 minutes at 37°C, causes a considerable stimulation in the enzyme activity with native E. coli DNA.

In order to determine the effect of brief trypsin treatment on DNA methylase activity bound to insoluble nuclear components, nuclear soluble and insoluble fractions were incubated with trypsin for 10 minutes at 37°C. The reaction was terminated with trypsin inhibitor prior to enzyme assay. The results presented in Table 13 show an increase in the enzyme activity of nuclear soluble fraction (soluble DNA methylase) with native E. coli DNA. The stimulation in the enzyme activity is 7-fold which is in close agreement with the results of Adams et al., 1983). On the other hand prior incubation of nuclear insoluble

Figure 11. The effect of increasing salt concentration on DNA methylation.

The assay mixture (140µl) contained 20µg protein of each fraction, 40µg of heat denatured E. coli DNA, 3.2µCi S-adeonsyl-L-(methyl-^{3}H)-methionine and buffer M containing various concentrations of salt. The assay mixture was incubated for 1 hour at 37^{o}C.

—O——O-Soluble DNA methylase activity.

—●——●-Bound DNA methylase activity.

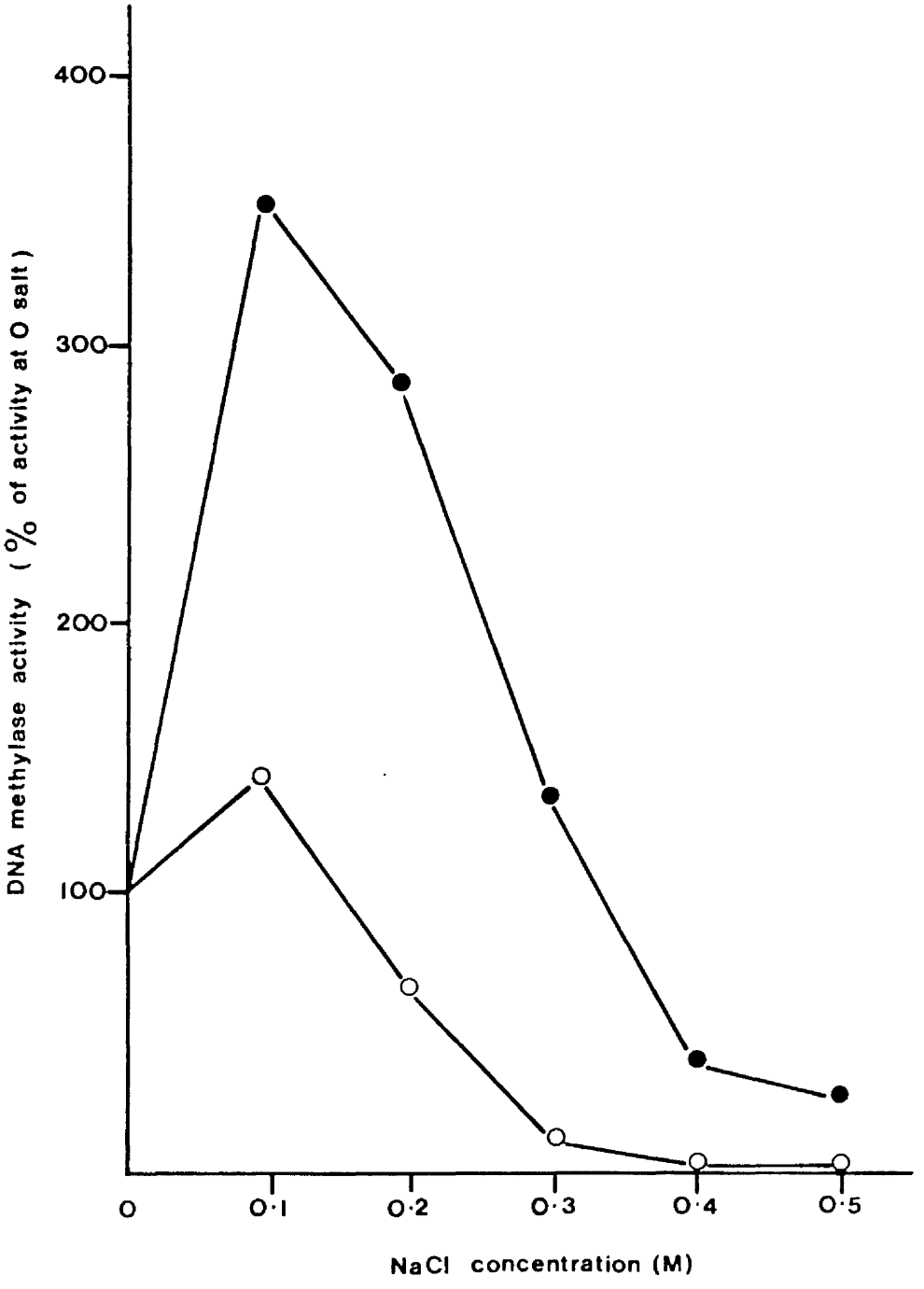


Figure 11. The effect of increasing salt concentration on DNA methylation.

fraction with trypsin leads in the reduction of enzyme activity in the bound form of DNA methylase.

4.2.4 Methylation of different DNA substrates by bound DNA methylase activity.

Different DNAs contain different base composition, so it is clear that the prevalence of 5-methylcytosine residues in DNA may be quite variable. In order to determine the rate of methylation, a number of DNAs from different organisms have been tested as substrate for DNA methylase bound to nuclear components (Figure 12). A comparison of the rates of methylation of different DNAs showed that the rate to some degree is correlated with GC content. M. luteus DNA which has GC content of 72% was methylated at a highest rate among other DNAs studied.

4.2.5 Effect of DNA concentration on activity of in vitro methylation by bound DNA methylase.

In order to determine, the extent of methylation of bound DNA methylase, the nuclear insoluble fraction was incubated with various concentrations of denatured E. coli DNA for 1 hour at 37°C. Figure 13 shows that despite the presence of endogenous DNA in the incubation the methylation of E. coli DNA with bound DNA methylase was almost linear up to the concentration of 3 µg DNA per µg protein but afterwards the increase was only slight.

5. Solubilisation of bound DNA methylase from nuclear insoluble fraction.

5.1 Digestion of nuclear insoluble fraction with micrococcal nuclease.

Creusot and Christman (1981) reported that under their conditions used for the release of DNA methylase from chromatin of Friend

Figure 12. Assay mixture (140µl) consisted of 20µg 'bound' DNA methylase and 40µg of different native DNA substrates, 3.3µCi S-adenosyl-L-(methyl-^{3}H)-methionine and buffer M containing 0.1M NaCl.

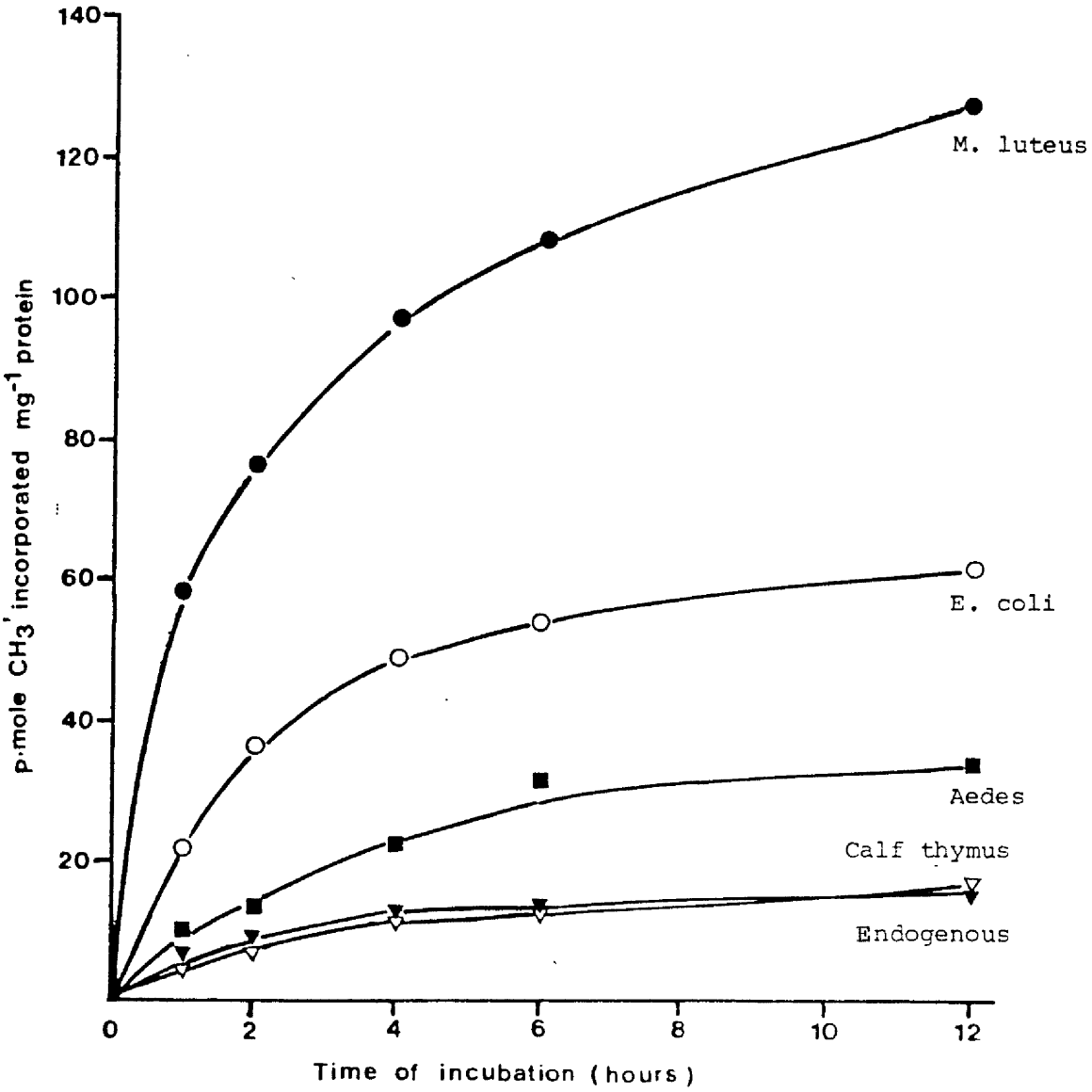


Figure 12. Methylation of different DNA substrates by bound DNA methylase activity.

