DNA METHYLATION IN BHK-21 CELLS

by

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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 (1978) 169 1-27) except the following:-

BHK-21/C13	Baby Hamster Kidney cells (clone 13)
BHK-21/PyY	Polyoma virus transformed baby hamster kidney cells
BrdUrd	Bromodeoxyuridine
BSS	Balanced Salt Solution
DNase	Deoxyribonuclease
HAP	Hydroxyapatite
5MC or m^5Cyt	5-Methyl Cytosine
mRNA	Messenger Ribonucleic Acid
rDNA	Ribosomal Deoxyribonucleic Acid
R-M system	Restriction-modification System
RSB	Reticulocyte Standard Buffer
SAM	S-adenosyl-L-methionine
SAH	S-adenosyl-L-homocysteine
SDS	Sodium dodecyl sulphate
SSC	0.15M NaCl, 0.015M trisodium citrate pH7.0

Except in the case of possible ambiguity the 'd' (deoxy-) prefix and the phosphate residues in the 3', 5'-linkages have been omitted in DNA sequences.

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SUMMARY

The level of 5-methyl cytosine residues is higher in the DNA of polyoma-virus-transformed BHK-21/PyY cells than in the DNA of the non-transformed BHK-21/C13 cells. The 5-methyl cytosine residues arise as a result of the transfer of methyl groups from S-adenosyl-L- methionine to DNA cytosine moieties by DNA methylases located in nuclei of these cells.

DNA methylases have been isolated and partially purified from nuclei of BHK-21/C13 and BHK-21/PyY cells and most of the properties of these two enzymes appear to be identical. However slight differences exist in the sequences these enzymes methylate in substrate E. coli DNA as shown by studies of the level of methylation of pyrimidine isostichs fractionated from the in vitro methylated substrate. These differences can neither account for the differences in the in vivo level of DNA methylation in the two hamster cell lines nor can they provide any evidence to suggest that the higher level of methylation in the BHK-21/PyY DNA may be due to the presence of a virus-coded DNA methylase(s) in these cells. It seems that the two BHK-21 cell genomes may be organised differently. Further studies have shown differences in the arrangements of the two genomes with respect to 5-methyl cytosine residues. The highly repetitive region of the BHK-21/PyY cell DNA is twice

as methylated as the corresponding region of the BHK-21/C13 cell DNA. The higher level of methylation of the BHK-21/PyY cell DNA may therefore be due to possible reiteration of certain sequences containing 5-methyl cytosine which may simply occur less frequently in the BHK-21/C13 cell genome.

The role of eukaryotic DNA methylation is as yet uncertain. It has been shown in this work that nuclei of BHK-21/C13 cells contain a SAM-dependent nuclease which appears to degrade unmethylated DNA but inactive towards DNA previously methylated by the BHK-21/PyY DNA methylase. The biological significance of this enzyme is unknown and it is not yet certain whether or not this enzyme is analogous to any of the bacterial restriction enzymes.

INTRODUCTION

1. OCCURRENCE OF METHYLATED BASES IN DNA

1.1 Historical Background

The nitrogeneous constituents of DNA were thought to be only adenine, thymine, guanine and cytosine until 1925 when a derivative of cytosine, a "5-methyl cytosine" was discovered among hydrolytic products of tuberculinic acid, the nucleic acid of the tubercle bacillus (Johnson and Coghill, 1925). Twenty five years later, 5-methyl cytosine was established as a definite constituent of DNA of mammalian, fish and insect origin (Wyatt, 1950; Wyatt, 1951) and in 1955, another constituent of DNA, a 6-methyl adenine was discovered in the DNA of bacteria (Dunn, 1955 a; b). Since these bases (5-methyl cytosine and 6-methyl adenine) occur in low amounts in DNA, they are often referred to in the literature as "rare" or "minor" bases.

1.2 Minor Bases and Base-Pairing

Since the publication of Watson and Crick's model for the structure of DNA (Watson and Crick, 1955 a; b), it had been taken for granted that minor bases take part in normal base pairing and do not disrupt the DNA duplex (Bessman et al, 1958). Recent work has however shown that whilst the above statement is true for 5-methyl cytosine, 6-methyl adenine residues disrupt the DNA helix.

X-ray crystallographic analyses of 6-methyl adenine and its analogues have shown that the exocyclic amino group is coplanar with the purine ring and that the C(6)-N(6) bond

bond has a partial double bond character (Sternglanz and Bugg, 1973 a; b). This means that the methyl group can either point to (as in Fig 1a) or away (as in Fig 1b) from the imidazole ring.

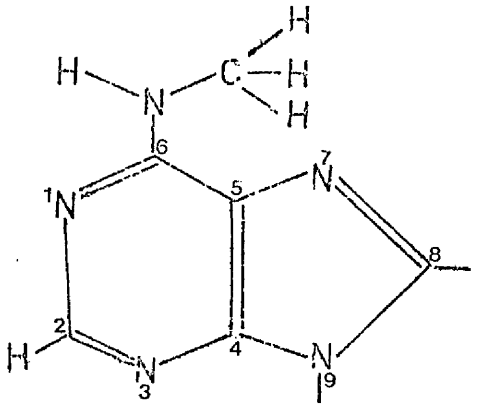


Fig 1a. 6-methyl adenine molecule (methyl group is pointing to imidazole ring).

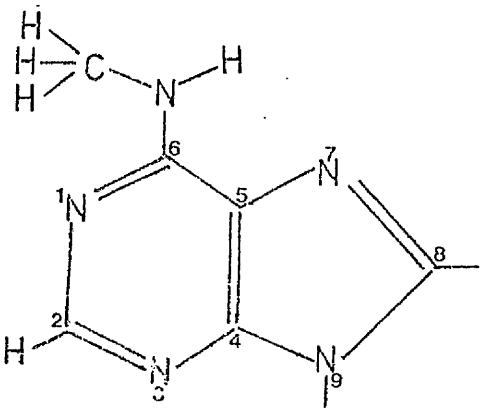


Fig 1b. 6-methyl adenine molecule (methyl group is pointing away from imidazole ring).

The latter conformation is preferred and this has been confirmed by high resolution n.m.r. studies (Engles and Von Hippel, 1974). In this arrangement however, the methyl group interferes with hydrogen bonding to thymine and consequently destabilises the DNA helix locally. In view of this interference by the methyl group, it is therefore not surprising that the Tm of poly(6MeA)-poly(U) is 41°C lower than the Tm of poly(A)-poly(U) (Ikeda et al, 1970).

By contrast, 5-methyl cytosine residues have been shown to form perfect Watson-Crick hydrogen bonds with guanine.

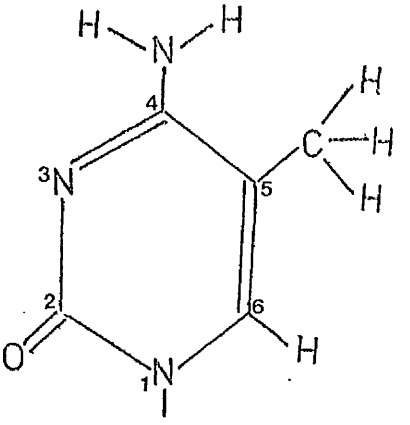


Fig 2. 5-methyl cytosine molecule.

Synthetic DNAs and homopolymer pairs containing cytosine substituted at the 5(c)-position by a methyl group (Fig 2) invariably melt at higher temperatures than do isomeric DNA lacking cytosine methylation. Furthermore, buoyant density studies have shown that methylation interferes with hydration in the major groove of helical DNA thereby contributing to helix stabilisation (Gill et al, 1974).

1.3 Distribution of Methylated Bases in DNA

1.3.1 In Prokaryotes

The minor base, 6-methyl adenine was first discovered in the DNA of bacteria (Dunn, 1955 a; b; Dunn and Smith, 1958) and for sometime it was thought that 6-methyl adenine was a characteristic modification of bacterial DNA. This idea was discarded when it became apparent that this minor base occurs in the DNA of other micro-organisms (Kaye et al, 1967) and in certain organisms it is not even detectable (Oda and Marmur, 1966; Vanyushin, 1968). Nevertheless 6-methyl adenine residues remain widespread as minor

bases in the DNA of many a bacterium cell. The discovery of 5-methyl cytosine in the DNA of bacteria (Doskočil and Šormorá, 1965 a; b) dealt the final blow to the notion of a qualitative difference in the methylation of bacterial and animal DNA.

1.3.2 In Eukaryotes

5-methyl cytosine residues occur in most eukaryotic DNA (Wyatt, 1950; Dunn and Smith, 1958; Craddock, 1971; Lawley et al, 1972). But the DNA of the blue green alga Plectonema boryanum contains 6-methyl adenine in addition to 5-methyl cytosine residues (Kaye et al, 1967) and in the DNAs of the micro- and macro-nuclei of Tetrahymena pyriformis, 6-methyl adenine residues are the only methylated bases detected (Gorovsky et al, 1973).

Plant DNAs are more methylated than animal DNAs and the relatively high amount of 5-methyl cytosine in plant DNA seems to be a specific phylogenetic acquisition as Archegonitae develop into contemporary forms of Angiosperms (Vanyushin, 1968). Thus wheat germ DNA contains 27% of its cytosine residues as 5-methyl cytosine (Chargaff and Davidson, 1955).

In mammals the level of DNA cytosine methylation vary from organism to organism in the range of 0.5-2.0 mole%. In fish this level is about 1.5-2.0 moles per 100 moles of cytosine residues whilst in some insects and crustacea, the level is very much lower - only 0.1 mole% (Vanyushin et al, 1970). In Drosophila, methylated bases seem to be

found in the satellite DNA sequences (Dawid, I.R., personal communication).

1.3.3 In Viruses and Bacteriophages

Phage DNA, like bacterial and animal DNAs contain methylated bases. 6-methyl adenine residues appear in the DNAs of bacteriophage T_{2r+} and T_{2r} and the Salmonella C bacteriophage (Dunn and Smith, 1958). The DNAs of T_1, T_2, T_4 and T_7 possess both 6-methyl adenine and 5-methyl cytosine residues (Gold et al, 1964). However, bacteriophages T_3, T_5 and T_6 are devoid of detectable base methylation in their DNAs (Gefter et al, 1966).

The DNA of the bacteriophage ØX174 has only one methylated base (a 5-methyl cytosine residue) in its 5,375 nucleotide genome and this methylated base plays an important role in the final steps of virus maturation (Razin et at, 1975; Friedman and Razin, 1976). In the DNA of the λ phage, the minor base appears as 5-methyl cytosine (0.08 mole%) (Ledinko, 1964).

The DNAs of the animal viruses polyoma and Herpes simplex have no detectable methylated bases (Kaye and Winocour, 1967; Low et al, 1969). Recently in 1977 Sharma and Biswal confirmed that no detectable 5-methyl cytosine residues are present in mature Herpes Simplex type 1 virions. However 5-methyl cytosine residues exist in the intracellular replicating viral DNA. These 5-methyl cytosine residues were not contributed by RNA or cellular DNA contamination. It has therefore been suggested that methylation might be necessary for replication of viral DNA and that mature

virions may have an enzyme which destroys the methyl donor pool (Sharma and Biswal, 1977).

1.4 The Relationship of DNA Methylation to DNA Synthesis

In prokaryotes, DNA synthesis is almost immediately followed by methylation (Billen, 1968; Lark, 1968). DNA methylation was therefore thought to be responsible for keeping the stability of nascent DNA during replication. However this may not be the case as *E. coli* grown in methionine-free medium continued for some time to synthesize DNA that was undermethylated (Lark, 1968).

In eukaryotes, Burdon and Adams, 1969 reported a delay between DNA synthesis and methylation and experiments by Kappler in 1970 suggested that this delay might be between 1 and 2 minutes in mouse adrenal cultured cells. Studies done on DNA methylation during the mitotic cycle of *Physarum polycephalum*, a myxomycetes whose nuclei undergo natural synchronous division, have shown that methylation of DNA occurs not only during the S-period but during the G_2 period, a time when essentially no synthesis of DNA takes place (Evans and Evans, 1970). Previously in 1969, Burdon and Adams had made a similar report from studies on methylation of cytosine residues in synchronously dividing mouse fibroblasts L929.

Events in the S period of the cell cycle have been well studied. Early replicating DNA is more methylated than late replicating DNA (Adams, 1971; Comings, 1972; Schneiderman and Billen, 1973) and rapidly reannealing DNA

synthesized very early in the S-period of the cell cycle (Comings and Mattoccia, 1970) is highly methylated (Brahic and Fraser, 1971; Sneiderman and Billen, 1973). Methylation in the S-period of the cell cycle has been examined by a visual DNA fibre autoradiography (Molitor et al, 1976). When mouse L929 cells received short pulses of radioactivity from ^{3}H-thymidine and L-[methyl-^{3}H] methionine, their DNAs after autoradiography using the fibre spreading technique showed that the pattern of replication was identical to that of methylation. However when these cells were labelled throughout the S-period the DNA fibre was uniformly labelled with ^{3}H-thymidine whilst the label with L-[methyl-^{3}H] methionine appeared to show distinct intervals of 8-20μm in the autoradiographic pattern. These gaps corresponding to 23-50 Kilo base-pairs (and 10% of the total genome) escaped methylation.

2. DNA METHYLASES

2.1 DNA Methylases in Prokaryotes

2.1.1 DNA Methylation at the Polynucleotide Level.

The discovery of minor bases in DNA (Wyatt, 1950; Wyatt, 1951; Dunn, 1955 a; b) stimulated interests in studies on DNA methylation and the search for a mechanism of methylation. Although it had been known since 1958 that the deoxyribonucleotide triphosphate of 5-methyl cytosine could quantitatively replace dCTP as a substrate for the enzyme DNA polymerase (Bessman et al, 1958), it was not until after the discovery of glucosylation of DNA of the T-even bacteriophage at the polynucleotide level, that it was suggested

that the incorporation of methyl groups might take place after polymerisation in the biosynthesis of DNA (Kornberg et al, 1961). Later studies with bacterial cells irradiated with ultraviolet light at a dosage which was sufficiently high to inhibit DNA synthesis, showed that the incorporation of methyl groups continued at a significant rate even after the inhibition of DNA synthesis (Ryan and Borek, 1964; Srinivasan and Borek, 1964).

2.1.2 Properties of Prokaryotic DNA Methylases

The 5-methyl cytosine residues in DNA arise as a result of the donation of methyl groups from S-adenosyl-L-methionine to DNA cytosine moieties and this reaction is catalysed by the enzyme DNA methylase(S-adenosyl-L-methionine : DNA (6-aminopurine) methyl transferase, EC 2.1.1.37) (Gold et al, 1963). The trivial name DNA methylase will be used throughout this Introduction.

$$\text{DNA} + \text{S-adenosyl-L-methionine} \xrightarrow[\text{methylase}]{\text{DNA}} \text{methyl-DNA} + \text{S-adenosyl-L-homocysteine}$$

The enzymes catalysing the methylation of DNA have been purified from Escherichia coli strain W (Gold and Hurwitz, 1964 a; b) and Bacillus subtilis strain 6633 (Oda and Marmur, 1966). The methylation reaction does not require the presence of divalent cations and the requirement of S-adenosyl-L-methionine (SAM) as a methyl donor cannot be met by other methyl donors. S-adenosyl-L-homocysteine is a potent inhibitor of this reaction. The substrate

specificity of the DNA methylase isolated from B. subtilis 6633 cells is quite different from that of E. coli W cells. The former enzyme transfers methyl groups from S-adenosyl-L-methionine to cytosine residues in native and heat denatured DNA (Oda and Marmur, 1966). The DNA methylase from E. coli W cells however, methylates adenine and cytosine residues in heat denatured DNA only (Gold and Hurwitz, 1964 a; b).