Table 13. The effect of brief trypsin digestion on DNA methylase activity.

80µl of each nuclear soluble and insoluble fractions were digested with 20µl of trypsin (70µg/ml buffer M*) for 10 minutes at 37°C. The reaction was terminated with 20µl of trypsin inhibitor (2.5 mg/ml of buffer M*) and then assayed for DNA methylase activity.

Buffer M* = Buffer M without PMSF.

Table 13. The effect of brief trypsin digestion on DNA methylase activity.

DNA methylase	Trypsin digestion	dpm incorporated into DNA hr^{-1} heat denatured *E. coli* DNA	dpm incorporated into DNA hr^{-1} Native *E. coli* DNA
'Soluble' DNA methylase	+	1392	2152
"	-	1504	320
'Bound' DNA methylase	+	48	20
"	-	268	140
'DNA released' DNA methylase*	+	0	0
"	-	144	52

* See Section 6

Assay mixture (70µl) consisted of 20µl of each samples, 20µg of E. coli DNA, 1.6µCi of S-adenosyl-L-(methyl-^{3}H)methionine and 30µl buffer M. Assays with denatured *E. coli* DNA were carried out in the presence of 0.1M NaCl.

Figure 13. Assay mixture (140μl) consisted of 24μg protein of NIF, 3.2μ Ci S-adenolsy-L-(methyl-^{3}H)-methionine, varying amounts of denatured E. coli DNA and buffer M containing 0.1M NaCl.

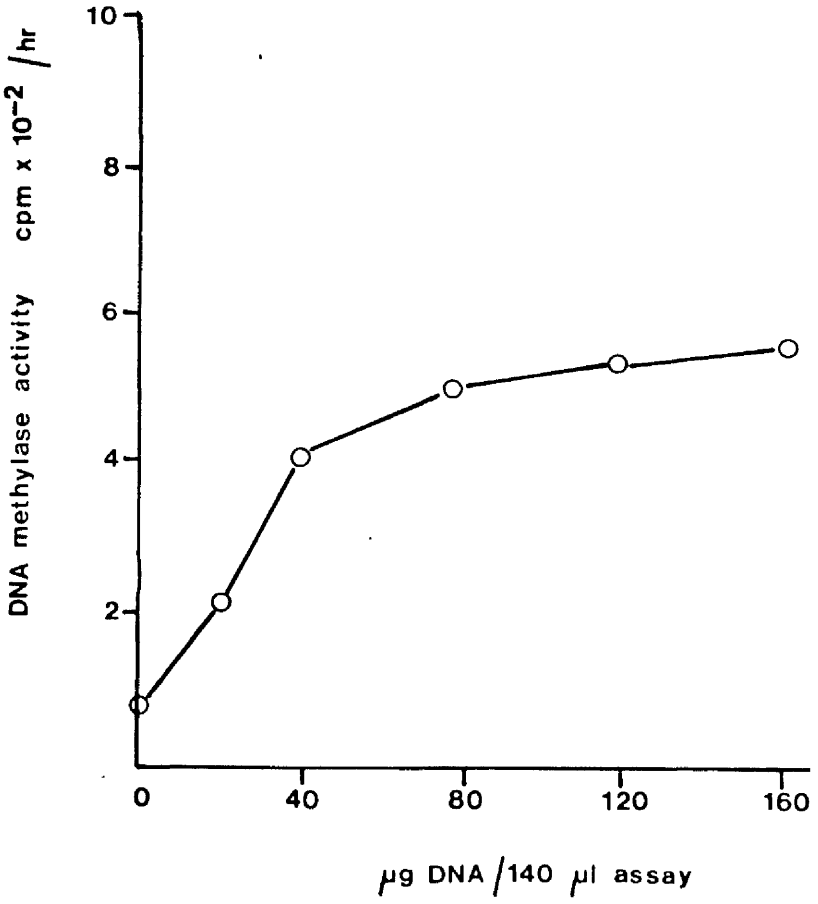


Figure 13. Effect of DNA concentration on activity of in vitro methylation by bound DNA methylase.

erythroleukemia cells with micrococcal nuclease (30 units/ml, incubation for 2 minutes at 37°C) approximately 50-60% of the DNA methylase was found in the soluble form. They suggest that the enzyme solubilised with micrococcal nuclease was still bound to chromatin. Similar findings have been reported from Chinese hamster ovary cells (Adams et al., 1977), where most of the nuclear protein (including 80% of the DNA methylase activity) was released from the nuclei in initial stages of nuclease (300 units/ml) treatment.

In order to solubilise the remaining activity bound to nuclear components (bound form of DNA methylase), when nuclear insoluble fraction was incubated with moderate levels of micrococcal nuclease (180 units/100µg chromatin DNA) for 40 minutes at 37°C and the digested material was assayed for DNA methylase activity in the presence of S-adenosyl-L-(methy-^{3}H)-methionine and added substrate DNA, most of the enzyme activity remained associated with the insoluble nuclear components methylating endogenous DNA (results not shown). In the next experiment digestion with much higher levels of micrococcal nuclease was carried out. Figure 14 shows that when nuclear insoluble fraction was incubated with micrococcal nuclease (2140 units/100µg chromatin DNA) for 40 minutes at 37°C, DNA methylase is responded to added substrate DNA for activity. Similar results are evident (Figure 15), when such nuclease digested material was first centrifuged at 12000 g for 30 minutes at 4°C and the supernatant collected, was incubated in the presence of S-adenosyl-L-(methyl-^{3}H)-methionine and added substrate DNA.

6. Abstraction of DNA methylase from nuclear insoluble fraction with DNA substrate.

6.1 Effect of E. coli DNA on the abstraction of DNA methylase from nuclear insoluble fraction.

Figure 14. DNA methylase activity in the nuclear insoluble fraction after micrococcal nuclease digestion.

Staphylococcal nuclease digestion. It was carried out according to the procedure of Dunn et al., 1980 with slight modifications. Incubation mixture (3.0 ml) consisted of 0.5 ml nuclear insoluble fraction, 1.2 ml of buffer M without EDTA, 0.14 ml 20mM $CaCl_2$, and 1.0 ml of micrococcal nuclease (2140 units/100µg chromatin DNA) was incubated for 40 minutes at 37°C. The reaction was terminated with 0.16 ml of 50mM EDTA (pH 7.5). The mixture was chilled on ice and used for DNA methylase activity (equal amount of digested and undigested nuclear insoluble fractin was used in each assay).

Assay mixture (210µl) contained 23µg protein of each, digested and undigested nuclear insoluble fraction, 40µg denatured calf thymus DNA, 3.2µCi S-adenosyl-L-(methyl-^{3}H)-methionine and buffer containing 0.1M NaCl.

—O——O— Activity of nuclear insoluble fraction without added substrate DNA

—●——●— Activity of nuclear insoluble fraction digested with micrococcal nuclease and assayed without added substrate DNA.

—▲——▲— Activity of nuclear insoluble fraction digested with micrococcal nuclease and assayed with added substrate DNA (den. calf thymus DNA).

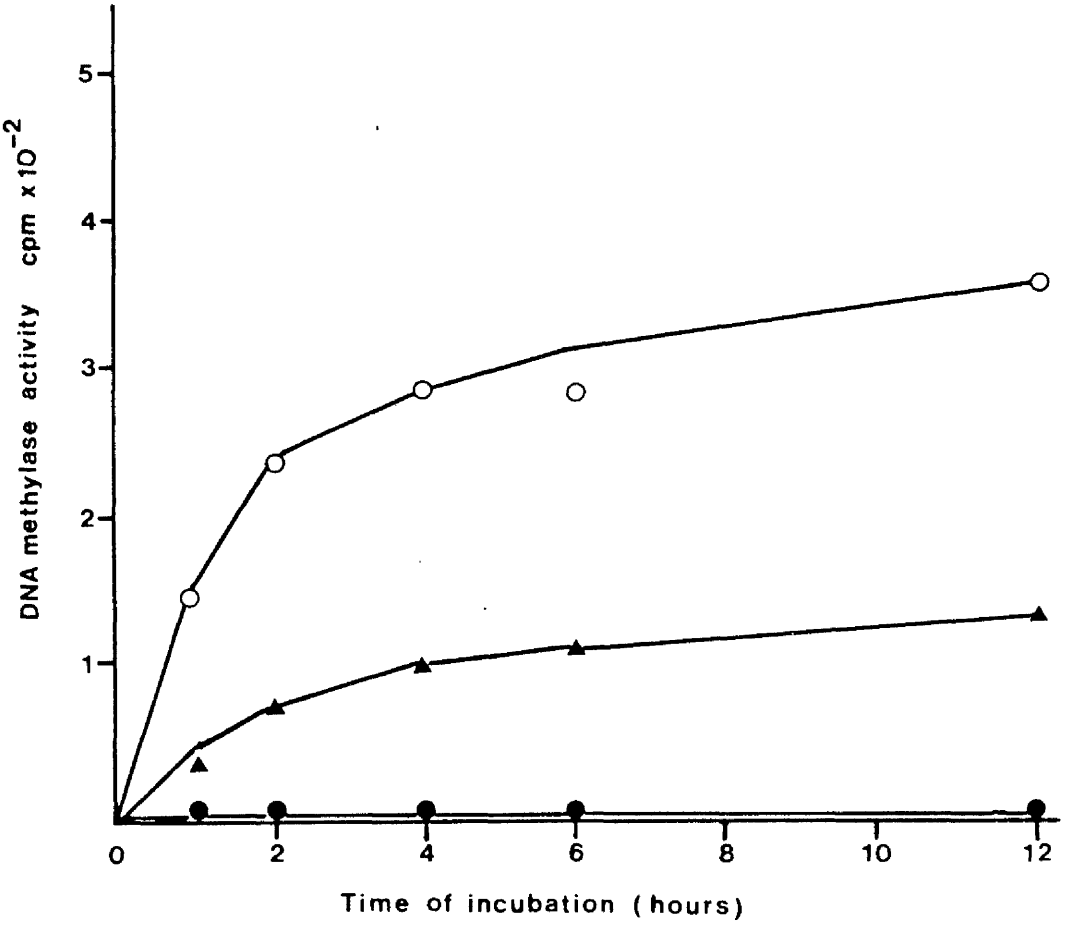


Figure 14. DNA methylase activity in the nuclear insoluble fraction after micrococcal nuclease digestion.

Figure 15. DNA methylase activity in the supernatant of nuclear insoluble fraction after micrococcal nuclease digestion.

The digestion of nuclear insoluble fractin was carried out as described in Figure 14. The digested nuclear insoluble fraction was then centrifuged at 12,000 g for 30 minutes at $4^{\circ}C$ and the supernatant was used for DNA methylase assay.

Assay mixture (210µl) contained 23µg protein of nuclear insoluble fraction and 19µg protein of the supernatant, 40µg den. E. coli DNA, 3.2µCi S-adenosyl-L-(methyl-^{3}H)-methionine and buffer M containing 0.1M NaCl.

—△——△— Undigested nuclear insoluble fraction with den. E. coli DNA.

—▲——▲— Undigested nuclear insoluble fraction without added substrate DNA.

—○——○— Supernatant of digested nuclear insoluble fraction with den. E. coli DNA.

—●——●— Supernatant of digested nuclear insoluble fraction without added substrate DNA.

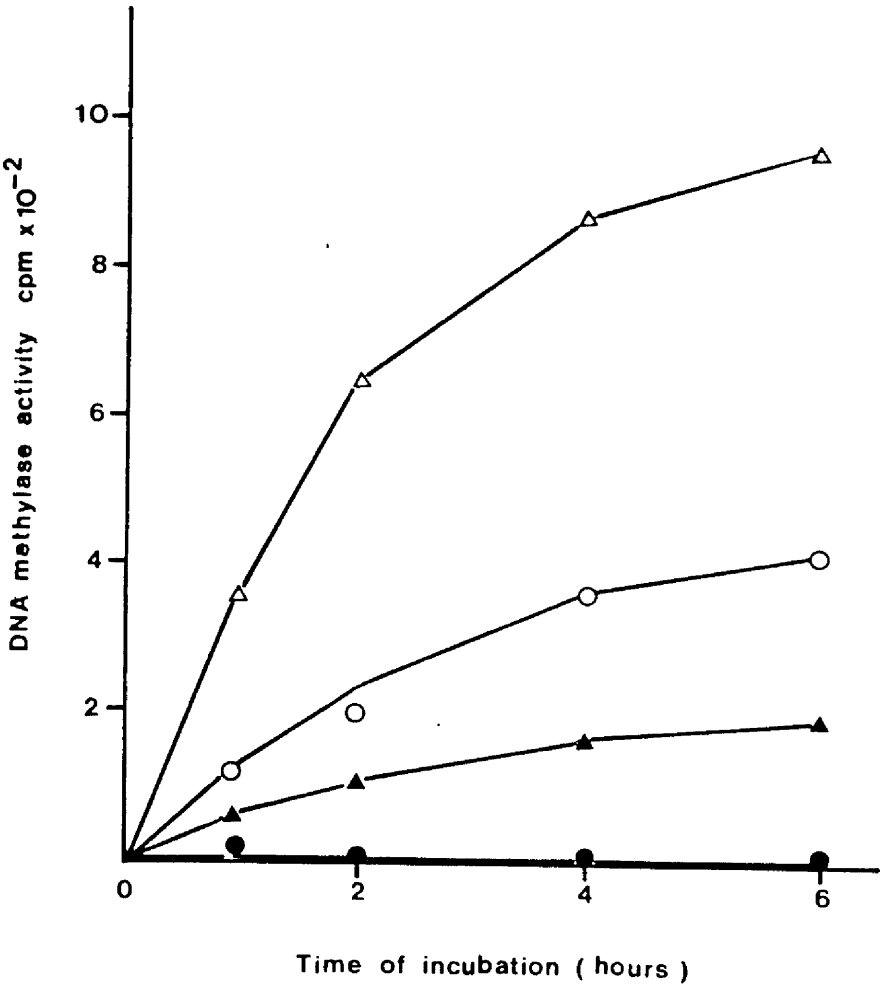


Figure 15. DNA methylase activity in the supernatant of nuclear insoluble fraction after micrococcal nuclease digestion.

The results presented in table 14 (experiment-B) show that prior incubation of nuclear insoluble fraction without added substrate DNA either at $0^{o}C$ or at $37^{o}C$ for two hours in the presence of S-adenosyl-L-(methyl-^{3}H)-methionine, did not release any significant amount of DNA methylase activity from insoluble nuclear components into a soluble form. However incubation with E. coli DNA at $37^{o}C$ (experiment-A) released a considerable amount of enzyme activity into the supernatant, which on further incubation (without any addition to incubation mixture) at $37^{o}C$ was able to transfer methyl groups into the DNA substrate to which it was associated.

6.2 Comparison between the effect of calf thymus DNA and E. coli DNA on the abstraction of DNA methylase from nuclear insoluble fraction.

From table 15, it can be seen that when nuclear insoluble fraction was incubated in the presence of calf thymus DNA for 2 hours at $37^{o}C$, no DNA methylase activity has been abstracted with the DNA whilst prior incubation with E. coli DNA for 2 hours has released a considerable amount of DNA methylase activity from insoluble nuclear components into a soluble form. A possible explanation of the inability of calf thymus DNA to abstract DNA methylase from insoluble nuclear components, may be its lower frequency of unmethylated sites available to DNA methylase for binding.

6.3 Effect of native and denatured E. coli DNA on the abstraction of DNA methylase from nuclear insoluble fraction.

Adams et al., 1979, have reported that their partially purified enzyme from Krebs II ascites cells (Soluble form of DNA methylase) can interact with native DNA from prokaryotes in the presence of lower

Table 14. Abstraction of DNA methylase from nuclear insoluble fraction with denatured E. coli DNA.

Experiment-A: 200µl of nuclear insoluble fraction in a total incubation mixture of 700µl, was incubated with 200µg heat denatured E. coli DNA, 16µCi S-adenosyl-L-(methyl-^{3}H)-methionine and buffer M containing 0.1M NaCl, at 37^{o}C for 2 hours. The mixture was then centrifuged at 12000 g for 30 minutes at 4^{o}C. The pellet obtained after centrifugation was resuspended in buffer M to the equal volume of the supernatant.

Experiment-B: The reaction was carried out as above, without heat denatured E. coli DNA.

Experiment-C: The reaction mixture without added DNA was incubated at 0^{o}C for 2 hours and then centrifuged as above.

Table 14. Abstraction of DNA methylase from nuclear insoluble fraction with denatured E. coli DNA.

Experiment	Fraction	dpm incorporated/2hr
Experiment-A	Supernatant *	1040
	Pellet	492
Experiment-B	Supernatant	336
	Pellet	540
Experiment-C	Supernatant	408
	Pellet	N.D+

+ N.D. = not determined

* when the supernatant fraction was incubated for a further 1 hour the incorporation of tritium counts rose to 1408 dpm.

Table 15. Abstraction of bound DNA methylase with different DNA substrate from nuclear insoluble function.

1. "Calf thymus" DNA released DNA methylase fraction was prepared by incubation of 100µl NIF with 100µg heat denatured calf thymus DNA and 50µl buffer M containing salt to the final concentration of 0.1M, for 2 hours at 37°C. No S-adenosyl-L-(methyl-^{3}H)-methionine was used in the incubation. The mixture was centrifuged at 12000 g for 30 minutes at 4°C. Supernatant was used for enzyme assay.

2. "*E. coli* DNA" released DNA methylase fraction: It was prepared as above.

Table 15. Abstraction of bound DNA methylase with different DNA substrates from nuclear insoluble fraction.

Fraction	DNA substrate used	activity (units hr^{-1} mg^{-1}DNA)
Nuclear insoluble fraction	None	3.2
" " "	20µg den.calf thymus	2.9
" " "	20µg den. E. coli	6.9
1. Nuclear insoluble fraction pre-incubated with calf thymus DNA and then centrifuged to yeild supernatant fraction.	None	0
2. Nuclear insoluble fraction pre-incubated with E. coli DNA and then centrifuged to yield supernatant fraction.	None	4.9

Total incubation mixture (70µl) contained 20µl of nuclear insoluble fraction or 40µl of supernatant fraction of NIF (nuclear insoluble fraction) preincubated with DNAs, 1.6µCi S-adenosyl-L-(methyl-^{3}H)-methionine and buffer M containing 0.1M NaCl.

concentration of salt and can form a salt resistant complex. This interaction may involve transient breathing of the DNA at certain sites to which enzyme can bind and travel along. But they did not find such complex formation with native DNAs from mammalian and amphebian sources.

The next experiement was designed to find out whether native DNA of E. coli can serve as a better reagent to bring about methylase abstraction from insoluble nuclear components. However the results given in table 16 show that no DNA methylase activity was recovered when the nuclear insoluble fraction was incubated with native DNA. The apparent inability of native E. coli DNA interaction with bound DNA methylase may indicate that the enzyme did not find particular sites for initial binding in the breathing area of the native DNA. However, the true explanataion remains obscure.

6.4 The effect of temperature on the release of bound DNA methylase in the presence of denatured E. coli DNA.

The time curves for the abstraction of DNA methylase activity at $0^{o}C$ and at $37^{o}C$ are given in Figure 16. The rate of abstraction is greater at $37^{o}C$ but even at $0^{o}C$ the enzyme is readily abstracted and the double reciprocal plot (Figure 17) shows that eventually equal amounts of enzyme would be abstracted at both temperatures.

Therefore, the question arises, if DNA methylase can bind to denatured E. coli DNA at $0^{o}C$, can it methylate at $0^{o}C$. Results presented in table 17 show that while no methylation of E. coli DNA was found when incubated with soluble DNA methylase at $0^{o}C$, the bound form of DNA methylase was able to incorporate methyl groups into the DNA at $0^{o}C$ albeit slowly.

Table 16. Effect of denatured and native E. coli DNA on the solubilisation of DNA methylase of nuclear insoluble fraction.

Supernatant	DNA methylase activity dpm incorporated hr^{-1}
Supernatant-1	00
Supernatant-2	148
Supernatant-3	228

Supernatant-1	100µl of nuclear insoluble fraction incubated with 50µg native E. coli DNA for 1 hour at 37°C in the absence of NaCl.
Supernatant-2	100µl of nuclear insoluble fraction incubated with 50µg denatured E. coli DNA for 1 hour at 37°C in the absence of NaCl.
Supernatant-3	100µl of nuclear insoluble fraction incubated with 50µg den. E. coli DNA for 2 hours at 37°C in the absence of NaCl.