2.1.3 Binary Nature of Prokaryotic DNA Methylase

In section 2.1.2, E. coli W cell DNA methylase has been shown to methylate cytosine and adenine residues in substrate DNA. From theoretical considerations, it seems unlikely that only one DNA methylase can achieve the methylation of cytosine (which involves formation of carbon-carbon bond) and the methylation of adenine residues (which requires a nitrogen-carbon bond). It is therefore conceivable that two different DNA methylases exist in bacterial cells.

In fact, evidence for the binary nature of bacterial DNA methylases has been cited by Fujimoto et al (1965) who showed that heterologous methylation with extracts of E. coli B cells can lead to incorporation of methyl groups in adenine bases in substrate DNA whilst extracts of E. coli W and K12 cells methylate both adenine and cytosine residues in any substrate DNA including E. coli B DNA which naturally lacks 5-methyl cytosine.

Another evidence for the binary nature of bacterial DNA methylases stems from studies with DNA methylases from T_2 bacteriophage infected cells. An increase in host DNA methylase activity has been reported after infection by T_2 bacteriophage (Gold and Hurwitz, 1964 c). This report has been confirmed by Fujimoto et al (1965) who discovered that when extracts of T_2 infected E. coli B cells were employed as methylating enzyme with either homologous or heterologous DNA as substrate, there was an elevation of adenine residues methylated over the level produced by extracts from non-infected cells. But extracts of T_2 infected E. coli K12 cells caused a profound change in the ratio of adenine to cytosine bases methylated. This change was initiated by a 10-fold increase in the activity of the adenine methylase. Thus it is only the activity of the adenine methylase that is enhanced by the bacteriophage infection.

2.1.4 Study of Phage Induced DNA Methylase

E. coli B cells are useful and important "tools" in the study of the presence of a phage-directed DNA cytosine-methylase in bacterial cells. E. coli B cells have no methylated cytosine residues and they are devoid of DNA cytosine methylases (Doskočil and Šormorá, 1965 a; b; Fujimoto et al, 1965). The detection of this enzyme after phage infection is therefore an obvious proof of the

presence of a phage-directed methylase. However, E. coli B cells can be made to synthesize DNA cytosine methylase by introducing into them the genes controlling the methylation of cytosine from E. coli K12 cells. It was only after the transfer of such genes that the DNA cytosine methylase was observed in the hybrid recombinant cell (Mamalak and Boyer, 1970). A high level of 5-methyl cytosine was found in the DNA of E. coli B cells after the transfer into these cells of the (N3) drug resistant factor (Hattmannet al, 1972). The appearance of the cytosine methylase in the hybrid E. coli B (N3) cells has been confirmed by in vitro studies which show that extracts from these cells methylate adenine and cytosine residues in substrate DNA whilst extracts of E. coli B cells (without the N3 drug factor) methylate only adenine residues.

2.1.5 Specific Phage-Directed DNA Methylases

Hausmann and Gold (1966) reported a 100-fold increase in the methylating capacity of extracts of E. coli B cells, 6 minutes after infection by T_2 bacteriophage. De novo protein synthesis is necessary for this increase to occur and the properties of the DNA methylase of the phage T_2-infected bacteria suggest that it is a new phage directed DNA methylase. T_4 and T_1 phage-infected E. coli B cells showed increases in host DNA methylase activity but these were smaller increases compared with the increase in the T_2 phage infected cells. T_7 and λ bacteriophages had no effect on the host DNA methylases (Hausmann and Gold, 1966).

Bacteriophage T_3 induces in E. coli cells the synthesis of a specific enzyme which breaks down S-adenosyl-methionine into thio-methyl adenosine and homoserine (Gefter et al, 1966). Hence infection by this bacteriophage leads to the suppression of DNA methylation.

The presence of a specific phage-directed DNA cytosine methylase has been observed in ØX174 bacteriophage infected E. coli B cells. After infection of these cells, 5-methyl cytosine residues appeared in the host DNA which before had only 6-methyl adenine residues. A DNA methylase isolated and partially purified from these infected cells methylated DNA from uninfected cells but not DNA from infected cells as all the potential sites in the latter DNA had been methylated already in vivo (Razin et al, 1970).

When Xpl2 bacteriophage infects Xanthomonas oryzae cells, it induces the activity of an enzyme that is responsible for the unusual occurrence of methylated bases in Xpl2 phage DNA (Tsong-Teh and Jenn, 1976). All the cytosine residues in Xpl2 phage DNA are methylated (Ehrlich et al, 1975; Tsong-Teh and Jenn, 1976). However unlike DNA methylases, this enzyme methylates cytosine residues at the nucleotide level before polymerisation of the DNA. The methyl donor in this enzymic reaction is not derived from the thio-methyl carbon of methionine but rather the 3-carbon of serine (Tsong-Teh and Jenn, 1976). Based on these characteristics it cannot be classified as a DNA methylase.

2.2 DNA Methylases from Mammalian Sources

2.2.1 Solubilisation of DNA Methylases from Chromatin

Burdon et al in 1967 were the first to report that DNA methylase in mammalian cells is associated with nuclear chromatin. They assayed the enzyme activity in chromatin isolated from Krebs II ascites tumour cells. In 1968, Sheid et al and Kalousek and Morris working independently with cell free homogenate of rat liver and rat spleen confirmed that DNA methylase is associated with the nuclear insoluble fraction - chromatin.

Later work with rat liver nuclei showed that DNA methylase can be solubilized from chromatin but only under stringent extraction conditions involving freezing and thawing a number of times followed by streptomycin sulphate precipitation (Kalousek and Morris, 1969 b). This achievement has led to the partial purification and studies of the nature of interaction with substrate DNA of DNA methylases from a number of mammalian sources (Drahovský and Morris, 1971 a, b; Roy and Weissbach, 1975; Sneider, et al, 1975; Turnbull and Adams, 1976; Cato and Burdon, 1977)

2.2.2 Purification of Mammalian DNA Methylases

DNA methylases have been purified from a number of sources ranging from rat spleen and liver cells (Kalousek and Morris, 1969 b; Morris and Pih, 1971), Krebs II ascites cells

(Turnbull and Adams, 1976) to cultured cells such as Novikoff rat heptoma cells (Sneider et al, 1975) HeLa cells (Roy and Weissbach, 1975) and BHK-21 cells (Cato and Burdon, 1977). The purification steps employed are fairly similar and they involve ammonium sulphate precipitation, gel filtration, DEAE-cellulose, phosphocellulose and hydroxyapatite column chromatography. Hitherto, no report of a DNA methylase purification to apparent homogeneity has been made by any group of workers. SDS polyacrylamide gels of the enzyme at the final purification step are heavily contaminated with other proteins (Sneider et al, 1975; Turnbull and Adams, 1975). The estimated molecular weight of the DNA methylase from HeLa cell nuclei was 120,000 (Roy and Weissbach, 1975). DNA methylase from Krebs II ascites cells when passed through Ultragel ACA34 Column produced a number of peaks of enzymic activity, the most prominent of which corresponded to a molecular weight of 240,000. When this peak activity was examined further on SDS polyacrylamide gels a prominent band corresponding to molecular weight of 80,000 was obtained (Turnbull and Adams, 1976). It has therefore been suggested that the active form of the DNA methylase might be a dimer or possibly a trimer (Turnbull and Adams, 1976).

2.2.3 Properties of DNA Methylases from Mammalian Sources

The methyl groups incoporated into cytosine residues in animal DNAs like methylated bases (5-methyl cytosine and 6-methyl adenine) in bacterial DNA, are derived from methionine (Brown and Attardi, 1965; Winocour et al, 1965

Burdon, 1966; Sneider and Potter, 1969).

The DNA methylases isolated so far from mammalian sources have the ability to methylate both single and double-stranded DNAs (Roy and Weissbach, 1975; Turnbull and Adams, 1976; Sneider et al, 1975) but heat-denatured DNAs (i.e. single-stranded DNAs) are much better methyl acceptors than native DNA. Generally, homologous DNA is a poorer substrate than heterologous DNA (Roy and Weissbach, 1975; Sneider et al, 1975; Turnbull and Adams, 1976; Kudryashova and Vanyushin, 1976). In vitro studies with isolated DNA methylases show that synthetic copolymers $(dC)_n$, $(dG)_n.(dC)_n$ are not methylated (Sneider et al, 1975). However the alternating double-stranded $(dG.dC)_n.(dG.dC)_n$ and the random single-stranded $(dG.dC)_n$ are methylated to a small extent (Roy and Weissbach, 1975; Sneider et al, 1975).

2.2.4 Sequences Methylated by Mammalian DNA Methylases

5-methyl cytosine does not occur randomly in mammalian DNA. It tends to be flanked by purines (Smith and Markham, 1952; Shapiro and Chargaff, 1960). In mammals, cytosine residues are preferentially methylated when in the doublet CpG (Doskočil and Šorm, 1962; Grippo et al, 1968). But the level of methylation seems to depend not simply on the proportion of CpG pairs. One report exists of a low level of methylation of cytosines in the sequence CpT (Sneider, 1972). DNA methylase isolated from mammalian sources have been used to modify cytosine residues in substrate DNA and

analyses of the methylated products reveal identical pattern of methylation of pyrimidine fragments as obtained from studies of *in vivo* methylation of a number of vertebrate DNAs (Browne and Burdon, 1977; Browne *et al*, 1977).

Difficulties arising from the sequencing of a whole eukaryotic genome have hampered the search for the site and sequence specificity of DNA methylases. So far, studies done in this field comprise analyses of the pyrimidine isostichs methylated *in vivo* or *in vitro* by DNA methylase preparations (Sneider, 1972; Tosi *et al*, 1972; Nass, 1973; Vanyushin and Kirnos, 1977). Recently the oocyte 5S DNA in *Xenopus laevis* has been sequenced and all the CpG doublets are probably methylated (G. Brownlee, private communication).

Nearest neighbour bases to the 5-methyl cytosine residue have been determined on *Micrococcus luteus* DNA methylated by HeLa cell DNA methylase and the results obtained have been compared with base analyses of *in vivo* methylated HeLa cell DNA (Roy and Weissbach, 1975). The 5' neighbours can be either G or C while the 3' neighbour is always a G. The sequence worked out is thus p(G/C)pmCpG. It has not been established whether this represents an ambiguity in recognition site or whether the trinucleotides pGpmCpG and pCpmCpG occur on opposite strands within a non palindromic sequence.

In fact in the conditions (Bollum, 1966) used in that experiment the pancreatic DNase digested products consisted of only

40-50% dinucleotides, the remainder being mostly tri-nucleotides and mononucleotides.

2.2.5 The Mechanism of Action of DNA Methylase from Mammalian Sources

Various attempts have been made to work out a mechanism of action of DNA methylase on substrate DNA but the proposals put forward have been unsuccessful in explaining the action of the enzyme at the molecular level.

The distribution of 5-methyl cytosine in mammalian DNA is not random (Smith and Markham, 1952; Shapiro and Chargaff, 1960). The DNA methylases transfer methyl groups to specific deoxyribonucleotide residues in DNA (Gold and Hurwitz, 1963; Yarus, 1969). Against this background, and from results of investigations on binding of DNA methylase from rat liver to substrate *E. coli* DNA, a mechanism of action of DNA methylases has been proposed (Drahovský and Morris, 1971 a; b). This mechanism explains how DNA methylase binds to DNA and appears to move down the DNA helix, scanning for methylation sites. Once a methylation site is reached, the enzyme undergoes a conformational change; thus exposing the binding site for the transfer of the methyl group from S-adenosyl-L-methionine. After the appropriate cytosine is methylated the enzyme returns to its previous state and continues the scan (Drahovský and Morris, 1971 b).

Other workers have not been successful in demonstrating this

mode of action of DNA methylases isolated either from rat liver nuclei (Adams, R.L.P. personal communication) or Krebs II ascites cells (Turnbull and Adams, 1976).

2.3 DNA Methylases in Plants

Kalousek and Morris (1969 a) have shown that nuclear extracts of pea seedling can be used as source of DNA methylase to methylate DNA in the crude extract.

3. BIOLOGICAL ROLE FOR DNA METHYLATION

3.1 Different Levels of DNA Methylation

3.1.1 Tissue and Species Specific Variation

In the DNA of higher animals, there is a species variation as well as a tissue specific difference in the 5-methyl cytosine content (Vanyushin et al, 1970, 1973; Kappler, 1971). This variation is independent of the cytosine plus guanine content of the DNA (Vanyushin et al, 1973). The significance of this tissue and species variation in the level of 5-methyl cytosine content of the DNA is uncertain. It could be a result of a variable delay between the synthesis of DNA and its methylation or it might reflect a role of 5-methyl cytosine in cellular differentiation.

3.1.2 Differences in 5-methyl Cytosine Levels During Development

There is a lower degree of methylation of sperm DNA as compared with somatic cell DNA in fish and mammals (Vanyushin et al, 1970) and since DNAs of sperm and somatic cells have identical base composition in terms of pyrimidine clusters

and nearest neighbour base sequence, it might be that sperm DNA of vertebrates are actually under methylated and that the deficiency in methyl groups is made up on or after oocyte fertilization. This observation if confirmed may be relevant in the interpretation of the role of methylation in the expression of genomic activity since it is assumed that the sperm genome is totally repressed (Rossi et al, 1975).

The methylation of DNA during embryogenesis of sea urchin has been studied in detail by Scarano who showed striking changes in the rate of methionine uptake by embryos of Sphaerechinus granularis and Parencentrotus lividus at various stages of development (Scarano, 1969). These changes have been linked indirectly to changes in 5-methyl cytosine content in the DNA of the developing sea urchin embryos. Tosi and Scarano, 1973 have reported that nuclei of the developing sea urchin Parencentrotus lividus embryos will methylate their own DNA in vitro but methylation is stimulated 20-fold by trypsin. This effect is noticeable at the blastula stage but not the gastula. This could therefore account for a different arrangement of DNA histone proteins during development. This view is supported by studies which show that between fertilization and the blastula stage, 40-60% of the newly synthesised proteins are rapidly transported to the nucleus where they associate with chromosomes (Kedes et al, 1969). The trypsin activation may therefore be a mere unmasking of DNA methylation sites as trypsin is known to digest peptide bonds adjacent to

basic amino acids in DNA-histone complexes (Whitlock and Simpson, 1977). Alternatively, the differential methylation at various stages in the development of the sea urchin embryo can be accounted for by the synthesis of specific DNA methylases during embryogenesis; some of which are sensitive to tryptic digestion.