After incubation, all incubation mixtures were centrifuged at 12000 g for 30 minutes at 4°C and then the supernatant was assayed for DNA methylase activity in the absence of substrate DNA.

Figure 16. Effect of temperature on release of bound DNA methylase activity in the presence of den. E. coli DNA.

200µl assay mixture contained 100µl of NIF, 100µg den. E. Coli DNA and 50µl buffer M was incubated at 37°C for various time periods. The centrifugation was carried out at 12,000 g for 30 minutes at 4°C. The supernatant was collected.

200µl assay mixture contained 100µl of NIF, 100µg den. E. coli DNA and 50µl buffer M was kept in ice for various time periods. The centrifugation was carried out as above.

DNA methylase assay:

Incubation mixture (140µl) contained 80µl of each sample, 3.2µCi S-adenosyl-L-(methyl-^{3}H)-methionine and 40µl of buffer M containing salt at the final concentration of 0.1M. The reaction was carried out for 2 hours at 37°C.

—O——O— DNA methylase activity released at 37°C.

—●——●— DNA methylase activity released at 0°C.

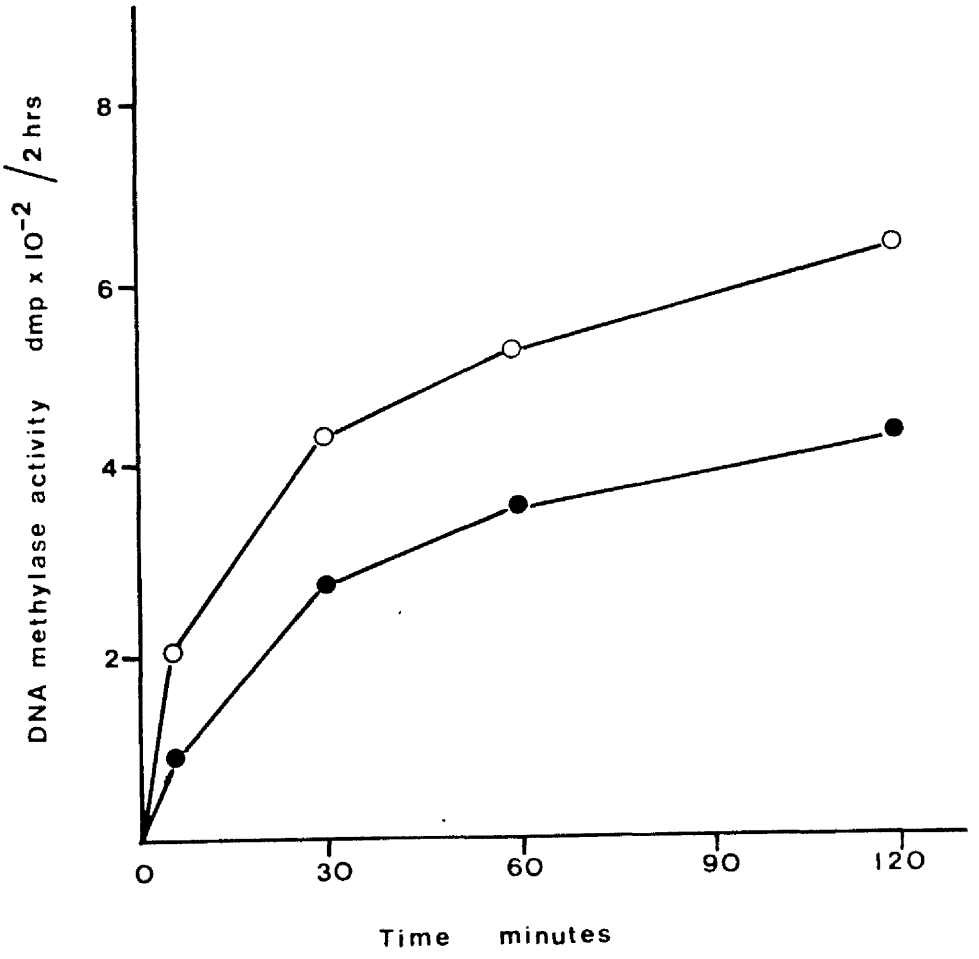


Figure 16. Effect of temperature on release of 'bound' DNA methylase activity in the presence of denatured E. coli DNA.

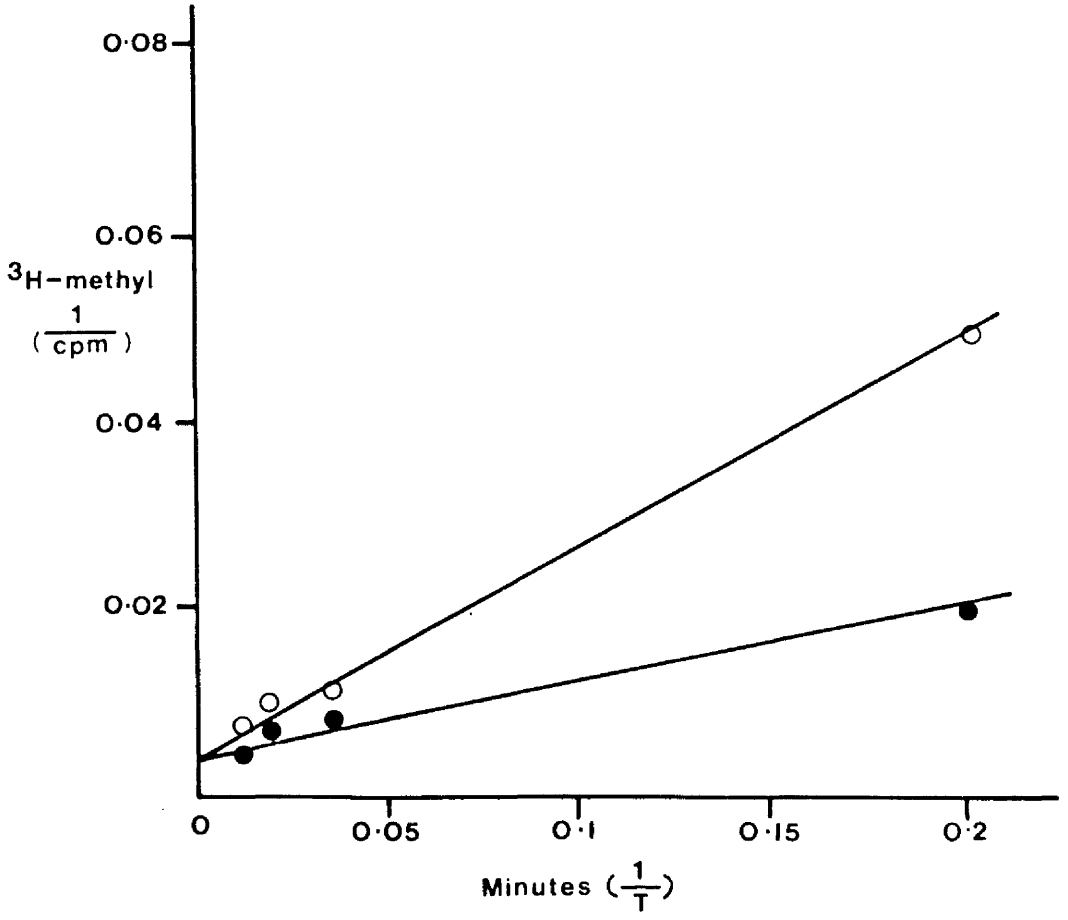


Figure 17. Double reciprocal plot of Figure 16.

7. Attempts to release DNA methylase from DNA after abstraction from nuclear insoluble fraction.

7.1 Treatment of DNA-released DNA methylase with streptomycin sulphate.

Kalousek and Morris (1969) reported that when their chromatin from rat spleen nuclei was treated with streptomycin sulphate, most of the enzyme activity was released in the supernatant as a soluble form. In order to dissociate DNA methylase from E. coli DNA, after abstraction procedure the supernatant obtained was gently stirred with streptomycin sulphate for 10 minutes at $0^{\circ}C$. Results given in Table 18 show that DNA methylase and E. coli DNA both remain in the supernatant.

7.2 Solubilisation using ammonium sulphate

Turnbull and Adams (1976) have shown that while purification of their DNA methylase (soluble DNA methylase) from Krebs II ascites cells, about 96% of the DNA methylase activity was precipitated at 60% saturation with respect to ammonium sulphate, a very useful purification was achieved. In order to separate DNA methylase from E. coli DNA after the abstraction procedure, the supernatant fraction was subject to 30% saturation with ammonium sulphate. After centrifugation at 12,000 g for 20 minutes at $4^{\circ}C$ the supernatant collected was further saturated to 60% with ammonium sulphate and centrifuged as above. The precipitate was collected and dissolved in minimum volume of buffer M. The results given in Table 19 show that while most of the enzyme activity was dissociated from E. coli DNA, there was still some activity associated to DNA. In view of above observation new experiment was designed and nuclear insoluble fraction was used for ammonium sulphate precipitation. Nuclear insoluble fraction subject to 30% saturation with ammonium sulphate was centrifuged overnight at 148,000 g in Beckman

Table 17. The effect of temperature on DNA methylase activity.

DNA methylase	DNA Substrate	Temp. of assay(°C)	DNA methylase activity dpm incorporated/2 hrs	% activity
Soluble DNA methylase	denatured. E. coli	37	8220	100
Soluble DNA methylase	"	0	32	0.38
DNA methylase released after incubation with E. coli DNA	None	37	1092	100
DNA methylase released after incubation with E. coli DNA	None	0	172	15.7

'DNA released' DNA methylase: Nuclear insoluble fraction was incubated with den. E. Coli DNA for 2 hours at 37°C, as described in Figure 16:

Assay mixture (140µl) contained 40µl of soluble DNA methylase and 80µl of DNA released DNA methylase preparations. 3.2µCi S-adenosyl-L-(methyl-^{3}H)-methionine, 40µg denatured E. coli DNA in the incubations with soluble DNA methylase. No substrate DNA used with DNA released DNA methylase as it already contained E. coli DNA. And buffer M containing 0.1M NaCl.

Table 18. Solubilisation of 'DNA released' DNA methylase with Streptomycin sulphate.