No differences exist in the level of 5-methyl cytosine residues in DNA during developmental stages after blastula in Xenopus laevis compared with the 1.6 mole% found in the liver DNA of the adult frog (Baur and Kröger, 1976). However when limb buds incubated 11 days after gestation differentiate from blasteme into recognisable cartilaginous bone, anlagen, differences in 5-methyl cytosine level were found in the DNA isolated from these stages of late development (Baur and Kröger, 1976). Other interesting information on DNA methylation has been obtained from studies on Xenopus laevis oocytes. Amplified, extra-chromosomal DNAs coding for ribosomal RNA in the amphibian oocytes, which are transcriptionally active are not methylated whereas the corresponding somatic rDNA is highly methylated (4.5 mole%) (Dawid et al, 1970). However both DNAs have the same general structure (Brown and Dawid, 1968; Gall, 1968) and both comprise of alternating transcribed regions of constant length and non-transcribed "spacers" of variable length (Wellauer et al, 1974).

3.1.3 Deamination of 5-methyl Cytosine at the Polynucleotide Level

It has been suggested that methylation of cytosine at the 5-carbon position followed by deamination at the 6-carbon position could give a thymine residue and thus a GC pair could be changed into an AT pair after replication. Such base modifications if they occur in promotor regions can lead to heritable changes in base sequence and could control the activity of structural genes (Scarano, 1971; Scarano et al, 1977). In vivo experiments with developing sea urchin embryos cultured in the presence of L-[CD_3] methionine showed that a proportion of DNA thymine is synthesized at the DNA polymer level (Scarano, 1971) This therefore provides circumstantial evidence that heritable changes occur in base sequence during embryonic development. Other workers have not been successful in demonstrating the transition of cytosine to thymine by methylation and deamination (Burdon and Adams, 1969; Sneider, 1973; Geraci et al, 1974).

3.1.4 Different Levels of 5-methyl Cytosine in DNA During Aging

Investigation of the 5-methyl cytosine level in the DNA of salmon has shown that it changes directionally in all the somatic tissues (liver, kidney, spleen, muscles) and falls 1.5 to 2 times with age (Berdyshev et al, 1967). The change in the content of 5-methyl cytosine is not coupled with changes in the composition and primary structure of

DNA. In view of these changes the acceptor capacity of nuclear DNAs of young (1 month) and old (28 months) rats were studied *in vitro*. DNA methylases from cells of young rats methylate DNA from nuclei of brain of old rats considerably more than homologous DNA from these young rats (Kudryashova and Vanyushin, 1976). According to Holliday and Pugh's hypothesis, the methylation of recurring sequences in DNA may be a unique biological clock which successively, replication after replication, countsoff the number of cell divisions (Holliday and Pugh, 1975). Based on this hypothesis one would expect DNA methylation to increase noticeably with age. But studies mentioned above point in the opposite direction.

3.1.5 Induced Changes in 5-methyl Cytosine Level in DNA

DNA methylation has been reported to respond to hormonal regulation (Romanov *et al*, 1976). Eight hours after interperitoneal injection of hydrocortisone the level of 5-methyl cytosine in rat liver DNA rose by 70% over the normal even though the molecular population of hydrocortisone-treated and non-treated liver DNAs were identical. It has also been reported that hydrocortisone stimulates the transcription of DNA (Lang, 1968). It has therefore been suggested that induced hyper-methylation of DNA under the influence of hydrocortisone might take part in the functional activity of the cell as the hyper-methylation concerns not merely the structural but apparently the

regulatory gene (Romanov et al, 1976).

The level of DNA methylation of a polyoma virus transformed baby hamster kidney cells is higher by 2-fold than the level of DNA methylation in the non-transformed hamster cells (Rubery and Newton, 1973; Nass, 1973; Browne and Burdon, 1977). DNA of baby hamster kidney cells transformed by adenovirus type 12 (Ad 12); the HA12/7 cell line, is highly methylated than the non-transformed hamster cell DNA (Günthert et al, 1976) and so is the RNA rouse sarcoma virus transformed BHK-21 cells (Nass, 1973). Recent studies have shown that the level of 5-methyl cytosine in rat hepatoma cell DNA is the same as that in rat liver DNA (2.4 mole%) (Singer et al, 1977). It has therefore been argued that the abnormal methylation of DNA is not necessarily correlated with cancerous growth - a view shared by Grant et al (1973) who showed that the 5-methyl cytosine content in a mouse non-neoplastic cell line is the same as that of the neoplastic line derived from it.

3.1.6 Changes in DNA Methylation of Brain Cells During Development of a Conditioned Reflex

During the development of a simple feeding conditioned reflex, there was a 15-26% increase in the level of 5-methyl cytosine residues of the DNA of brain cells. This increase was temporarily linked to the activation of the cell genome in different brain regions. In the case of long-term memory, there was a 10% increase in the level of 5-methyl cytosine in the DNA of those regions (cortex, hyppocampus) which are

directly associated with the formation of a conditioned reflex (Vanyushin et al, 1977). These differences were reported to reflect a change in the level of DNA methylation during learning. The drawback to this experiment is primarily the method used to determine the 5-methyl cytosine level. This consists of perchloric acid digestion of un-labelled DNA and subsequent separation of the bases on thin-layer chromatographic plates and elution of the spots followed by analyses by U.V spectroscopy. This technique gives results that are non-reproducible. In fact the differences in 5-methyl cytosine level might only be due to errors introduced by the technique used. Secondly if the levels reported are real, it might be necessary to classify the character of DNA methylation during a more prolonged development such as "automisation" of conditioned reflexes, forgetting and acute fading of developed habits.

3.1.7 Methylation of Mitochondrial DNA

Contrasting reports have been made about the level of methylation o mitochondrial DNA. Whilst Nass, 1973 reported that the level of 5-methyl cytosine residues in mitochondrial DNA of mouse L cells and variants of BHK cells studied were one-fourth to one-fourteenth the level in nuclear DNA of these cells, Dawid, 1974 reported that no 5-methyl cytosine residues exist in the mitochondrial DNA of HeLa and Xenopus laevis cells. On the other hand, Vanyushin and Kirnos (1976, 1977) after studying the base composition, pyrimidine

clusters and character of methylation of mitochondrial and nuclear DNAs from mammals, fish and birds, reported that despite species differences in G+C content and low pyrimidine clustering of mitochondrial DNAs, on the whole, the methylation of mitochondrial DNA surpass by 1.5 - 2 times the methylation in nuclear DNA. In the studies described, the extraction of mitochondrial DNA was carried out under the most meticulous conditions to avoid nuclear DNA contamination. Examination of the final mitochondrial DNA preparation under the electron microscope showed circular shapes of contour lengths 4.5 - 5.5μm which fit in with known shapes and lengths of mitochondrial DNA (Vanyushin and Kirnos, 1976; 1977). From the above data, it is difficult to establish whether mitochondrial DNAs are more/less methylated than nuclear DNA. However, like nuclear DNA methylation, mitochondrial DNAs are species specific in their gross content of 5-methyl cytosine residues. It can therefore be suggested that this might have some biological significance. Sager and Kitchin, 1975 have postulated that mitochondrial DNA might be involved in restriction-modification mechanism.

3.2 Restriction-Modification (R-M) Mechanism in Bacterial Cells

3.2.1 The R-M Mechanism

Studies on bacteriophage-cell interaction have revealed that bacterial cells contain on the one hand restriction enzymes which recognise specific set of nucleotides within DNA and make a double-stranded cleavage, and on the other hand,

modification enzymeswhich recognise the same nucleotide sequence and modify them to prevent them from being cleaved (Arber and Dussoix, 1962; Dussoix and Arber, 1962; Arber, 1974). Paradoxical though this might sound, it simply means that an unmodified DNA entering a bacterial cell is destroyed by the cell's restriction enzyme unless this invading DNA has been modified at the restriction sites. The whole mechanism of restriction and modification can therefore be viewed as a defence mechanism whereby bacterial cells defend themselves against the intrusion of foreign DNA - be it a phage particle or naked DNA. But bacterial cells do not destroy their own DNA by harbouring a site specific restriction enzyme and this is because these sites have all been modified (Arber, 1974). However, not all modification sites are sites for the restriction enzyme (Hattmann et al, 1973).

3.2.2 R-M Nomenclature

The nomenclatural system used in this Introduction is essentially that used by Smith and Nathans, 1973. This system abbreviates the name of the organism (e.g. E. coli becomes Eco) and the enzyme specified by a virus or plasmid identified with a suffix (e.g. Eco P1 or Eco R1). Eco K is determined by the genome of E. coli K12, Eco B by the genome E. coli B; Eco P1 by the phage P1; R1 by the resistant factors R124 and RY-5; and RII by several resistant factors e.g. R factor N3. The system hsd

(host specificity for DNA) is used for the genes determining DNA restriction and modification activities.

3.2.3 The Products of Modification

The products of modification usually consists of methylation of the N-6 amino group of adenine residues in a specific DNA sequence (Meselson and Yuan, 1968; Haberman et al, 1972; Hedgpeth et al, 1972; Haberman, 1974). For example in the E co R1 system the sequence below is recognised and methylated

5' - A/TpGpAp$\overset{*}{A}$pTpTpCpT/A - 3'

3' - T/ApCpTpTpA$\underset{*}{p}$ApGpA/T - 5'

* shows methylated bases.

If the adenine residues are not methylated, the restriction endonuclease cleaves as shown below:-

5' - A/TpG$\overset{\downarrow}{p}$ApApTpTpCpT/A - 3'

3' - T/ApCpTpTpApA$\underset{\uparrow}{p}$GpA/T - 5'

⟵ indicates cleavage sites.

In the E co RII system methylation is at the 5-carbon of cytosine residues (Bigger et al, 1973; Boyer et al, 1973). Another type of modification is glucosylation of phage DNA

which protects it from degradation by specific nucleases in E. coli cells (Revel, 1967). In this Introduction the products of modification will be limited to methylation.

3.2.4 Classes of Restriction Endonucleases

Restriction depends on an endonuclease that recognises the same specific DNA sequence as the modification methylase and provided it is unmodified it introduces successive cleavages in each strand resulting in a double-stranded break at that sequence or elsewhere in the DNA. The sites of cleavage are symmetrical. They exhibit two-fold rotational symmetry about an axis perpendicular to the helical axis of the DNA. The restriction endonucleases can be divided into two classes:-

The Class I-type enzymes require ATP, Mg^{2+} and S-adenosyl methionine as cofactors for their activity. They recognise specific sites on DNA, however the points of cleavage appear to be random and the cleavage products are heterogeneous. Thus no specific fragments can be detected by gel electrophoresis. Following cleavage the restriction endonuclease is converted into an efficient ATPase. This class embodies the E. coli B and K restriction systems and the bacteriophage P1 restriction system (Meselson and Yuan, 1968; Haberman et al, 1972; Arber, 1974; Haberman, 1974).

The Class II-type enzymes utilise Mg^{2+} as cofactor and make a double stranded cleavage at a defined site which is also the site recognised by the corresponding modification

methylase. This class consists of the E co Rl and E co RII systems (Hedgpeth et al, 1972; Metz and Davis, 1972; Bigger et al, 1973; Boyer et al, 1973).

Below is shown a summary of the properties of the two classes:-

Table 1: Classes of Restriction endonucleases

Properties	Class I	Class II
Cofactor requirements	ATP, Mg^{2+}, SAM	Mg^{2+}
Molecular Weight	200,000-450,000	80,000-100,000
Subunits	3 γ - hsdS α - hsdR β - dsdM	2
Points of cleavage	Remote from recognition site	At recognition site
Products of Restriction	Heterogeneous fragments	Discrete fragments
Examples	E co B E co K E co P1	E co R1, E co RII

from Arber, 1974

3.2.5 The "hs Genetic Model"

A model which explains the R-M system at the genetic level has been proposed. This model postulates that the gene hsdS (S stands for specificity) codes for a polypeptide which is used in both restriction and modification in the recognition of sites. The products of another gene hsdR (R stands for restriction) is required for restriction reaction but probably not the modification activity and yet another gene hsdM (M stands for modification) codes for proteins required for modification only (Arber and Linn, 1969; Boyer and Roulland Dussoix, 1969; Glover and Colson, 1969; Glover, 1970) . Hence a modification deficient (m-) mutant could be r^-m^- if the mutation affects the hsdS gene and this could be lethal in haploid cells as the restriction enzymes will destroy the unmodified cellular DNA. In fact this has been found to be the case (Arber, 1965; Arber and Linn, 1969; Boyer, 1971). The hs genetic model has been supported by genetic complementation studies which suggest that in the *E. coli* B and K R-M system, three closely linked genes code for three diffusable proteins for restriction, modification and specificity (Eskin and Linn, 1972). In the E co B system the largest of the three gene products, the α-subunit (see Table 1) is required for restriction but not modification. Hence recognition must be by one of the smaller subunits (the γ subunit which is the product of the hsdS gene) (Arber, 1974). In contrast,

in the R-M system R1 and RII, coincident recognition in restriction and modification is made by a dimeric form of a single gene product which also exerts the cleavage or the modification functions respectively (Arber, 1974).

3.3. Other Roles of DNA Methylation in Prokaryotes

DNA methylation in bacterial cells protects DNA from being degraded by strain specific restriction enzymes (see section 3.2). But only a limited number of methylated bases are involved in the R-M system. Despite extensive studies there is no evidence that the 6-methyl adenine and 5-methyl cytosine modifications by potent and specific methylases are related to any other function. Experiments in which a phage induced enzyme destroys the cell's S-adenosyl methionine supply virtually eliminating 6-methyl adenine and 5-methyl cytosine from the DNA, did not show any defect or alteration in function of the cell (Gefter *et al*, 1966).

A number of experiments have been carried out to determine other roles for methylation but the data available are so variable that it is not possible to reach any further conclusions. Greenberg and Krasna, 1976 showed that *in vitro* enzymically methylated DNA from *Bacillus subtilis* and bacteriophage Ø105 were not different from the unmethylated DNAs in terms of physiochemical properties and template activity for *in vitro* assays involving DNA and

RNA polymerases. Nevertheless methylation of these DNAs led to 50% decrease in transforming ability in two strains of B. subtilis tested. Furthermore the ability of Ø105 DNA to rescue a defective phage strain was decreased 50% by methylation (Greenberg and Krasna, 1976). Marinus and Morris, 1974 reported that the DNA isolated from E. coli with a mutation in DNA adenine methylase was sensitive to U.V. and mutagens and contained single-stranded breaks. The 6-methyl adenine content of the DNA from this mutant was 0.08 mol% in contrast to the wild-type which contained 0.5 mol% 6-methyl adenine residues. They suggested that the role of 6-methyl adenine residues is to protect DNA from nuclease degradation. The occurrence in Diplococcus pneumoniae of R.Dpn I, an endonuclease specific for methylated DNA in addition to R.Dpn II another endonuclease specific for unmethylated DNA (Lacks and Greenberg, 1975) suggests that in these cells control and regulation of DNA methylation is very important and that DNA methylation might have a role other than that of protecting DNA against endonucleolytic action.