DNA methylase	Streptomycin sulphate treatment	Extra DNA substrate	DNA methylase activity (dpm incorporated hr^{-1})
'DNA released' DNA methylase	-	-	868
'DNA released' DNA methylase	-	40µg den. *E. coli*	964
'DNA released' DNA methylase	+	-	568
'DNA released' DNA methylase	+	40µg den. *E. coli*	760

750µl 'DNA released' methylase fraction were gently stirred with 5µl of 5% streptomycin sulphate for 10 minutes. Centrifugation was carried out at 12000 g for 20 minutes at 4°C and supernatant was used for methylase assay.

Assay mixture (140µl) consisted of 50µl of each fraction, 3.2µCi S-adenosyl-L-(methyl-^{3}H)-methionine and buffer M containing NaCl (0.1M).

Table 19. Solubilisation of E. coli DNA released DNA methylase with ammonium sulphate.

Fraction	DNA substrate	DNA methylase activity p.mole CH_3 incorporated $hr^{-1}mg^{-1}$ protein
E. coli DNA released DNA methylase fraction	None	10.6
30-60% ammonium sulphate precipitate fraction	den. E. coli (20µg)	3.5
30-60% ammonium sulphate precipitate fraction	None	0.2

Assay mixture (70µl) contained 12µg protein of each fraction, 1.6µ Ci S-adenosyl-L-(methyl-^{3}H)-methionine and buffer M, 30-60% ammonium sulphate fraction was assayed in the presence of 20µg heat denatured E. coli DNA released DNA methylase fraction already contained DNA.

Table 20. Solubilisation of DNA methylase of nuclear insoluble fraction with ammonium sulphate.

Fraction	exogenous DNA substrate	p.mole CH_3 incorporated $hr^{-1}mg^{-1}$ protein
Nuclear insoluble fraction (NIF)	None	6.36
Nuclear insoluble fraction (NIF)	20µg den. E. coli	21.8
30-60% ammonium sulphate precipitate derived activity from NIF	None	0
30-60% ammonium sulphate precipitate derived activity from NIF	20µg den. E. coli	111.0

Total assay mixture (70µl) contained 20µl of each fraction, 1.6µCi S-adenosyl-L-(methyl-^{3}H)-methionine and buffer M containing 0.1 M Na l.

SW50.1 rotor at 4°C. This yielded a clear supernatant which was subject to 60% saturation with ammonium sulphate and centrifuged as above for 5 hours. The precipitate was then dissolved in minimum volume of buffer M. From Table 20 it can be seen that DNA methylase can be dissociate from insoluble nuclear components (including DNA) in a free form with high yield.

8. Size and subunit composition of DNA methylase derived from nuclear insoluble fraction by ammonium sulphate precipitation.

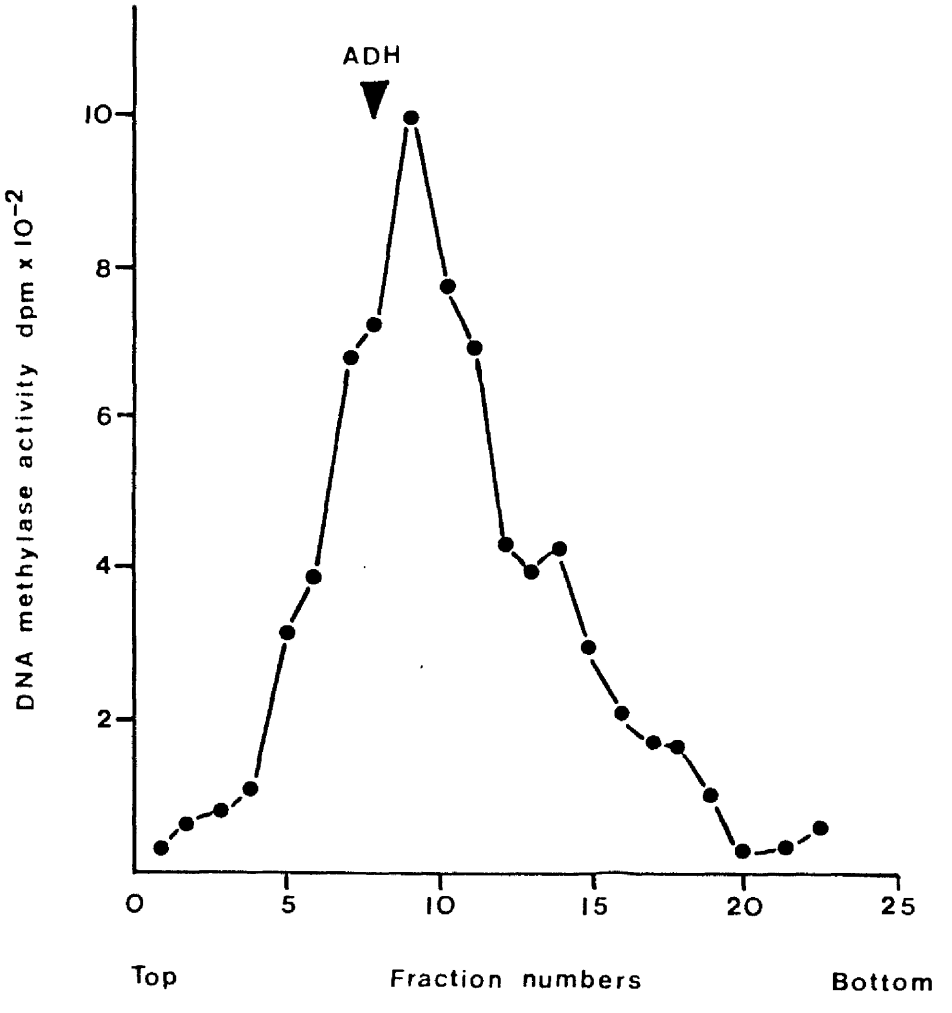
8.1 Glycerol density gradient analysis.

Figure 18 shows the gradient profile obtained when redissolved ammonium sulphate precipitate of nuclear insoluble fraction was subject to sedimentation in glycerol gradients (10-25%). The gradients were carried out in the presence of 0.2M Nacl to avoid aggregation of proteins DNA methylase activity emerged as a single well defined peak, eluted just after the marker alcohol dehydrogenase (molecular weight 150,000 (7.1S).

Adams et al., 1979 have reported that when their DNA methylase (soluble form of DNA methylase) was subjected on glycerol gradients it appeared to have a size of about 8.3S corresponding to the molecular weight of 180,000. This value for the size of the enzyme was found only after the purification was carried out in the presence of protease inhibitor, phenyl methyl sulphonyl fluoride (PMSF). Whilst early studies with the same enzyme (Turnbull and Adams 1976) showed a smaller size (approximately 7.5S). They suggested that the enzyme eluted at this size is a monomer. The well defined peak of the bound form of DNA methylase also showed the similar size i.e. 7.5S, corresponding to the molecular weight of 160,000 as reported by Turnbull and Adams (1976).

Figure. 18. Glycerol density gradient analysis of DNA methylase released from nuclear insoluble fraction by ammonium sulphate.

0.2 ml of ammonium sulphate fraction of nuclear insoluble fraction was applied onto a 10-25% glycerol gradient made up in buffer M containing 200mM NaCl. Yeast alcohol dehydrogenase (ADH) was used as molecular weight marker. The gradient was centrifuged at 78000 g in the Beckman SW50.1 rotor, for 16 hours at 4°C. 8 drop fractions were collected starting from the top of the gradient.


ADH
10
8
6
4
2
DNA methylase activity dpm x 10^{-2}
0
5
10
15
20
25
Top
Fraction numbers
Bottom

DISCUSSION

1. General

It has been shown in the Results section (Sec. 1) that whilst most of the DNA methylase activity can be readily solubilised at low salt concentration from mouse cell nuclei (either from Krebs II ascites cells of L-929 cells), there remains a proportion of DNA methylase activity associated with insoluble nuclear components. Even 2M NaCl in the extraction buffer failed to solubilise this activity from such nuclear material. The importance of this enzyme activity remaining associated with these insoluble nuclear structure and its possible role in maintaining and/or changing of DNA methylation pattern compared to the properties of the DNA methylase readily solubilised at low salt concentration, will be discussed.

1.1. Maintenance and alteration of DNA methylation patterns.

In mammalian DNA, cytosine residues are preferentially methylated when in the dinucleotide CpG (Doskocil and Sorm 1962, Grippo et al., 1968). Other dinucleotides, such as CpC, CpT and CpA have also been shown to contain 5-methyl cytosine but in rare instances (Sneider 1980, Greunbaum et al., 1981b, Burdon and Adams 1980). However, not all CpG sites are methylated *in vivo*. With the help of restriction enzymes and specific labelling techniques, it has been estimated that depending upon the specific organism, from 50-80% of these sites are modified in a particular cell (Cedar et al., 1979, Vander-Ploeg and Flavell 1980, Greunbaum et al., 1981b). Each methylated site has been shown to contain methyl groups positions on both complementary strands $\begin{matrix}-mCG-\\-GmC-\end{matrix}$ (Bird 1978, Cedar et al., 1979). The methylation state of these sites in specific genes have been detected using certain restriction enzymes such as Hpa II, Hha I and Sma I which are inhibited by methylation at

their restriction site CpG (Gautier et al., 1977, Bird and Southern 1978, Waalwijk and Flavell 1978a). Such studies have revealed a tissue specific variation in methylation pattern. For example, β-globin gene in rabbit liver, 50% of the Hpa II sites are methylated while 100% methylated in sperm DNA and 80% methylated in brain DNA (Waalwijk and Flavell 1978a). Genes, in avain tissues, coding for ovalbumin, conalbumin and ovamucoid are less methylated in the oviduct of laying hen whereas in the sperm DNA they are found highly methylated (Mandel and Chambon 1979).

The methylation of DNA occurs shortly after synthesis (Burdon and Adams 1969, Kappler 1970, Adams 1971) and would appear to be mediated by methylase which acts preferentially on hemimethylated sequences leading to the maintenance of methylation pattern on the DNA following replication (Bird 1978). DNA mediated gene transfer techniques have provided further evidence that the pattern of DNA methylation can be inherited through many cell divisions within one cell type (Pollock et al., 1978, Wigler et al., 1981 Stein et al., 1982).

Studies on DNA methylation in viral system on the other hand have provided convincing evidence for the existence of de novo methylation in mammalian cells. For example, the DNA of adenoviruses, which is normally unmethylated or methylated to a very limited extent i.e. 0.04 and 0.05% 5-methyl cytosine per cytosine for Ad. type 2 and type 12 respectively, is found methylated when integrated in to the genome of transformed hamster or hamster tumour cells (Gunthert et al., 1976, Kuhlman and Doerfler 1982, Doerfler et al., 1982). Thus, viral DNA must be methylated de novo at some time, probably during or after insertion to the host genome. It is not yet clear, however, whether patterns of DNA methylation are maintained and altered by the same enzyme or whether

there are distinct DNA methylase activities. If these functions are performed by the same enzyme, then are there cellular factors that might modulate their properties?

1.2 Properties of soluble DNA methylase and relation to the problem of maintenance and alteration of DNA methylation patterns.

DNA methylases isolated so far from various mammalian sources have been shown to methylate unmethylated single stranded DNA and double stranded DNA. DNAs from bacterial cells are excellent methyl group acceptors for these activities. E. coli DNA is a good substrate but M. luteus DNA seems even better suggesting that DNAs of high G+C content serve as best substrate. Presumably this reflects a corresponding high level of unmethylated CpG dinucleotides. On the other hand hemimethylated duplex DNA has really served the best substrate for DNA methylase activity (Greunbaum et al., 1982, Adams et al., 1979, Jones and Taylor 1981).

It has been suggested that the ability of DNA methylase from mammalian cells, to methylate denatured or single stranded DNA may be due to the fact that these enzymes do not have to make bilateral symmet ric contact in a duplex site (Smith 1979) i.e. their recognition involves asymmetric contact with only one strand of DNA. But when denatured hemimethylated DNA was used as a substrate for DNA methylase, the rate of methylation was greatly reduced (Adams et al., 1979, Taylor and Jones 1982). The nature of binding of DNA methylase appears to vary with the substrate DNA. The interaction of DNA methylase with substrate DNAs has been shown to occur randomly on a first come first served basis (Taylor and Jones 1982, Pfeifer et al., 1983). The interaction of DNA methylases with DNAs irrespective of whether the substrate is a poor or good acceptor of methyl group appear complicated. Moreover, from these

properties of soluble enzymes as far established, it is difficult to conclude that the enzyme is responsible for either maintenance or alteration of DNA methylation patterns. The possibility of the other auxilliary regulatory proteins can not be excluded. These might alter the properties of the enzyme according to the need to maintain or alter DNA methylation patterns.

DNA methylase isolated from nuclei of Krebs II ascites cells at low salt concentration seems to express properties similar to those reported for other mammalian DNA methylases (Drahovsky and Morris 1971a, b, Sneider et al., 1975, Roy and Weissbach 1975, Simon et al., 1978). Whilst this enzyme methylates single stranded and completely unmethylated double stranded DNA (Turnbull and Adams 1976, Adams et al., 1979), hemimethylated double stranded DNA again served as the best substrate (Adams et al., 1979). The overall amount of enzyme activity however, is noteably low. Thus it is not unreasonable to suppose that it might merely play a role in the alteration of DNA methylation when only a few modifications may be required to alter a particular pattern of gene expression. This of course still leaves the question of maintenance of DNA methylation patterns.

Another possibility is that there is a multiplicity of DNA methylases. The occurrence of methylated CpG dinucleotides in different sequences is suggestive of the existence of more than one DNA methylases. However, such differences may still reflect the activity of single enzyme. Except in the case of Novikoff hepatoma cells, where three peaks of enzyme activity were detected following electrophoresis of DNA methylase on native polyacrylamide gels (Sneider et al., 1975), no evidence is yet available for the existence of multiple methylase species in mammalian cells.

DNA methylases, once solubilised from their sources, have been further purified. Several steps have been shown to involve in their purification (for review, see Adams and Burdon 1982b). DNA methylase solubilised from Krebs II ascites cells, when passed through ultragel AcA34 column produced a number of peaks of enzyme activity which on further purification gave one sharp peak (on glycerol gradients) (Turnbull and Adams 1976). Therefore the possibility, can not be excluded that additional DNA methylase activities, except one, have been lost during purification process.

In early studies, it has been reported that cellular factors other than DNA and the enzyme can affect DNA methylation (Burdon and Douglas 1974). A chromatin like preparation prepared by extracting a nuclei of Krebs II ascites cells in low salt buffer catalysed incorporation of methyl groups into cellular extract, cytoplasm or cytosol fractions (none of which contain detectable DNA methylase activity) causes methylation to be stimulated. It was further shown that the factors from nuclei causing stimulation of enzyme activity were heat sensitive while from cytoplasm were stable. Thus the importance of auxilliary cellular factors which might modulate the enzyme activities in the cell nucleus can not be ruled out. Such factors might regulate the activity of DNA methylase such as to promote maintenance activity or de novo activity.

In present study, a brief search for soluble factors was carried out. The soluble DNA methylase extracted at low salt concentration, was further purified on phosphocellulose column. All the enzyme activity was eluted at 0.5M NaCl. The proteins remaining on the column were eluted by washing the column at 1M NaCl. When these proteins were incubated with partially purified soluble DNA methylase, an inhibition of

DNA methylation was observed (Table 2). Later it was found that the protein eluted at 1M NaCl from phosphocellulose column was a mixture of DNA binding proteins and considerable amount of nuclease activity (Results not shown) which made further analysis difficult.

Such studies still leave open the question of additional DNA methylase activity in the nucleus.

1.3 Methylation, replication and the nuclear matrix.

In bacteria, methylation of DNA occurs only on the nascent DNA close to the growing replication fork (Billen 1968). Although methylation occurs rapidly it probably is delayed until Okazaki fragments have been ligated (Marinus 1976). In eukaryotes, most of the methylation of DNA occurs shortly after synthesis (Burdon and Adams 1969, Adams 1971). The cells and tissues which are synthesising DNA have also shown the highest levels of DNA methylation (Figure 5, Adams 1971). A short delay before detectable methylation of newly synthesised DNA has also been reported (Kappler 1970, Hotta and Hecht 1971, Adams 1974). However, it is recently shown that in L-929 cells the methylation of DNA occurs within each replicon (Molitor et al., 1976).

In a number of tissues and cell types, for example regenerating liver (Berezney and Coffey 1976, 1977), lung (Hemminki 1977), prostate (Shaper et al., 1979) isolated bovine liver cells (Dijkwel et al., 1979), 3T3 cells (Pardoll et al., 1980) and in slime mould Physarum polycephalum (Wille and Steffens 1979), the newly replicated DNA has been shown associated with the nuclear matrix. Results from sedimentation studies have also showed that newly replicated DNA is associated with nuclear matrix (Benyajati and Worcel 1976, Wanka et al., 1977). In some of these cases this DNA is shown to be highly constrained or attached at

both ends to a supporting matrix (Benyajati and Worcel 1976). A fixed matrix model of DNA replication was then proposed (Pardoll et al., 1980).

It has been shown that DNA replication in eukaryotes proceeds discontinuously in subunits on the long chromosomal DNA called replicons. Each chromosome contains up to thousands of replicons which replicate at different times in S phase (Edenberg and Huberman 1975). Moreover, the sequence of replicon synthesis along the DNA is not shown continuously progressive from one end of the DNA molecule to the other (Faken and Hancock 1974, Edenberg and Huberman 1975). However 0.1 to 2% of the total DNA has been shown associated with the nuclear matrix (Cook and Brazell 1975, Berezney and Buchholtz 1981), and the DNA anchored to the matrix is shown about 80 kb in length ($\backsim$25μm) close to the approximate average size of the completed replicon (Huberman and Riggs 1968). A similar size has been estimated for the DNA loops of the folded Drosophila genome (Benyajati and Worcel 1976) as well as for the DNA loops anchored to the chromosomal scaffold (Paulson and Laemmli 1977).

Like prokaryotic DNA replication, a large number of enzymes have been known to be involved in eukaryotic replication. The enzymes which have shown to increase during periods of cellular DNA synthesis include thymidine kinase, thymidylate kinase, deoxycytidine kinase (Brent et al., 1965, Littlefield 1966), deoxycytidylate deaminase (Gelbard et al., 1966), ribosyl diphosphate reductase (Hwang et al., 1966), DNA polymerase-α(Chang et al., 1973) DNA polymerase-δ(Spadari and Weissbach 1974 a,b). The nuclear matrix prepared by treating chinese hamster embryo fibroblast (CHEF/18) cells with 2M Nacl is reported to have a number of these enzymes, ribonucleotide diphosphate reductase, thymidylate synthetase, dihydrofolate reductase, topoisomerase and DNA polymerase-α(Noguchi et al., 1983). DNA methylase activity has also

been reported in such nuclear structure (Noguchi et al., 1983). Since the process of DNA replication has been shown associated with nuclear matrix structure (Pardoll et al., 1980, Berezney and Bachholtz 1981) and the methylation of DNA shortly after synthesis of nascent DNA strand, the occurrence of bound DNA methylase (Table 2) in the nuclear structure which resists solubilisation with 2M Nacl (the concentration at which almost all histones and most of the nonhistone proteins can be dissociated from chromatin) may suggest that it is a part of replication complex and involved in maintenance of the DNA methylation patterns after DNA replication (Qureshi et al., 1982).

2. Properties of 'bound' compared with 'soluble' DNA methylase.

2.1 Enzymic properties.

2.1.1 Methylation of heterologous DNAs.

Earlier studies with soluble DNA methylase (Turnbull and Adams 1976) using different DNA substrates have shown different levels of methylation of DNAs by the enzyme. This level appeared to be related to the number of G+C content in these DNAs. E. coli DNA which contain 52% G+C content was shown to be an efficient acceptor of methyl groups. Similar results have been reported from other mammalian DNA methylases (Roy and Weissbach 1975, Sneider et al., 1975, Simon et al., 1978). The DNA of M. luteus (72% G+C content) has shown even better substrate for these activities (Roy and Weissbach 1975, Simon et al., 1978). The alternating double stranded poly (dG-dC)-poly(dG-dC) and random single stranded poly(dG,dC) synthetic molecules have also been shown to be highly effective acceptors (Sneider et al., 1975, Roy and Weissbach 1975). Recently, Adams and Gibb, (1981) have reported that despite the similar size of SV40 DNA conformation to ϕX174 DNA, the SV40 DNA was

found much poorer acceptor of methyl groups than ØX174 DNA. The poorer acceptance of methyl groups by SV40 DNA may be related to the lower G+C content of the DNA. Whilst the bound DNA methylase could methylate the endogenous DNA with which it was associated, which possibly being newly replicated is deficient in methyl groups. However, like the soluble enzyme it utilizes exogenous substrates provided they were of high G+C content and contained unmethylated CpGs e.g. E. coli and M. luteus DNA. Calf thymus DNA is not a good exogenous substrate presumably due to both its lower G+C content and the fact that most of its CpGs will already be methylated.