A biological role of DNA methylation has been established in the maturation of ØX174 bacteriophage. The ØX174 DNA contains only one methylated base, a 5-methyl cytosine, in the 5,375 nucleotide genome. The methylation process occurs at a late stage in the phage DNA maturation and it is catalysed by a phage-induced DNA methylase. It has been postulated that the 5-methyl cytosine moiety in ØX174

bacteriophage DNA is recognised by a specific endonuclease which mediates the excision of one-genome long viral DNA which is followed by a ligase activity in the maturation of the bacteriophage (Friedman et al, 1977, Friedman and Razin, 1976).

3.4 R-M Mechanism in Eukaryotes

3.4.1 Fate of Undermethylated DNA in Eukaryotic Cells

Nuclear DNA synthesis is unaffected for several hours in mouse 3T3 cells or SV40-transformed 3T3 cells deprived of methionine (Culp and Black, 1971). However the newly synthesised DNAs become 30-40% deficient in their normal complement of 5-methyl cytosine residues. This methyl-deficient DNAs appear to be stable in vivo and are not hydrolysed to acid soluble nucleotides.

The fact that methyl-deficient DNAs are not degraded in mammalian cells in contrast to their lability in E. coli $15T^-$ cells (Lark, 1968) perhaps points to the presence of a highly organised complex of nucleoprotein (chromatin) in mammalian cells which might protect the DNA from the extensively organised nucleolytic breakdown seen in bacteria. Also the methyl-deficient DNAs described above are hemimethylated (i.e. only one strand of the duplex DNA is deprived of methyl groups) and if R-M mechanism occurs in eukaryotes as described in bacteria, then such DNA would be protected against endonucleolytic cleavages (Arber, 1974).

3.4.2 Selective Silencing of Eukaryotic DNA

While it is debatable whether R-M mechanism exists in eukaryotic cells, a number of developmental processes have been observed in eukaryotic organisms for which no mechanisms are known. These are the processes which have been recently implicated in R-M mechanism in eukaryotes in a review by Sager and Kitchin, 1975. These processes range from selective (uniparental) inheritance of chloroplasts (Sager, 1972; Sager and Lane, 1972; Sager and Ramanis, 1974 a; b) and mitochondrial DNAs (Bolotin et al, 1971; Perlman and Birky, 1974), chromosomal elimination in interspecies somatic cell hybrids (Weiss and Ephrussi, 1966; Carlile, 1972) to X-chromosome inactivation in placental mammals (Lyon, 1961; 1968; 1972; Brown and Chandra, 1973; Riggs, 1975).

3.4.3 Selective Destruction of DNA in Chlamydomonas

From the above mentioned (section 3.4.2) set of genetic phenomena, the most extensively studied is the selective destruction of DNA in Chlamydomonas. In the sexual alga Chlamydomonas, inheritance of the chloroplast genome is non-Mendelian (maternal) as a result there is selective degradation of the chloroplast DNA of paternal origin that occurs in the zygote (Sager and Lane, 1972). It has been postulated that the molecular mechanism of this degradation is a restriction-modification process (Sager and Lane, 1972; Sager and Ramanis, 1974 a; b). The strongest experimental

evidence yet for R-M system in Chlamydomonas is from experiments demonstrating modification of maternal DNA (Sager, 1972; Sager and Lane, 1972; Schlanger and Sager, 1974). Since maternal DNA is modified it is retained but restriction-type enzymes degrade the paternal DNA which is unmodified.

Recently a deoxyribonuclease activity has been detected in extracts of Chlamydomonas vegetative cells (Burton et al, 1977). This enzyme has some of the properties of a restriction endonuclease, namely that, upon cleavage of adenovirus-2 DNA it produces a discrete and specific fragmentation pattern (Burton et al, 1977).

AIMS OF THE PRESENT STUDY

In view of the species and in particular tissue specific variation in the level of DNA methylation in eukaryotes, one of the aims of this study was to determine likely explanations for the extensive differences in DNA methylation in two BHK-21 cells routinely grown in this Department. These two BHK-21 cells are BHK-21/C13 and BHK-21/PyY.

A second objective, a more general one, was to determine whether DNA methylation in eukaryotic cells, like DNA methylation in prokaryotes, is part of a modification-cleavage mechanism.

MATERIALS

1. BIOLOGICAL

1.1 Cells

Hamster BHK-21/clone C13 cells (Macpherson and Stoker, 1962), and hamster BHK-21/PyY cells (Stoker, 1964) are cell lines maintained routinely in this Department.

1.2 Deoxyribonucleic Acids

DNAs from calf thymus, salmon testes, E. coli type IV (strain B), Micrococcus lysodeikticus were purchased from Sigma, London Chemical Co. Ltd., Kingston-Upon-Thyme, Surrey.

Phage λ and ØX174 DNAs were purchased from Miles Laboratories Ltd., Stoke Poges, England. SV40 DNA was provided by Mr. John Logan of this Department and Dr. R.L.P. Adams, also of this Department, supplied ^{14}C-deoxycytidine labelled methyl-deficient mouse L929 DNA (Adams and Hogarth, 1973) and L-929 bromodeoxyuridine labelled DNA.

Aedes albopictus was kindly donated by Dr. R. L. P Adams.

1.3 Proteins and Enzymes

Bovine serum albumin	Armour Pharmaceutical Co., Ltd., Eastbourne, England.
S_1 nuclease	Calbiochem Ltd., Hereford, England.
Alkaline phosphatase	Whatman Biochemical Ltd., Kent.
Snake venom phosphodiesterase	Worthington Biochem Co., Freehold, New Jersey.
Calf serum	Bio-cult Laboratories Ltd., Paisley, Scotland.

2. CHEMICALS

2.1 Chemical Reagents

Most of the chemicals used were Analar reagents supplied by B.D.H. Chemical Ltd., Poole, Dorset, except for the following:-

Toluene (A.R.) grade	Koch-light Laboratories Ltd., Colnbrook, England.
2,5-diphenyloxazole (P.P.O) (scintillation grade)	"
Tween 80	"
Trichloroacetic acid (T.C.A.)	"
Hyamine hydroxide (1M Solution in methanol)	Fisons Scientific Apparatus Loughborough, England.
Trifluoroacetic acid (T.F.A.)	Aldrich Chemical Co. Ltd., Middlesex.
Amino acid mixture	Bio-cult Laboratories Ltd., Paisley, Scotland.
Vitamins	"
Penicillin	Glaxo Pharmaceuticals, London.
Streptomycin	Oxoid Ltd., London.
Tryptose phosphate broth	"
Kodirex KD 54T (35 x 43 cm)	Kodak Ltd., Manchester.
DX-80 developer	"
FX-40 X-ray liquid fixer	"
Cellulose acetate electrophoresis strips (90 x 25 cm)	Oxoid Ltd., London.
Polygram cel 300 DEAE	Macherey-Nagel and Co., Camlab, Cambridge.
Formamide (Fluka)	Fluorschem Ltd., Derbyshire, England.

Agarose (for electrophoresis)	Sigma London Chemical Co. Ltd., Kingston-Upon-Thyme, Surrey.
Ethidium bromide	"
Dithiothreitol	"
Thymine, adenine, guanine, cytosine and 5-methyl cytosine	"

2.2 Radiochemicals

^{32}P-orthophosphate (^{32}Pi 2mCi/ml) carrier free (supplied in a solution of dil HCl)	Radiochemical Centre, Amersham
L-[methyl-^{14}C] methionine (55 mCi/mmole)	"
[6-^{3}H] Thymidine (23.7 Ci/mmole)	"
S-adenosyl-L-[methyl-^{3}H] methionine (500 mCi/mmole; 11.4 Ci/mmole)	"

2.3 Chromatographic Materials

2.3.1 Paper Chromatographic Materials

Whatman No. 1	H. Reeve-Angel and Co. Ltd., London
Whatman 52	"
" 3MM	"

2.3.2 Column Chromatographic Materials

Sephadex G25 (medium)	Pharmacia Uppsala, Sweden.
Hydroxyapatite (Bio-gel H.T.P.)	Bio-Rad Laboratories, Richmond, California.
Hydroxyapatite (Bio-gel H.T.P.) DNA grade	"
DEAE-cellulose (pre swollen) microgranular	Whatman Biochem. Ltd., Kent.

3. STANDARD SOLUTIONS

3.1 Solutions for Cell Culture

These consist of a mixture of amino acids, vitamins, inorganic salts and antibiotics of the following composition:-

Table 2: Constituents of Eagles Minimum Essential Medium (M.E.M) As Used in the Biochemistry Department, Glasgow University (Busby et al, 1964)

M.E.M Amino Acid Formulation

	mg/litre
L-arginine	126.40
L-cysteine	24.00
L-glutamine	292.00
L-histidine HCl	38.30
L-isoleucine	52.50
L-leucine	52.50
L-lysine	73.10
L-methionine	14.90
L-phenylalamine	33.00
L-threonine	47.60
L-tryptophan	10.20
L-tyrosine	36.20
L-valine	46.90

M.E.M. Vitamins

	mg/litre
D-calcium pentothenate	2.0
Choline choride	2.0
folic acid	2.0
i-inositol	4.0
nicotinamide	2.0
pyridoxal HCl	2.0
riboflavin	0.2
thiamine HCl	2.0

Formulation of Inorganic Salts and Other Components (BSS) (Earle, 1943)

	mg/litre
$Cacl_2.6H_2O$	393.0
KCl	400.0
$MgSO_4.7H_2O$	200.0
NaCl	6800.0
$NaH_2PO_4.2H_2O$	140.0
glucose	4500.0
$NaHCO_3$	2240.0
phenol red	17.0
streptomycin	100.0
penicillin	100,000 units.

METHODS

1. CELL CULTURE TECHNIQUES

1.1 Growth of Cells

BHK-21/C13 and BHK-21/PyY cells were grown as monolayers in 80 oz. or 20 oz. bottles. The growth medium consisted of Glasgow's modification of Eagle's minimum essential medium supplemented by 10% (v/v) calf serum (Busby et al, 1964), 10% (v/v) tryptose phosphage broth (Difco), penicillin (100 units/ml) and streptomycin (100µg/ml). Each bottle was seeded with 3×10^7 cells and grown for 2 days at 37°C in an atmosphere containing 5% CO_2.

The stock cultures were routinely examined for contamination by fungi and yeasts using Sabourand's medium. Bacterial contamination was monitored with blood agar plates and mycoplasmal contamination using mycoplasma agar (Flow Laboratories).

1.2 Labelling of Cells

1.2.1 With $^{32}PO_4$ or [6-^{3}H] Thymidine

Cells were labelled 24 hours after seeding. The normal medium was poured off and fresh medium containing 2 mCi(^{32}P) orthophosphate or 100µCi [6-^{3}H] thymidine (26.4 Ci/mmole) were added. The cells were grown for 48 hours at 37°C in 5% CO_2 atmosphere.

1.2.2 With L[methyl-^{14}C] methionine

The method used was a modification of the method used by Nass, 1973. Cells growing in logarithmic phase were transferred into a medium deprived of methionine but containing 2% (v/v) calf serum and 1 μCi/ml L-[methyl-^{14}C] methionine (55 mCi/mmole) and grown for 2 days (approximately 2 cell generations). 1 mg/ml sodium formate was added to the medium to reduce the introduction of non-specific label into bases via the one-carbon pool (Nass, 1973).

1.3 Harvesting of Cells

The culture medium was decanted and the monolayer of cells was washed with ice cold BSS (see Materials 3.1). The cells were scraped off using a rubber wiper and collected by centrifugation at 1,500 rpm for 10 min at 4°C.

2. ISOLATION OF DNA

2.1 Preparation of Nuclei

Cells harvested as described above were first allowed to swell in RSB medium [10 mM-NaCl/10 mM Tris-HCl, pH7.4/1.5 mM $MgCl_2$] for 10 min. The cells were resuspended in 1% (v/v) Tween 80 in water and homogenised. The resulting nuclei were collected by centrifugation at 2,500 rpm for 10 min. and washed three times in RSB medium and stored as a pellet at -20°C for less than 4 weeks.

2.2 Extraction of DNA

DNA was prepared by the method of Hell et al, 1972. The nuclear pellet was suspended in 20 vol of MUP medium [8M urea/0.24M sodium phosphate buffer pH 6.8] containing 1% (w/v) sodium dodecyl sulphate, 0.01M EDTA, pH6.8. It was sheared in a sealed, filled container with a Waring blendor at full speed for 6 periods of 15 sec each, alternating with 30 sec cooling in ice. The homogenate was then poured into a thick slurry of hydroxyapatite (2g of HAP per 1g of nuclei) in MUP and the mixture was stirred gently at room temperature for 1 hour. The slurry was poured into a sintered glass funnel (Gallenkamp Sinta glass No. 3) and the liquid drawn under low suction. The hydroxyapatite was washed free of RNA and protein with MUP until no further material was eluted by E_{260} and E_{280} measurements on the effluent. Urea was removed by washing with 0.014M sodium phosphate buffer pH6.8. The removal of urea was monitored by refractive index measurements on the effluent. The DNA was eluted with 0.4M sodium phosphate pH6.8 and dialysed for 18 hr. against 10 vol distilled water at 4°C. Solid NaCl was added to a concentration of 0.2M and the DNA precipitated with 2 vol of absolute alcohol at -20°C for 18 hr. The DNA was collected by centrifugation at 16,000g for 30 min. and dried in air at room temperature. The DNA precipitate contained considerable salt and this can be removed by extensive dialysis in 0.05M NaCl. The DNA prepared this way has E_{260}/E_{280} of 1.8-1.9 and an average single-stranded

molecular weight of 1.1×10^6 daltons and contains 0.5% RNA and less than 1% protein.

2.3 Shearing of DNA

DNA in 0.1 x SSC (0.015M NaCl, 0.0015M trisodium citrate pH7.0) was sheared by sonication using a Dawe soniprobe. The DNA solution was placed in a glass vial and kept cold on ice. Twelve successive cycles of sonication of 15 sec at 7 amperes followed by 45 sec cooling were performed. The average number of base-pairs produced by this method was determined by Dr. A. Campbell of this Department using the Zimm plot technique (Campbell and Eason, 1975).

3. STUDIES ON BHK-21 CELL DNA

3.1 Reassociation Kinetics (Young et al, 1976)

The sheared DNA (about 200 base-pairs) was dialysed extensively in sterile distilled water. [6-^{3}H] thymidine labelled BHK-21 cell DNA was mixed with [^{14}C-methyl] labelled DNA in the proportion of 200:1 in terms of radioactivity. The mixture of DNAs was lyopilised and redissolved in buffer H [0.5M NaCl, 25mM Hepes, 0.5mM EDTA, 50% (v/v) formamide (fluka) pH6.8]. The DNAs were diluted with buffer H to the required concentration and 50µl portions were sealed in glass capillaries and heated at 65°C for 10 min. to denature the DNA and then incubated at 43°C for various lengths of time. The Cot values were computed on the basis that the reassociation of DNA in a concentration of 83µg/ml for 1 hour corresponds to a value of Cot 1 (Britten and Kohne, 1968).

The parameter Cot is expressed in moles of nucleotides times seconds per litre. The value will therefore depend on the concentration of DNA and the time of the reassociation reaction. After incubation for the required time, the contents of each capillary were flushed with 0.3ml of a buffer consisting of 0.07M sodium acetate (pH4.5), 2.8mM $ZnSO_4$, 0.14M NaCl. 50 units of S_1 nuclease were added and incubated for 3 hr. at 37°C. 0.1ml aliquots were taken before and after the S_1 nuclease digestion and precipitated onto Whatmann 3MM discs by cold 5% (w/v) trichloroacetic acid. The filters were washed twice in methylated spirit and once with ether and dried in air. The radioactivity was calculated by scintillation spectroscopy.

3.2 Melting Curve of DNA

The DNA was dissolved in cacodylate buffer (0.01M Na^+) pH7.0. The DNA solution was freed of dissolved air by evacuation and the quartz cuvettes were covered during the experiment to minimise evaporation. The actual melting was performed with a Unicam SP800A ultraviolet spectrophotometer with a Scalamp thermocouple galvanometer attachment. The rate of heating was 2°C/min.

3.3 Analysis of 5-methyl Cytosine Level in DNA by Mass Spectrometry

These studies were done in collaboration with Mr. Anthony Ritchie of the Department of Chemistry, University of Glasgow.

3.3.1 Preparation of Sample (Deutsch et al, 1976)

Samples of DNA (about 100µg) and 40µg of 5-methyl cytosine and thymine were treated separately with trifluoroacetic acid (TFA) in sealed ampoules at 180°C for 30 min. The trifluoroacetic acid was removed by vacuum evaporation and the digested products were dissolved in water and lyophilised. An equivalent of 20µg of the solid was dissolved in 10µl methanol (Analar grade) and taken up in a capillary tube which was sealed at one end.

3.3.2 Low Resolution Mass Spectrometry (MS 12)

Methanol was removed from the sample by vacuum evaporation at 25°C. The sample was heated in the ion-source by the probe heater from 25°C to 250°C. The conditions used were 8KV, 70eV, 500µA. Ion chamber temperature and pressure were 180°C and 0.133×10^{-3}Pa respectively. At various temperatures registered by the probe heater, mass spectra were recorded on an ultraviolet recorder in the mass range of 20-600. Figs 3a, b, c, d show the low resolution mass spectra of BHK-21/PyY DNA in the mass range of 80-180 at 25°C, 100°C, 130°C and 170°C.

3.3.3 High Resolution Mass Spectrometry (MS 902S)

Under the conditions used for the low resolution mass spectrometry studies certain amounts of (M-1) ion of thymine (<1%) were present at m/e 125 (i.e. at the expected position of the 5-methyl cytosine). Similarly carbon-13 isotope of

Fig 3 Mass spectra of trifluoroacetic acid treated BHK-21/PyY DNA

40μg BHK-21/PyY DNA was treated with trifluoroacetic acid in sealed ampoules at 180°C for 30 min. The trifluoroacetic acid was removed by vacuum evaporation and the digested products were dissolved in water and lyophilised. An equivalent of 20μg of the solid was dissolved in 10μl methanol (Analar grade) and taken up in a capillary tube sealed at one end. The methanol was removed from the sample by vacuum evaporation at 25°C and the mass spectra recorded on an ultraviolet recorder in the mass range 20-600. Part of this spectra (80-180) recorded when sample probe was at

(a) 25°C, (b) 100°C, (c) 130°C, (d) 170°C,

are presented.

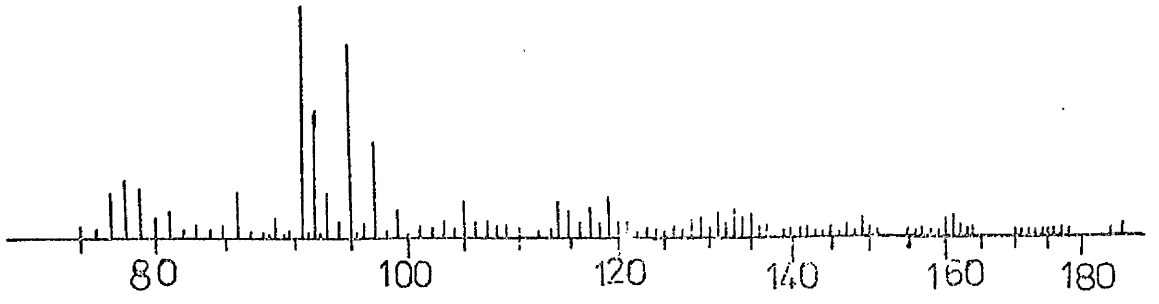


Fig3 (a) 25°C

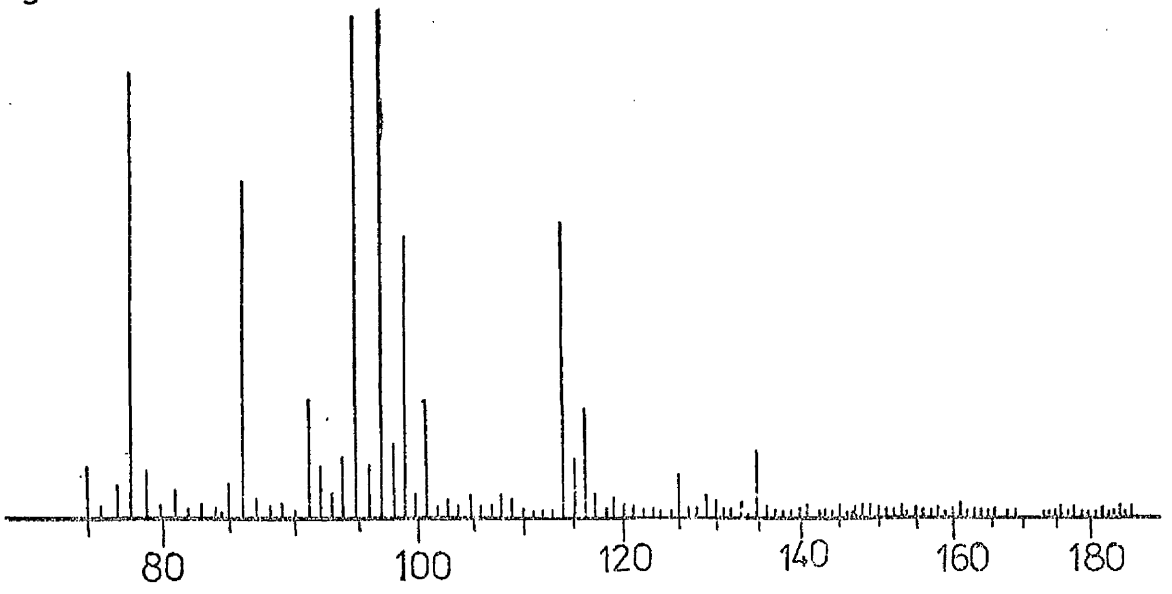


Fig 3 (b) 100°C

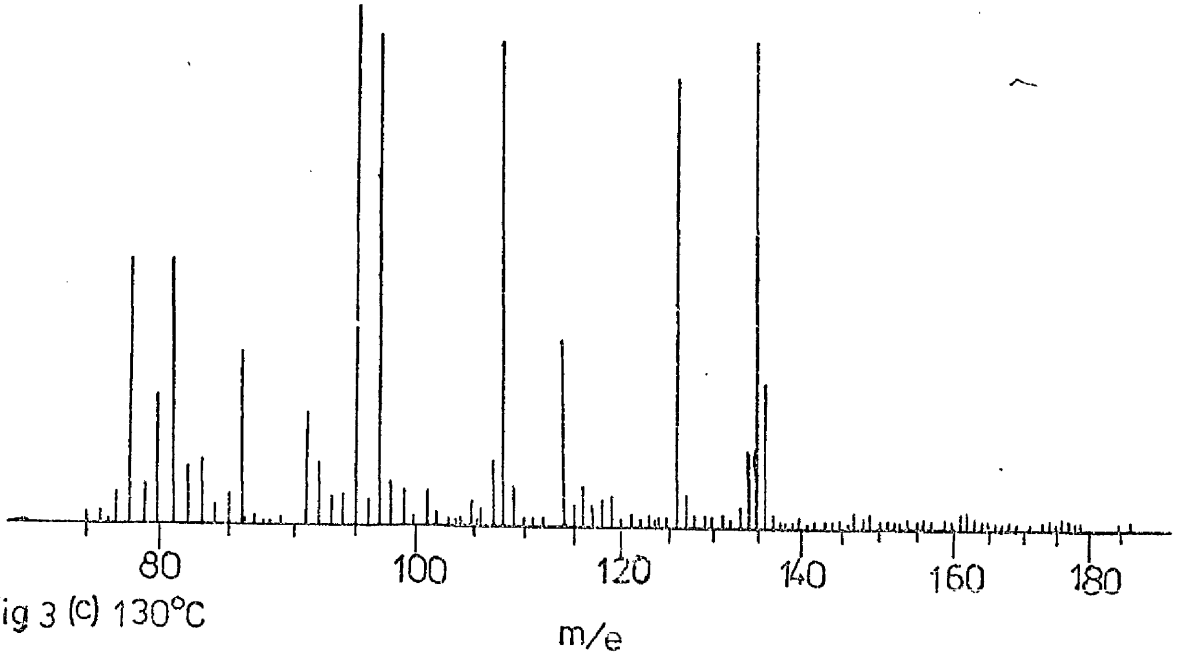


Fig 3 (c) 130°C

Fig.3(a),(b),(c) Mass Spectra of TFA-treated BHK-21/PyY DNA

Fig 3 (d) Mass spectra of trifluoroacetic acid treated BHK-21/PyY DNA

(See previous page for legend)

Fig 4 High resolution mass spectra of a mixture of 5-methyl cytosine and thymine.

A mixture of 5-methyl cytosine and thymine (20μg) treated with trifluoroacetic acid was put into the direct heated probe of the AEl MS 902S mass spectrometer and the mass spectra were recorded at 180°C. The positions of the various molecular ions were established at a precision of ±2ppm with thymine as the reference ion. The results were compared with calculated values in the "mass and abundance tables".

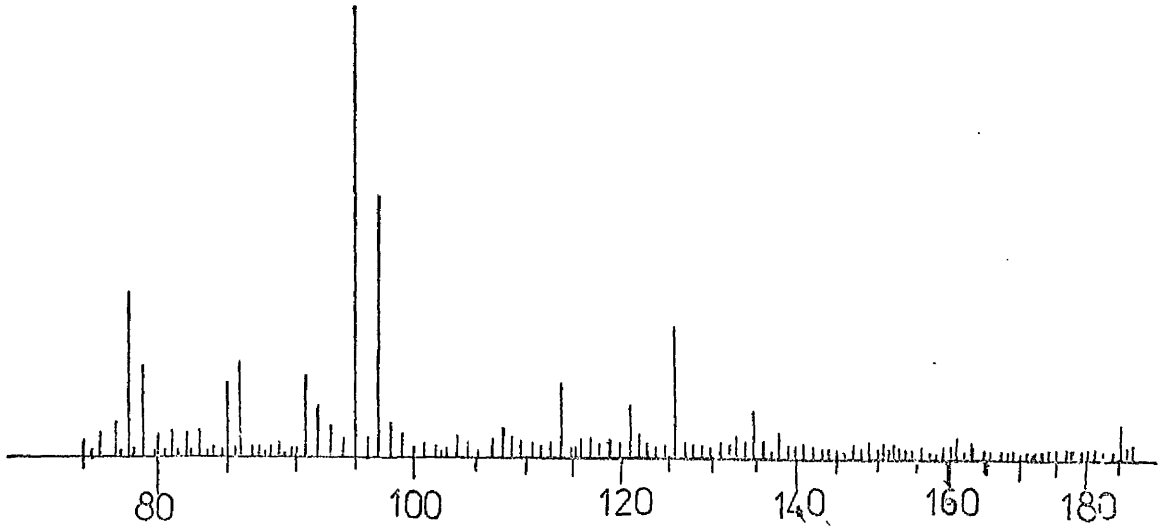
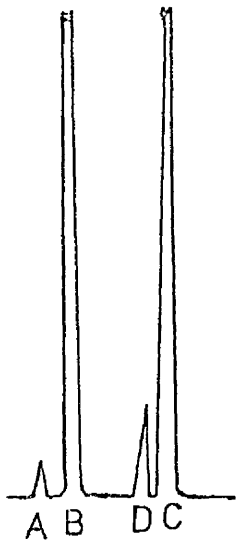


Fig 3 (d) 170°C

Fig 3(d). Mass Spectra of TFA-treated BHK/PyY DNA.



A = 125.03509 (M-1) Thymine

B = 125.05891 5-MC

C = 126.04292 Thymine

D = 126.06225 [^{13}C] 5-MC

Fig 4 High Resolution Mass Spectra of a mixture of 5-Methyl Cytosine (5-MC) and Thymine.

5-methyl cytosine which is one mass unit higher than the 5-methyl cytosine molecular ion, appeared at m/e 126, the expected position for thymine. High resolution (70ppm) showed complete separation of these peaks (Fig. 4) and the identity of the ion peaks were established using the peak matching unit with a precision of ± 2ppm using thymine as a reference ion and comparing the resulting masses with the calculated ones in the "mass and abundance table".

The samples were introduced into an AEl MS 902S mass spectrometer by means of the heated direct insertion probe. The methanol in the sample was removed by vacuum at 25°C. The conditions used in this study was similar to those employed for the MS 12 studies except that the resolution was 70ppm at 10% valley. The sample was heated manually in the ion source by the probe heater from 50°C to 270°C. The reference peak used was C_2F_5 to establish the position of the 5-methyl cytosine. Before every run, an additional reference peak C_3F_5 was used to check the position of the 5-methyl cytosine peak. C_3F_5 flow was stopped and the readings were taken on the collector meter for every 5°C rise in temperature by measuring the peak height of thymine and flicking over to measure the peak height of the 5-methyl cytosine. As the temperature was increased manually, the rate of increase in temperature varied from experiment to experiment.

Fig. 5 shows the molecular ion yield of thymine and 5-methyl cytosine of Aedes albopictus DNA treated with trifluoroacetic acid.

Fig 5 Total molecular ion yield of thymine and 5-methyl cytosine of Aedes albopictus DNA treated with trifluoroacetic acid.