2.1.2 Effect of salt on DNA methylation.

From early studies it is found that the common property of all mammalian DNA methylases is to methylate single stranded DNA more efficiently in the presence of salt (0.1M NaCl) whilst native DNA is methylated in the absence of salt. In the present study the methylation of single stranded E. coli DNA is carried out by bound DNA methylase in increasing salt concentration (Figure 11). Both forms of DNA methylases responed similarly.

2.1.3 Effect of pH on DNA methylase activity.

The reason for determining the pH optimum has already been described in Results section 4.2.1. The maximum activity of both forms of DNA methylase is shown at about the same pH.

2.1.4 Sedimentation.

Although, Turnbull and Adams (1976) have found, their less purified soluble DNA methylase eluted from Ultragel column in the same

place as catalase marker (240,000 mol. wt). Density gradient sedimentation of their highly purified enzyme, however, indicate that the enzyme might be monomer of an apparent molecular weight of about 160,000, which still posses methylase activity. The glycerol gradient analysis of bound DNA methylase (Figure 18) which was solubilised from 2M NaCl insoluble nuclear components using 30% ammonium sulphate (see sec 7.2), have shown similar sedimentation characteristics to those of the highly purified soluble DNA methylase. To avoid aggregation of proteins sedimentation was carried out in the presence of 0.2M Nacl.

From such studies, it seems that the soluble form of DNA methylase and that associated with the insoluble components of the nucleus have many properties that are similar i.e. response to Nacl, pH, DNA substrates and sedimentation. Such findings suggest that despite the different location, the methylase components may in fact be identical. More conclusive evidence might come from the use of antibodies.

2.2 Behaviour of DNA methylases in vivo.

2.2.1 Effect of hydroxyurea on intranuclear location of DNA methylases.

Early studies with hydroxyurea have suggested that although in mammalian cells (e.g. mouse fibroblast cells), DNA methylation and replication did not occur simultaneously, methylation of DNA is a process which only proceeds effeciently in the presence of continual supply of nascent DNA (Burdon and Adams 1969). It is also shown that hydroxyurea preferentially inhibits DNA synthesis on the continuous strand (Hunter and Franke 1975). Despite the inhibition of DNA synthesis, hydroxyurea has also shown to generate single stranded low molecular weight DNA fragments in mouse L-cells (Radford et al., 1982). The reduction in the level of the bound form of DNA methylase of hydroxyurea treated cells

(Figure 6) may suggest that its association to the nuclear matrix may require continued synthesis of nascent DNA. Since hydroxyurea does not stimulate protein production (Speck et al., 1982), an increase in the activity of soluble form (Figure 6) may reflect the release of bound DNA methylase from nuclear matrix when DNA synthesis was blocked. However, the possibility of other cellular factors involved in the association of DNA methylase to nuclear components can not be ruled out.

2.2.2 Effect of cycloheximide on intranuclear location of DNA methylases.

Pulse labelling experiments in the HeLa cells and other cell lines in the presence of variety of inhibitors of protein synthesis, for example cycloheximide, have confirmed the finding that protein synthesis is required for the continuation of DNA synthesis in mammalian cells (Littlefield and Jacobs 1965, Young 1966, Weiss 1969, Fujiwara 1972). A decrease in the rate of DNA fork progression after inhibition of protein synthesis in embryonic avain erythrocytes has also been shown (Weintraub and Holtzer 1972). In this system cycloheximide reduced incorporation of thymidine into DNA by 50% within 25 seconds of addition. In the yeast genome, all proteins required for one round of DNA replication are made in a time just before initiation of replication takes place (Hereford and Hartwell 1973). Furthermore Creusot et al., (1982) have shown that when Friend erythroleukemia cells were incubated in the presence of cytoheximide for 4 hours, the protein production of these cells was reduced to 13%. However, cycloheximide not merely inhibits the synthesis of protein but also affects the synthesis of DNA (Table 11), in some cases DNA synthesis is more inhibited than the protein synthesis (Creusot et al., 1982). Therefore, the increase in the

activity of soluble form (Figure 7) when the protein and DNA synthesis is blocked, shows that the association of bound DNA methylase in the nuclear matrix is dependent on the replication of DNA and also protein production either directly or indirectly. A possible model for the relationship between the bound and soluble forms of DNA methylase is shown in Figure 19.

2.2.3 The effect of 5-azadeoxycytidine on DNA methylases.

The use of 5-azacytidine or 5-azadeoxycytidine has been useful in a search for a role for DNA methylation in eukaryotes. The incorporation of these antileukemic drugs (Momparler and Gonzales 1978, Rivard et al., 1980) into DNA has led to induction of new phenotypes for treated murine cells (Jones and Taylor 1980, Taylor and Jones 1979). The induction of differentiation by 5-azadeoxycytidine has been suggested to be related to its inhibition of DNA methylation (Jones and Taylor 1980). Low levels of 5-azadeoxycytidine can produce hypomethylated DNA by blocking the activity of DNA methylase (Creusot et al., 1982, Taylor and Jones 1982, Adams et al., 1982). The mechanism of this effect is not yet clear but substitution of 5-azadeoxycytosine residues for 1 in every 300 cytosine residues can block all the DNA methylase activity in the cell (Creusot el al., 1982). This reduction occurred when FEL cells were grown for 10 hours in the presence of 1μM 5-azadeoxycytidine. Similarly, exposure of mouse L-cells to 1μM 5-azadeoxycytidine for 10 hours lead to the reduction of soluble form of DNA methylase activity to between 10 and 20% (Qureshi et al., 1983, Table 12). Creusot et al., (1982) were unable to recover DNA methylase activity from 5-azadeoxycytidine treated FEL cells with rigorous extraction with 1M NaCl. Even extraction with 2M NaCl did not release any further DNA

methylase activity from L-aza cells (L-929 cells treated with 5-azadeoxycytidine) in the soluble form (Results not shown). Further, since it had been reported that extensive digestion of chromatin of 5-azadeoxycytidine treated cells with micrococcal nuclease did not increase the yield of active enzyme (Creusot et al., 1982), we repeatd this approach by treating nuclear insoluble fraction from L-aza cells with very large amount of micrococcal nuclease (2500 units/100µg chromatin DNA). However no activity was released into a soluble form (Figure 9) while in control cells that concentration of micricoccal nuclease will of course release bound methylase from the matrix of cells. Where then is the DNA methylase in the 5-azadeoxycytidine treated cells? The experiments with cycloheximide amongst other things also indicated that the soluble DNA methylase does not appear to have a rapid metabolic turnover.

On the other hand it has recently been suggested that DNA methylase either becomes irreversibly bound to 2-deoxy 5-azacytosine residues in its substrate DNA or binds normally to these residues but has its catalytic activity destroyed during the process of transferring methyl groups (Creusot et al., 1982). The irreversible binding of DNA methylase to DNA in other 5-azacytidine treated mammalian cells has also been observed (Taylor and Jones 1982). In E. coli K12 cells, the inhibition of DNA methylase after 5-azacytidine treatment has also been reported (Friedman 1981). A model of the mechanism of inhibition of DNA methylase by 5-azadeoxycytidine has recently been proposed (Santi et al., 1983). This model is based on the mechanism of inhibition of other methylase activities, such as tRNA methylase (Lu et al., 1979, Lu and Randerath 1980). The general mechanism is described, as to how DNA methylase transfers methyl groups from S-adenosyl-L-methionine to the 5

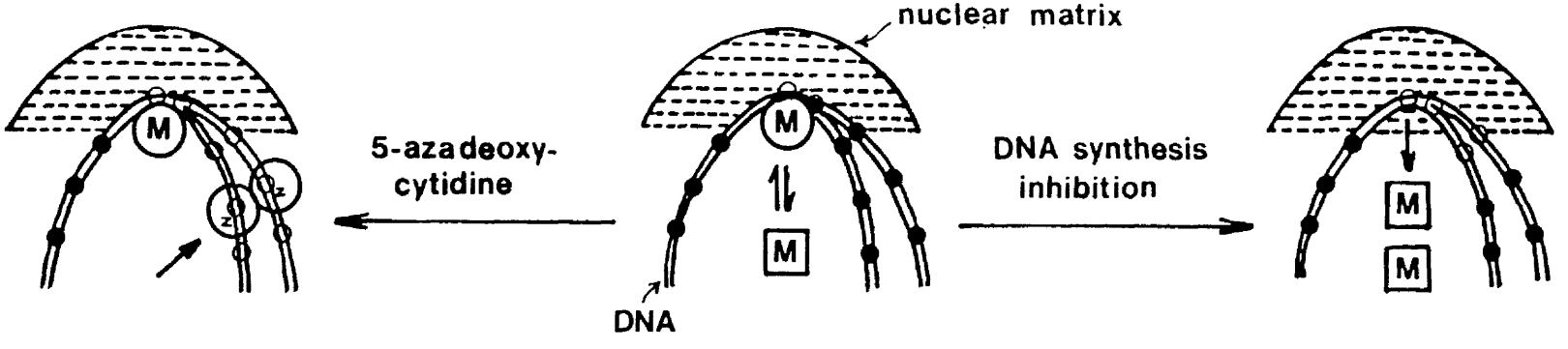


Fig.19 Translocation of DNA methylases in mouse cell nuclei.

- ● methylated sites
- ○ hemimethylated sites.
- O_z 5-aza deoxycytosine containing sites.