Trifluoroacetic acid-treated Aedes albopictus DNA was inserted into the heated insertion probe of the mass spectrometer AEl MS 902S. The sample was heated manually from 50°C to 270°C and the molecular ions were recorded every 5°C rise in temperature by taking the reading on the collector meter for thymine and flicking over to take reading for 5-methyl cytosine

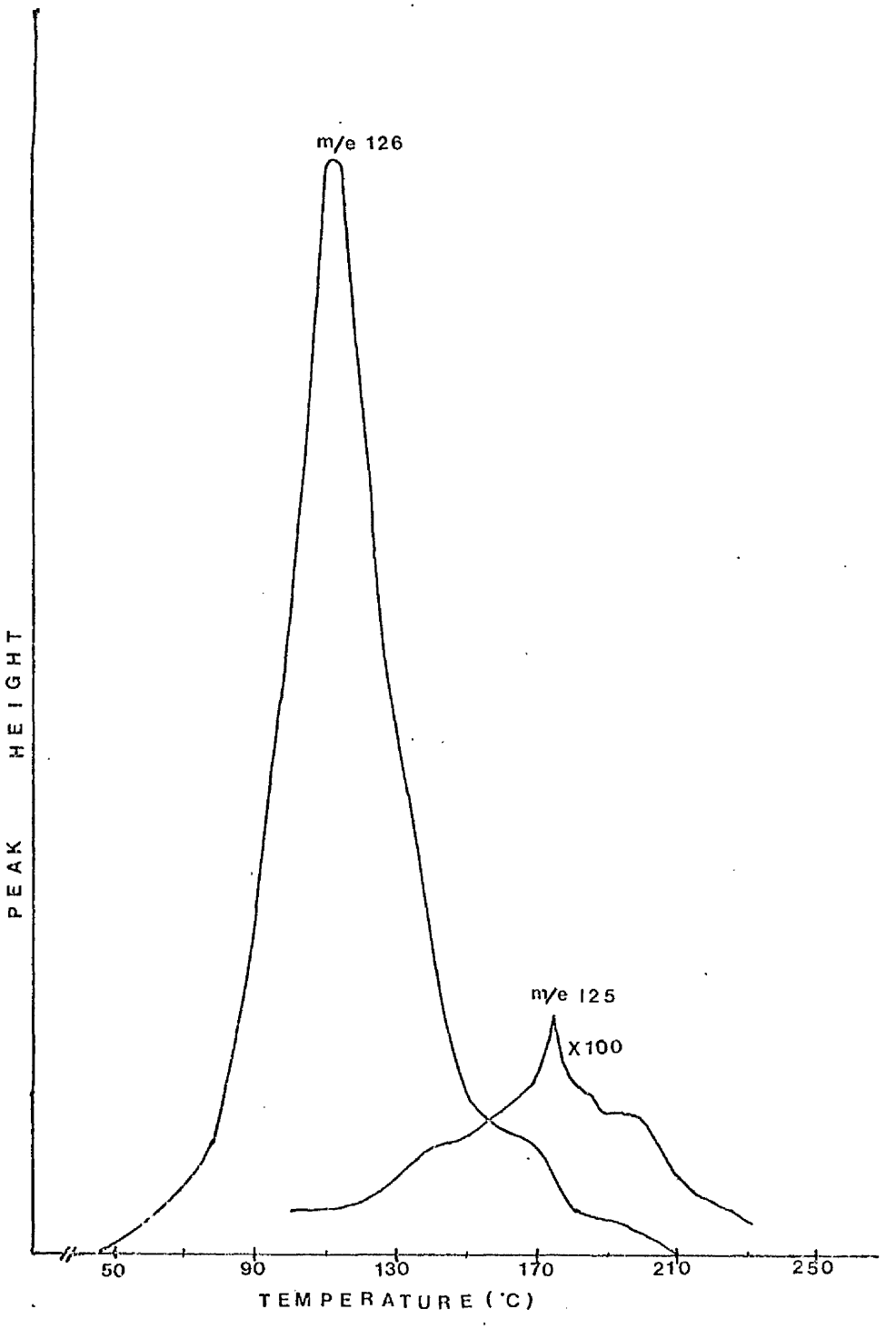


Fig 5 Total molecular ion yield of thymine and 5-methyl cytosine of Aedes albopictus DNA treated with trifluoroacetic acid

3.3.4 Processing of Results

To facilitate comparison of the different values of 5-methyl cytosine in DNA samples, the relative molecular ion yield (RMIY) was used for calculation.

RMIY = area of 5-methyl cytosine peak/area of thymine peak. This value times 100 and multiplied by the ratio of thymine to cytosine in the DNA gives the mole fraction of the cytosine that is methylated.

The molecular ratio of thymine to cytosine was obtained by the native and melting spectral analysis (Hirschman and Felsenfeld, 1966).

4. ENZYME STUDIES

4.1 DNA Methylase Preparation

(a) Nuclear soluble fraction

Nuclei obtained from BHK-21 cells (section 2.1) were resuspended in buffer M [50mM tris-HCl pH7.8; 1mM EDTA, 1mM DTT and 10% (v/v) glycerol] and made 0.4M with respect to NaCl. This was stirred gently for 15 min at 4°C and centrifuged for 1 hour at 12,000g to yield a preparation termed nuclear soluble fraction (NSF)

(b) Ammonium sulphate precipitation

NSF was dialysed for 4 hours against two changes of 100 vol of buffer M. The dialysed NSF was made 32.5%-62.5% saturated with ammonium sulphate according to the method of Noltman et al, 1961. The precipitate from this step was collected by centrifugation at 2,500 rpm for 30 min. The pellet was

dissolved in 10 vol. of buffer M and passed through a column of sephadex G25 (medium). 6 x 2.5cm and 3ml fractions were collected.

(c) DEAE-cellulose column chromatography

The enzyme fraction collected from the column of sephadex G25 (medium) was loaded onto DEAE-cellulose column (DE 52; 14 x 2.5cm) previously equilibrated by the passage through it of 10 vol. of buffer M. The column was washed with one vol. of buffer M followed by a linear gradient of buffer M containing 0 to 0.5M NaCl. 3ml fractions were collected and assayed for protein (OD_{280}) and DNA methylase activity. The DNA methylase was eluted as a single peak at 0.1M NaCl concentration. The pooled enzyme fraction from the DEAE-cellulose column was dialysed for 2 hours against 2 litres of buffer M.

(d) Hydroxyapatite column chromatography

The dialysed enzyme was loaded onto a 3ml hydroxyapatite column previously equilibrated with 100 vol. of buffer M and the column was washed with 5 vol. of this buffer and the enzyme eluted with a linear gradient of 0 to 1M potassium phosphate. The DNA methylase was eluted as a single peak at 0.3M K_2HPO_4. The pooled enzyme was again dialysed with two changes of 100 vol. of buffer M for 4 hours. The enzyme preparation was stored in 0.5ml portions in plastic ampoules at -70°C until ready to be used. This purification procedure sufficed for the DNA methylase from the BHK-21/C13 cells as well as the activity from the

BHK-21/PyY cells.

4.2 DNA Methylase Assay

The DNA methylase assay was done according to the method of Turnbull and Adams (1976). The standard assay mixture (140µl) contained 40µg of DNA, 3.3µCi S-adenosyl-L-[^{3}H-methyl] methionine (1µCi/nmole) (obtained by mixing different amounts of two different S-adenosyl-L-[^{3}H methyl] methionine of specific activities 500mCi/mmole and 10Ci/mmole respectively) and 100µl of enzyme protein in buffer M. The reaction was initiated by the addition of enzyme and incubated for 1 hour at 37°C (unless otherwise stated) and terminated by the addition of 2ml of a stopper solution made up of 0.25 M NaCl, 2mM EDTA, 1% (w/v) sodium dodecyl sulphate, 3% 4-amino salicylic acid (sodium salt), 5% (v/v) n-butanol and 0.5mg/ml of salmon testis DNA.

The reaction mixture was deproteinized by mixing with phenol/m-cresol/8-hydoxyquinoline solution 88%/12%/0.1% (v/v/w). After centrifugation at 2,500 rpm for 20 min. the upper (aqueous) layer was removed and the DNA extracted by centrifugation at 2,500 rpm for 20 min. after overlayering with 2 vol. absolute alcohol. The DNA was then treated with 0.2ml of 0.5M NaOH for 3 hr. at 37°C to remove RNA. (This step has been found to be unnecessary as RNA is not methylated by the enzyme preparation). 0.1ml aliquots of the NaOH treated DNA was applied onto filter discs (Whatman 3MM;

2.5cm diameter) and the DNA precipitated by 5% (w/v) cold trichloroacetic acid. The filters were washed four times with ice cold trichloroacetic acid followed by a wash with ethanol and ether. The discs were then dried in air and the radioactivity estimated by scintillation spectrometry. One unit of DNA methylase is defined as the amount of enzyme which under the standard assay conditions, catalyses the incorporation of 1 pmole of methyl group into E. coli DNA in 1 hour.

Protein concentration was estimated by the Lowry technique (Lowry et al, 1951).

4.3 Scintillation Spectrometry

Paper discs or strips and radioactive areas excised from DEAE-cellulose thin layer plates were placed in scintillation vials and treated with 0.5ml hyamine hydroxide (1M solution in methanol) and incubated for 20 min. at 60°C. The radioactivity was estimated in toluene scintillator (0.5% (w/v) diphenyloxazole in toluene).

4.4 S-adenosyl-L-methionine dependent DNase assay

4.4.1 Source of Enzyme

Nuclei from BHK-21/C13 cells were suspended in buffer R [50mM tris-HCl pH7.8, 1mM dithiothreitol, 12.5mM EDTA, 10% (v/v) glycerol] and made 0.4M with respect to KCl, stirred gently for 15 min. at 4°C and then centrifuged

Assay of the BHK-2I SAM-dependent nuclease Activity.

The assay mixture(20µl) consisted of 25mM Tris-HCl pH 7.8, 6mM EDTA, 0.5mM dithiothreitol, 55µM-66mM SAM, 5% $^{v}/_{v}$ glycerol, Iµg phage λ DNA and enzyme protein. The assay was initiated by the addition of enzyme and unless otherwise stated the reaction mixture was incubated for Ihr at 37°C.

at 12,000g for 60 min. The resulting supernatant was dialysed extensively against two changes of 100 vol of buffer R and subsequently used as source of enzyme. The enzyme was incubated with phage λ DNA or other substrate DNA and S-adenosyl-L-methionine (55μM to 66mM) at 37°C for 60 min.

4.4.2 Gel Electrophoresis

The assay outlined in section 4.4.1 was stopped by the addition of 20μl of a stopper solution made up of 20% (w/v) sucrose, 2% (w/v) sodium dodecyl sulphate, 100mM EDTA and 0.05% bromophenol blue to 10μl of assay mixture. The digested products were analysed by electrophoresis through 1.5% (w/v) agarose gel for 105 min at 60mA per gel. The gels were stained with 1μg/ml ethidium bromide and photographed under ultraviolet light.

4.4.3 Sucrose gradients

In some cases the products of the digestion were examined by velocity centrifugation. In such cases the reaction was stopped by the addition of an equal volume of 0.5M NaCl solution followed by quick freezing in dry-ice-alcohol mixture. The sample (80μl) was layered on 5-20% linear sucrose gradients in 10mM tris-HCl pH7.9, 1mM EDTA, 0.1M NaCl and centrifuged for 3 hr at 40,000 rpm in a SW 50.1 rotor at 20°C.

Three-drop fractions were collected on Whatman 3MM paper discs and the DNA precipitated by washing with ice cold 5% (w/v) trichloroacetic acid followed by two washes with absolute alcohol and a wash with ether. The paper discs were dried in air and the radioactivity estimated by scintillation spectrometry (section 4.3).

5. STUDIES ON METHYLATED SUBSTRATE DNA

5.1 Determination of S_1 nuclease sensitive DNA

This was done according to the method of Sutton, 1971 with slight modifications. The methylation reaction involving substrate *E. coli* DNA and enzyme preparation from BHK-21 cells was stopped as already described (section 4.2). However the stopper solution contained heat deratured sonicated calf thymus DNA instead of the carrier salmon testis DNA. The methylated DNA and carrier DNA were isolated and dissolved in a buffer (0.03M sodium acetate pH4.5, 3×10^{-5}M $ZnSO_4$, 0.01M NaCl) and 100 units/ml S_1 nuclease were added. The reaction was carried out at 50°C and at 1 min intervals 100µl aliquots were applied onto Whatman 3MM filters and the DNA was precipitated on the filters by ice cold 5% (w/v) trichloroacetic acid. This was followed by two washes with ethanol and one wash with ether and the paper discs were dried in air. The radioactivity was estimated by scintillation spectrometry (section 4.3).

5.2 Identification of the products of methylation

E. coli DNA methylated *in vitro* by the BHK-21 DNA methylases was hydrolysed in 12N perchloric acid (100°C, 60 min.) and the liberated bases were chromatographed in n-butanol/HCl/water (65/16.7/8.3; v/v/v) on Whatman No. 1 paper for 60 hrs. The chromatographic paper was cut into strips and the radioactivity estimated (section 4.3). More than 96% of the radioactivity co-chromatographed with a 5-methyl cytosine marker.

5.3 Pyrimidine Isostichs of *E. coli* DNA methylated *in vitro* by BHK-21 DNA methylases

5.3.1 Depurination of DNA

E. coli DNA (40µg) methylated *in vitro* by either BHK-21/C13 or BHK-21/PyY DNA methylases was mixed with $^{32}PO_4$ HeLa cell DNA (80µg). The purpose of the $^{32}PO_4$-labelled DNA is to help identify oligonucleotides fractionated by this method by means of autoradiography. Since the DNAs were collected by alcohol precipitation, they were contaminated with salt and they were therefore desalted by dialysis against 10 vol distilled water for 4 hr at 4°C. The DNAs were lyophilised and dissolved in 0.1ml distilled water and to this was added 0.2ml of freshly prepared solution of 3% diphenylamine (recrystallised from aqueous alcohol) in 98% formic acid (Burton and Petersen, 1960) and incubated for 18 hr at 37°C.

5.3.2 Fractionation of Oligonucleotides
(Ling, 1972; Browne and Burdon, 1977)

The depurination reaction was stopped by the addition of 0.3ml distilled water and the diphenylamine was extracted four times with 2ml of cold ether. The oligonucleotides obtained were lyophilised and fractionated by electrophoresis at pH3.5 on 2.5 x 95cm strips of cellulose acetate. The strips were first soaked in first dimension buffer (5% acetic acid-7M urea adjusted to pH3.5 with pyridine). The depurinated DNA was dissolved in 10µl of the same buffer and applied as a spot 15cm from one end of the strip and a spot of the marker dye [1% (w/v) Xylene cyanol FF (blue); 2% w/v) Orange G (Yellow); 1% (w/v) Acid fuschin (pink)] applied on either sides. The rest of the cellulose acetate strip was wiped clean of excess buffer before the strip was placed in position in an electrophoresis tank. Electrophoresis was carried out at 4.5 KV for 50 min. After electrophoresis the cellulose acetate strips were allowed to drip to remove excess white spirit. The section 3-4cm behind the blue dye and 3-4cm in front of the yellow dye was placed 3cm from one of the short ends of the DEAE-cellulose thin layer plates. Five strips of Whatman 3MM paper 2.5 x 30cm soaked in distilled water were carefully placed on top of the cellulose acetate strip on the DEAE-cellulose thin layer plate. A glass plate was placed on top of the wet Whatman 3MM strips to maintain efficient contact of the cellulose acetate strip with the DEAE-cellulose plate.

Water thus flowed into the layer transferring the oligonucleotides onto the DEAE-cellulose layer. The DEAE-cellulose thin layer plate was briefly chromatographed at 60°C with water until the front was about 5cm up the plate. This was designed to remove excess urea, transferred from the first dimension, away from the origin which otherwise interferes with the fractionation. Ascending chromatography was then conducted with 80ml of homomix C at 60°C (Brownlee and Sanger, 1969). The ascending front was soaked up by a wad of 2.5 x 30cm Whatman 3MM strips attached to the top of the thin layer plate. The homochromatography took 24-36 hr to reach completion depending on the batch of DEAE-cellulose plates used. Brownlee and Sanger, 1969 suggested the use of three different homomixes for the second dimension fractionation. In this study 5% homomix C was found to give the best seperation of fragments.

5.3.3 Autoradiography

The DEAE-cellulose plates were dried and marked with blue ink containing [^{35}S] sulphate and placed over a sheet of X-ray film in a lead-lined folder. These folders were stored for 1 week for $^{32}PO_4$ labelled DNA registering 50c.p.s. on a minimonitor. The films were developed for 4-6 min, washed with water and fixed for 10-12 min. Processing was performed in a Kodak P3 X-ray film processing unit. An autoradiograph of the fractionated oligonucleotides of a

mixture of E. coli DNA methylated by BHK-21/PyY DNA methylase and $^{32}PO_4$ HeLa cell DNA is shown in Fig 6. Fig 7 is the key to the autoradiograph in Fig 6.

5.3.4 Estimation of Radioactivity

Oligonucleotide containing areas were excised from the DEAE-cellulose thin layer plate after reference to autoradiographs (see fig 6), placed in scintillation vials and the radioactivity estimated as outlined in section 4.3.

5.3.5 Calculation of percentage of 5-methyl cytosine in each oligonucleotide fragment

The frequency of occurrence of 5-methyl cytosine as measured by the presence of tritium counts in each oligonucleotide fragment has been expressed relative to the amount of tritiated methyl label in the monopyrimidine (pCp) fragment expressed as 100%. Thus the percentage methylation of any fragment can be expressed as

$$\frac{N_x}{N_{cyt}} \times 100$$

where $N_x = {}^3H$ in oligonucleotide (x)

and $N_{cyt} = {}^3H$ in the pCp spot.

Tritium counts were corrected by substrating from the total ^3H counts the counts arising as a result of spill-over from the ^{32}P channel.

Fig 6 An autoradiograph of pyrimidine isostichs of a mixture of E. coli DNA methylated by BHK-21/PyY DNA methylase and $^{32}PO_4$-labelled HeLa-cell DNA

E. coli DNA (40μg) methylated in vitro by BHK-21/PyY DNA methylase was mixed with $^{32}PO_4$ HeLa-cell DNA (80μg). The two DNAs were depurinated and fractionated in two dimensions by electrophoresis on cellulose acetate strips at pH3.5 in one dimension followed by homochromatography in the other dimension. The spots were excised and their tritium content estimated by scintillation spectrometry. For details see Methods Section 5.3.

Fig 7 Diagram of a two-dimensional fractionation of pyrimidine isostichs

This is a diagram of the autoradiograph in Fig 6 showing the fractionation of pyrimidine isostichs in two-dimensions by electrophoresis at pH3.5 on cellulose acetate strips in one dimension and by homochromatography in the other dimension.


PO4
HOMOCHROMATOGRAPHY.
2
1
ELECTROPHORESIS.

Fig 6.

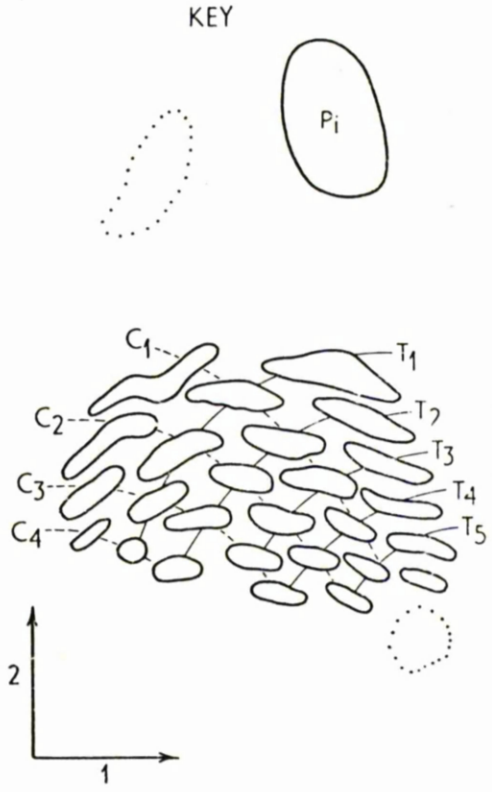

KEY
Pi
C1
C2
C3
C4
T1
T2
T3
T4
T5
2
1

Fig 7.

5.4 3' end analyses of methyl groups in dipyrimidine isostichs

5.4.1 Removal of dipyrimidine spots from DEAE-cellulose thin layer plates

The areas corresponding to the C_{2P_3} and $(CT)_{P_3}$ spots were scraped from the DEAE-cellulose thin layer plates, washed with absolute alcohol to remove urea, eluted with 30% (v/v) triethylamine carbonate (pH 10.0) and dried three times, each time re-wetting with water to effect complete removal of triethylamine carbonate.

5.4.2 Enzymic digestion of dipyrimidine fragments

The eluted oligonucleotides were dissolved in 10µl of buffer (0.1M tris-HCl pH8.9; 0.01M $MgCl_2$) containing 5µg bacterial alkaline phosphatase and incubated at 37°C for 30 min. The required amount of alkaline phosphatase was collected by centrifugation at 5,000g for 10 min from an ammonium sulphate solution. The pellet was dissolved in the above buffer (Ling, 1972). The reaction was stopped by making the reaction mixture 0.001M with respect to EDTA and heated for 3 min at 90°C. (Min-Jou and Fiers, 1969).

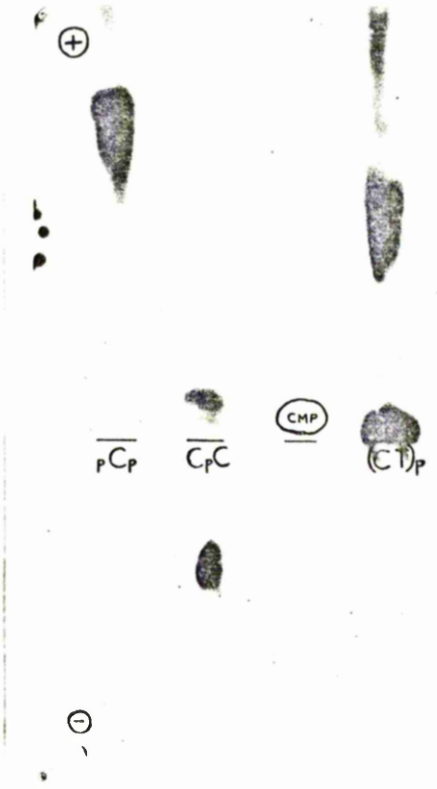
100µg snake venom phosphodiesterase and 10mM $MgCl_2$ in a total volume of 20µl were added and the digestion continued for 1 hr at 37°C. The snake venom phosphodiesterase purchased from Worthington was purified before use by

the method of Sulkowski and Laskowski, 1971. The snake venom phosphodiesterase (5mg) was dissolved in 0.6ml of water in the bottle in which it was purchased and 0.25ml of 1% acetic acid was added. The enzyme was incubated at 37°C for 3 hr and then transferred to an ice bath where 0.1ml of 1% NH_4OH and 0.05ml of 1.0M tris-HCl (pH9.0) were added. 100µl portions of the enzyme solution were stored in lyophilised state in small glass ampoules. This treatment removed 5'-nucleotidase activity by a factor between 100 and 1000-fold without loss of exonuclease activity (Sulkowski and Laskowski, 1969). Before use the lyophilised enzyme was dissolved in 0.1M tris-HCl pH8.9, 0.01M $MgCl_2$. Snake venom phosphodiesterase treatment of the dephosphorylated dipyrimidine fragments yielded 5' mononucleotides and these were seperated by electrophoresis in a buffer (5% acetic acid pH3.5 adjusted by addition of pyridine) at 80v/cm on Whatman 52 paper for 35 min.

5.4.3 Identification of 5' mononucleotides

The fractionated mononucleotides were identified by autoradiography as described in section 5.3.3. The spot was cut and the radioactivity estimated by scintillation spectrometry (section 4.3). Fig 8 shows the autoradiograph of the dipyrimidine fragments C_{2P_3} and $(CT)_{P_3}$ from Fig 6 fractionated into 5' mononucleotides after alkaline phosphatase and snake venom phosphodiesterase treatments.

Fig 8 An autoradiograph of alkaline phosphatase and snake venom phosphodiesterase digestion products of the pyrimidine isostichs C_2p_3 and $(CT)p_3$



The areas corresponding to the Cp_2, C_2p_3 and $(CT)p_3$ pyrimidine isostichs were scraped from the DEAE-cellulose thin layer plates (see Figs 6 and 7) and eluted with 30% (v/v) triethylamine carbonate (pH10.0). The C_2p_3 and $(CT)p_3$ pyrimidine isostichs were digested with alkaline phosphatase followed by snake venom phosphodiesterase. The digestion products were separated by electrophoresis at pH3.5 on Whatman 52 paper and identified by autoradiography. The 5'CMP marker was identified by viewing under U.V. light and the Cp_2 was run alongside the other samples as a marker.

EXPERIMENTAL

1. Level of DNA Methylation in BHK-21 Cells

1.1 Techniques for studying methylation of DNA

The general techniques available for determining the 5-methyl cytosine content of DNA samples are (1) the direct spectrophotometric analysis of 5-methyl cytosine residues isolated from a given DNA sample after hydrolysis and two-dimensional chromatography (Wyatt, 1951; Vanyushin et al, 1968) or (2) a comparison of radioactivity incorporated in vivo into 5-methyl cytosine from L-[methyl-^{14}C] methionine to some measure of DNA such as absorbance, colour development with diphenylamine or incorporation of radioactivity from isotopically labelled thymidine (Brown and Attardi, 1965; Salomon et al, 1969) or (3) by comparing the radioactivity from [2-^{14}C] deoxycytidine incorporated into DNA cytosine and 5-methyl cytosine residues (Kappler, 1971).

However whilst the first of these methods is direct it requires fairly large amounts of DNA and is subject to considerable error. The second and third methods on the other hand require much less DNA, but can only be used on cells that are dividing (or at least making DNA) as they rely on the in vivo incorporation of radioactive label from a precursor into DNA. Of these

methods the third is preferable to the second as the second is subject to errors arising from fluctuations in the intracellular "pool" size of say L-methionine or S-adenosyl-L-methionine. Clearly a sensitive method for analysis of 5-methyl cytosine in small samples of unlabelled DNA would be an advantage when comparing the different 5-methyl cytosine levels in say terminal differentiated cells or indeed germ cells. Recent techniques such as mass spectrometry (Deutsch et al, 1976), gas chromatography (Razin and Sedat, 1977) and high pressure liquid chromatography (Singer et al, 1977) would appear to be of use in this context.

The DNAs of two BHK-21 cell lines cultured in this department which have previously been reported to have different levels of methylation using in vivo labelling techniques (Browne and Burdon, 1977) were assayed again but this time with the mass spectrometer. This method is sensitive and it is reported to detect $1m^5cyt$ residue per 10 kilobases (Deutsch et al, 1976). The standard error using this technique was given as $\pm$0.2mol % by Razin and Cedar, 1977, and this is slightly better than the error of between $\pm$0.2 and $\pm$1.9mol % obtained by Nass, 1973 but similar to the standard error reported by Browne and Burdon, 1977 using radioactive labelling techniques.

1.2 Use of the Mass Spectrometer to measure 5-methyl cytosine residues in DNA of BHK-21 cells

DNA extracted from BHK-21/C13 and BHK-21/PyY cells was treated with 0.5M NaOH for 3 hr at 37°C to remove RNA and dialysed against two changes of 2 litres of distilled water and the DNA lyophilised. The DNA was then treated with trifluoroacetic acid and put in the direct heated probe of the mass spectrometer. Preliminary studies with low resolution mass spectrometer MS12 showed that the samples distil out of the probe at different rates depending on the temperature of the probe (see Methods 3.3.2). Studies conducted using high resolution mass spectrometer MS 902S have shown that it is possible to separate the (M-1) ion of thymine present at m/e 125 from the 5-methyl cytosine peak also at 125. Separation of thymine can also be achieved from a C13-isotope of 5-methyl cytosine both at m/e 126. Using high resolution mass spectrometry, no contribution of peaks from other bases, deoxyribose sugar or phosphate were hidden under the peaks studied (Deutsch et al, 1977).

Table 3 The level of 5-Methyl Cytosine in BHK-21 and *Aedes albopictus* DNAs

Source of DNA	No. of determinations	RMIY	% cytosine methylated (RMIY x thymine/cytosine)	Average % cytosine methylation
BHK-21/C13 cells	2	0.53 0.60	0.424 0.480	0.45
BHK-21/PyY cells	2	1.26 1.19	1.073 1.014	1.04
Aedes albopictus cells	2	0.15 0.16	* *	

The relative molecular ion yield (RMIY) of the TFA digested BHK-21 DNAs and *Aedes albopictus* DNA were calculated by constructing a graph of the peak heights against temperature (see Fig 5, Methods Section 3.3.4). The area under the 5-methyl cytosine curve divided by the area under the thymine curve times 100 gives RMIY. The percentage of cytosine methylated is the RMIY multiplied by the ratio of thymine to cytosine. The ratio of thymine to cytosine is 0.80 for BHK-21/C13 DNA and 0.85 for BHK-21/PyY DNA.

*This ratio was not calculated for *Aedes albopictus* DNA.

The levels of DNA methylation of BHK-21 cells determined by this technique were lower than those reported by either Nass, 1973 or Browne and Burdon, 1977. Bases other than those being monitored distil out of the direct heated probe of the MS 902S mass spectrometer and this results in a general build-up of pressure in the ion chamber which in turn slightly suppresses the molecular ion yield of the 5-methyl cytosine and thymine molecules. A mass spectra at 130°C (see Fig 3c) show that Adenine m/e 135 and other unidentifiable peaks about m/e 108 distil from the probe at the temperature the thymine peak m/e 126 is at its maximum. Attempts to minimise the pressure rise (sometimes about four times the starting pressure of 0.133×10^{-3}Pa) by keeping the temperature steady for a few minutes to allow the pressure to fall were unsuccessful as the pressure rise in some cases lasted for an appreciable length of time. Perhaps this might explain why the values of BHK-21 DNA methylation obtained were lower than those reported elsewhere (Nass, 1973; Browne and Burdon, 1977).