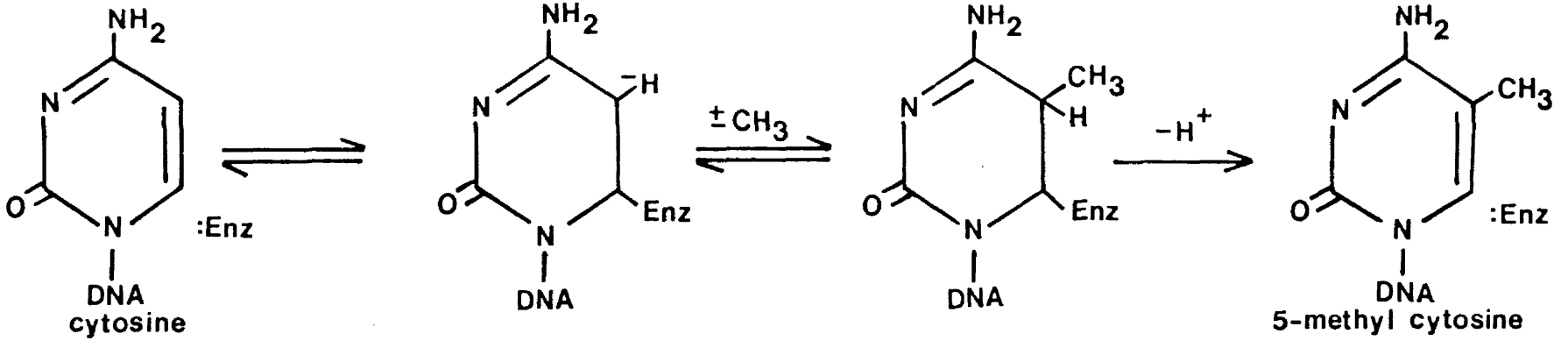
- [M] soluble DNA methylase.
- (M) bound DNA methylase.

position of cytosine ring by attaching itself to the 6th carbon of the cytosine base by eliminating proton from the 5th carbon of the cytosine residue and then releases itself in the active form. Similar mechanisms have been argued for DNA methylase from E. coli K12 and Hpa II methylase (Friedman 1981). Other enzymes thought to operate by this mechanism are dTMP synthase, dUMP hydroxymethylase and dCMP hydroxymethylase (Pogolotti and Santi 1977, Kunitani and Santi 1980, Yeh and Greenberg 1967). In the case of 5-azadeoxycytidine substituted DNA, due to the nitrogen on the 5th position of 5-azacytosine residues, the DNA methylase cannot eliminate the proton from that position and remains inactive. Covalent complex formation between 5-azadeoxycytosine and DNA methylase may then occur (Santi et al., 1983) (See also Figure 20).

As already mentioned, since the treatment of cells with cycloheximide or hydroxyurea may cause the release of DNA methylase from the bound form. This approach was repeated but with the cells previously treated with 5-azadeoxycytidine. The results from cycloheximide experiment (Table 13) and from hydroxyurea (Dr. R.L.P Adams, personal communication), however suggests that the DNA methylase may indeed be inactivated in the 5-azadeoxycytidine treated cells or be in a form which cannot be released by cycloheximide and hydroxyurea.

Since, the loss of the soluble DNA methylase may be related to covalent complex formation between the enzyme and 5-azadeoxycytosine residues in the DNA at the nuclear matrix (Santi et al., 1983), the slight increase in the activity of bound DNA methylase from 5-azadeoxycytidine treated cells (Figure 8) may reflect increase in DNA methylase bound but not yet inactivated. Figure 19 also indicates a model showing the outcome of 5-azadeoxycytidine treatment whereas the methylase could become irreversible bound to DNA at the matrix site.

(1)



(2)

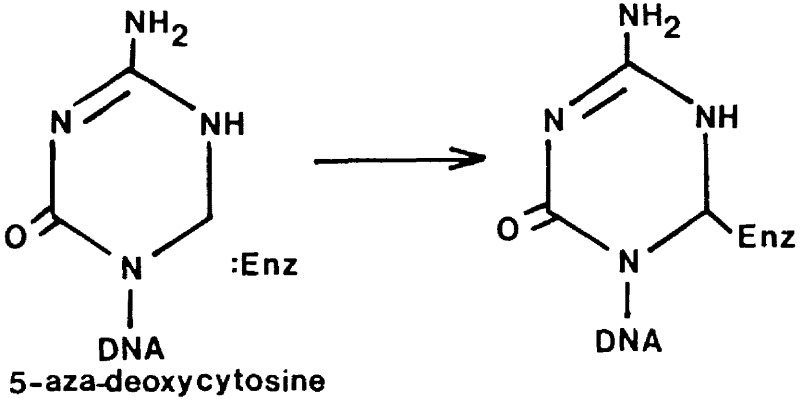


Fig.20 Mechanism of the action of DNA methylase.

3. Nature of bound DNA methylase and attempts to solubilise it.

3.1 Micrococcal nuclease digestion.

Digestion of chromatin with low levels of micrococcal nuclease has shown to release DNA methylase activity into a soluble form in a number of eukaryotic cells. For example, when CHO cells were treated with very low levels of micrococcal nuclease, most of the nuclear protein including 80% of the DNA methylase activity was released from the nuclei in the initial stages of the nuclease treatment (Adams et al., 1977). About 65% of the enzyme activity has also been solubilised from FEL cells, and this percent of solubilisation was achieved only by incubating cells for 5 minutes with 30 units/ml of micrococcal nuclease (Creusot and Christman 1981). Since micrococcal nuclease preferentially attacks the DNA fibres connecting adjacent nucleosomes (Noll 1974, Axel 1975), the enzyme activity which was released by digestion with low levels of micrococcal nuclease probably represents that associated with linker regions of the chromatin DNA.

As already mentioned, from 80-85% of the enzyme activity can be solubilised from nuclei at low salt concentration (0.2-0.4 NaCl) (Turnbull and Adams 1976, Creusot and Christman 1981), therefore, the amount of enzyme solubilised either by low levels of micrococcal nuclease or with low salt concentration is almost the same. Further investigations for the solubilisation of the remaining activity in the chromatin was not carried out by those groups of workers. However, when nuclear insoluble fraction (NIF) from Krebs II ascites cells was digested with normal levels of micrococcal nuclease (180 units/100µg chromatin DNA) none of the bound enzyme activity was released in the soluble fraction (results not shown). It seems that the DNA methylase bound to insoluble nuclear components may be directly associated with the nuclear matrix in such a way that normal levels of micrococcal nuclease could not dissociate it.

However, it has recently been reported that tightly bound nonhistone chromosomal proteins can be dissociated by digestion of chromatin with large amount of micrococcal nuclease. For example, the tightly bound nonhistone chromosomal protein antigen from HeLa chromosomal scaffold was dissociated by using about 2000 units of micrococcal nuclease per 100µg chromatin DNA. The immuno activity, which was abolished by digestion with micrococcal nuclease was shown recovered by the subsequent addition of HeLa DNA to reconstitute the immune complex (Dunn et al., 1980). This approach was used in an attempt to solubilise the DNA methylase bound to insoluble nuclear components. The incubation of chromatin of Krebs II mouse ascites cells (after removing about 85% of the enzyme activity by repeated extraction at low salt concentration) with extremely large amount of micrococcal nuclease, results the release of at least some of the bound enzyme into a soluble fraction (Figure 15). Whilst there remained no further activity in the bound fraction after this treatment, the amount of activity that was made soluble was about half that anticipated. The reasons for this incomplete recovery are not clear. The Ca^{+2} necessary to activate the micrococcal nuclease is unlikely to be the cause. Concentration up to 4mM have no effect on soluble DNA methylase (Adams and Burdon 1982b), However the high levels of EDTA required to chelate the Ca^{+2} may be to blame.

3.2 "Abstraction" of DNA methylase with DNA substrate.

Since such extremely large amount of micrococcal nuclease were necessary to solubilise even small amounts of 'bound' DNA methylase, alternative procedures were necessary simply to reduce expenditure.

In early reports the interaction of enzyme DNA *in vitro* and its scanning on the DNA substrate for methylation has already been

established by number of workers (Drahovsky and Morris 1971a, b, Simon et al., 1978, Adams and Gibb, 1981, Taylor and Jones 1982, Pfeifer et al., 1983). Similarly, the tight and loose binding of DNA methylase with DNA substrate in the presence of salt at different temperatures i.e. tight complex formation at 37°C and loose binding at 0°C has also been reported (Drahovsky and Morris 1971a,b). Taking into account, these properties of soluble mammalian DNA methylases, the effect of simply incubating the nuclear insoluble fraction with some unmethylated DNA was attempted.

It will be recalled that unmethylated DNAs of high G+C content of E. coli seemed to act as excellent substrate when added to the bound DNA methylase preparation. This raised the question of whether the 'bound' enzyme might relocate itself on to the added substrate DNA. In practice incubation with E. coli DNA actually abstracted about 75% of the enzyme from its bound form. Mammalian DNAs such as calf thmus DNA, which were poor exogenous substrates, were also poor at abstracting the DNA methylase from the bound matrix form (tables 14 and 15). In short the more unmethylated CpG sites in a DNA the more efficient it appears at abstracting DNA methylase from the bound form.

Furthermore, from figures 16 and 17, it can be seen that abstracting of DNA methylase with E. coli DNA does not require incubation at 37°C. The rate of abstraction at 37°C is higher than at 0°C but the same amount of enzyme activity can be abstracted at 0°C, if the length of incubation is increased. It seems that the binding of enzyme molecule to the exogenous DNA containing unmethylated cytosine molecules does not require higher temperature, but the mechanism remains obscure. A problem however, that the DNA methylase abstracted from nuclear components is still bound to the abstracting DNA. Thus a true "solubilisation" has not been achieved unless the DNA can be removed from the enzyme.

3.3 Solubilisation using ammonium sulphate.

Because of its high solubility and low interference to enzyme activity, ammonium sulphate has widely been used in the selective precipitation of enzymes from their aqueous extracts. Indeed ammonium sulphate has been employed as a step in the fractional purification of soluble DNA methylase (Turnbull and Adams 1976, Cato et al., 1978, Adams et al., 1979).

Treatment of the bound matrix material with 30% saturation of ammonium sulplate released DNA methylase into a soluble form (table 20). The reasons for the efficient dissociation of the DNA methylase from its bound complex is not clear. However, the binding site of the enzyme molecule, which may be attached to the phosphate backbone of the DNA molecule, may be so firm that even 2M NaCl fails to dissociate enzyme molecule from DNA. On the other hand either the high ionic strength possibly with ammonium sulphate or even the presence of sulphate ions may seem to loosen the matrix DNA associations.

Whatever the explanation the fact that 60% ammonium sulphate is already known to precipitate soluble DNA methylase, can be used to advantage in as much as the bound enzyme solubilised at 30% ammonium sulphate, can then be precipitated at 60% ammonium sulphate. This approach provided high yield of previously bound DNA methylase in a soluble form. This will permit further studies on the relationship between the various nuclear forms of DNA methylase.

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