Alternatively these low levels of 5-methyl cytosine residues may be due to the values obtained for the molecular ratio of thymine to cytosine for the two BHK-21 cell DNAs as determined from their native and melting spectral data. (Hirschman and Felsenfeld, 1966)

2. Isolation of DNA Methylase from BHK-21 cells

2.1 Preliminary Investigation

2.1.1 Localisation of DNA methylase activity in nuclei

The distribution of DNA methylase activity in cultured eukaryotic cells is known to vary with the growth of the cells [maximum DNA methylase activity being found in mid-log phase whilst late-log or early stationary phase cells have less enzyme activity (Sneider et al, 1975)]. In this study BHK-21 cells were harvested after mid-log phase. The division time of BHK-21/C13 and BHK-21/PyY cells is similar although the latter cells are less contact inhibited (Stoker, 1962).

BHK-21 DNA contains 5-methyl cytosine residues (see Experimental 1.2) and the methyl groups are presumably incorporated into cytosine residues by one or more specific DNA methylases. Although DNA methylase can methylate homologous DNA to some extent (Kalousek and Morris, 1969b) most of the potential DNA methylase sites are probably already methylated in vivo. A foreign DNA (E. coli DNA or Micrococcus lysodiekticus DNA) was chosen for the assay of DNA methylase activity from BHK-21 cells as DNAs from E. coli B and M. lysodiekticus have no detectable 5-methyl cytosine residues (Fujimoto et al, 1965; Vanyushin et al, 1968). The conditions for the methylase assay was adapted from that used by Turnbull and Adams, 1977 for

Krebs II ascites DNA methylase. The reaction required S-adenosyl-L-[methyl-^{3}H] methionine (23.6μM) as a methyl donor and was carried out in 50mM tris-HCl buffer (pH7.8), 1mM dithiothreitol, 1mM EDTA and 10% (v/v) glycerol. DNA methylase activity was found predominantly in nuclei of the BHK-21 cells studied (Table 4). Although enzyme activity was found in the cytoplasm this was not investigated further and might have arisen as a result of leakage from the nuclei.

Table 4 The occurrence of DNA methylase activity in nuclei of BHK-21 cells

Source of Enzyme	Specific Activity (Units/mg Protein)	
	BHK-21/C13 cells	BHK-21/PyY cells
Nuclei	0.390	1.388
Cytoplasm	0.098	0.476

The standard assay mixture (140μl) consisted of 40μg *M. lysodiekticus* DNA, 3.3μCi S-adenosyl-L-[methyl-^{3}H] methionine (1μCi/nmole). The reaction was initiated by the addition of nuclei or cytoplasmic protein in buffer M (50mM tris-HCl pH7.8, 1mM dithiothreitol, 1mM EDTA and 10% (v/v) glycerol. For details of assay conditions see Methods Section 4.2.

2.1.2 Identification of the methylated base

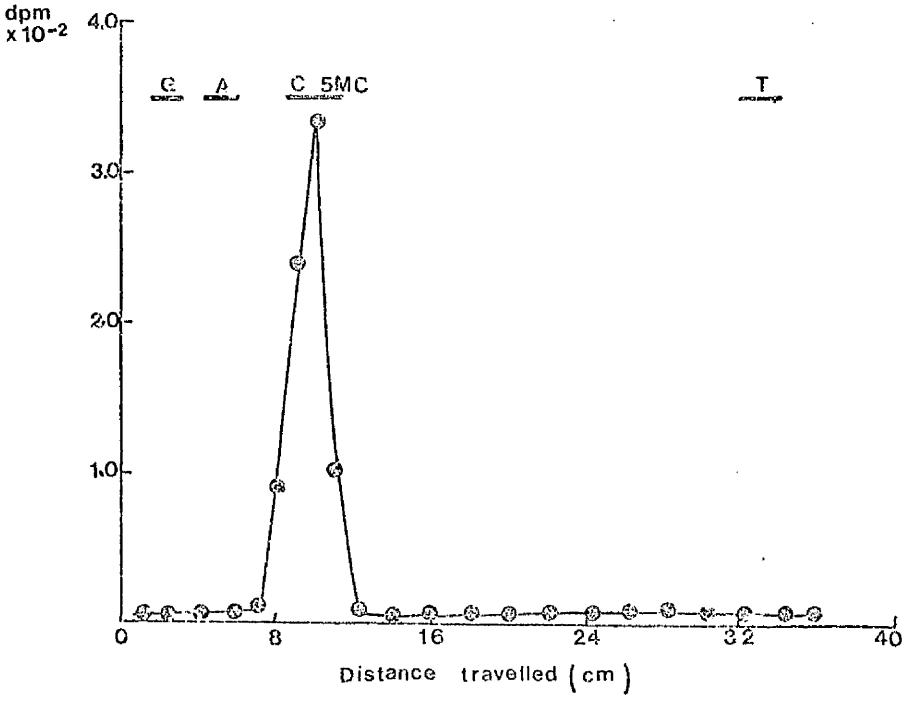
While *in vivo* labelling of cells with [methyl-^{3}H] or [methyl-^{14}C] methionine may lead to some incorporation of non-specific label in bases via the one-carbon pool, *in vitro* methylation with S-adenosyl-L-[methyl-^{3}H] methionine and enzyme should produce only the product of methylation of the substrate DNA. Nuclear extracts (NSF) of BHK-21/Cl3 and BHK-21/PyY cells were used as sources of enzyme to methylate substrate *E. coli* DNA. The isolated methylated DNA was digested with formic acid and the bases chromatographed on paper. The only base methylated by the two nuclear extracts was cytosine. Fig 9 shows the distribution of radioactive label in *E. coli* DNA methylated by nuclear extracts of BHK-21/PyY cells. This result agrees with other experiments using DNA methylases prepared from HeLa cells and Krebs II ascites cells to methylate substrate DNA (Roy and Weissbach, 1975; Turnbull and Adams, 1977).

3. Purification of DNA Methylases from BHK-21 cells

3.1 Nuclear Soluble Fraction

BHK-21 nuclei were suspended in buffer M [50mM tris-HCl pH7.8; 1mM EDTA, 1mM DTT, 10% (v/v) glycerol] and made 0.4M with respect to NaCl. This was stirred gently for 15 min at 4^{o}C and centrifuged for 1 hr at 12,000g. The

Fig 9 Identification of the products of methylation



E. coli DNA (40µg) methylated by nuclear extracts of BHK-21/PyY cells was hydrolysed in 12N perchloric acid (100°C, 60 min) and the liberated bases chromatographed in n-butanol/HCl/H_2O (65%/16.7%/8.3%; v/v/v) on Whatman No. 1 paper for 60 hr. The paper was cut into 0.5cm strips and the radioactivity estimated by scintillation spectrometry.

methylase activity was found as a soluble form in the supernatant, and is thus separated from the majority of nucleic acids and nucleoprotein by the centrifugation. The nuclear extract was then dialysed and purified by ammonium sulphate precipitation and by column chromatography.

3.2 Ammonium Sulphate Fractionation

Nuclear soluble extracts from BHK-21 cells were made 32.5%-62.5% saturated with ammonium sulphate according to the method of Noltman et al, 1961. Eighty per cent of the BHK-21/PyY cell DNA methylase activity and 85% of the BHK-21/C13 cell DNA methylase preparation were associated with the above ammonium sulphate cut.

3.3 Column Chromatography

DNA methylase from both cell lines behaved similarly on DEAE-cellulose and hydroxyapatite columns. The DNA methylase elutes as a single peak from these columns. Fig 10 a, b are DEAE-cellulose column and hydroxyapatite column chromatographys of DNA methylase from BHK-21/PyY cells. Tables 5 and 6 show the purification of BHK-21/PyY and BHK-21/C13 DNA methylases.

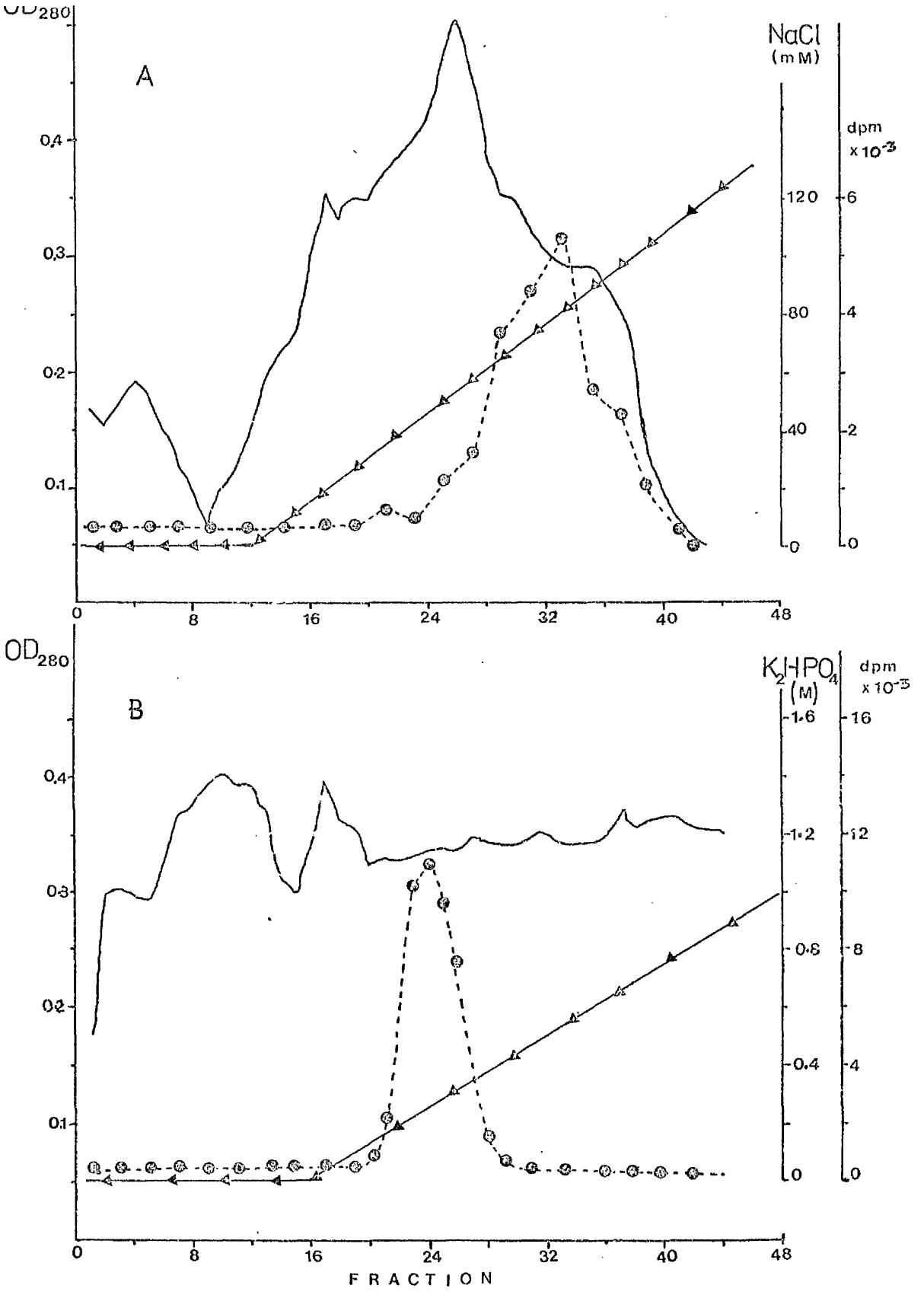


Fig 10 DEAE-cellulose (A) and hydroxyapatite (B) chromatograms of BHK-21/PyY DNA methylase.

Elutions were carried out as described in Methods Sections 4.1 (c) and 4.1 (d) (———) optical density (⊙---⊙) DNA methylase activity and (◂——◂) conductivity in milliohms.

Table 5 : Purification of BHK-21/PyY DNA methylase

Step	Volume (ml)	Protein (mg)	Activity x 10^{-2} * pmoles/hr	Specific Activity (units/mg protein)	Purification
I Nuclei	24	2280.0	6.98	0.31	1
II Nuclear soluble fraction	21	579.6	4.03	0.70	2.3
III 32.5%-62.5% Ammonium sulphate fraction	60	246.0	55.80	20.22	66.1
IV DE-52 peak	40	64.0	17.00	26.36	86.8
V Hydroxyapatite peak	25	17.5	10.74	61.38	200.5

Table 6 : Purification of BHK-21/C13 DNA methylase

Steps	Volume (ml)	Protein (mg)	Activity x 10^{-2} * pmoles/hr	Specific Activity (units/mg protein)	Purification
I Nuclei	25	1425.0	6.82	0.48	1
II Nuclear soluble fraction	18	437.4	4.56	1.04	2.2
III 32-5%-62-5% Ammonium sulphate fraction	60	228.0	25.59	11.22	23.4
IV DE-52 peak	50	55.0	20.13	36.6	76.3
V Hydroxyapatite peak	25	32.5	18.12	55.7	116.2

* E. coli DNA was used for the assay of the activity of the enzyme at various steps in the purification.

3.4 Properties of the partially purified DNA methylase from BHK-21 cells

In vitro studies of the properties of DNA methylases from various organs in rat have shown tissue specificity of the enzymes studied (Kudryashova and Vanyushin, 1976). The properties of DNA methylases from BHK-21 cells were studied with a view of finding any differences in specificity between BHK-21/C13 and BHK-21/PyY DNA methylases.

The two enzymes have similar pH profiles (Fig 11) and both have a Km for S-adenosyl-L-methionine of 16μM (Fig 12). The two enzymes however seem to differ in their requirement for substrate *E. coli* DNA. The BHK-21/C13 DNA methylase preparation appear to be saturated with lower amounts of DNA than the BHK-21/PyY enzyme (Fig 13). The methylation of *E. coli* DNA (40μg in standard assay reaction) catalysed by both enzymes showed a linear relationship with time for more than 1 hour. In an attempt to methylate all the potential methylation sites in *E. coli* DNA, more enzyme and S-adenosyl-L-methionine were added after 40, 80 and 120 minutes but the reaction proceeded for more than 2 hours (Fig 14), so long as there was enough enzyme and SAM to replenish the decreasing concentration of these substances. The optimum temperature for the two enzymes was found to be 44°C (Fig 15).

Fig 11 The Effect of pH on the methylation of *E. coli* DNA by the BHK-21/C13 and BHK-21/PyY DNA methylases

The methylase assay mixture (140µl) consisted of 50mM tris-HCl (pH6.8-9.1), 1mM EDTA, 1mM dithiotreitol, 10% (v/v) glycerol. 40µg *E. coli* DNA, 3.3µCi S-adenosyl-L-[methyl-^{3}H] methionine (1µCi/nmole) and

O———O 3.6mg BHK-21/C13 DNA methylase (NSF)

●———● 2.2mg BHK-21/PyY DNA methylase (NSF)

For details of the assay see Methods Section 4.2.

Fig 12 Lineweaver-Burk plot of substrate S-adenosyl-L-methionine

The methylase assay mixture (140µl) contained 40µg *E. coli* DNA (6.25µM to 50.0µM) S-adenosyl-L-[methyl-^{3}H] methionine (1µCi/nmole) and

O 3.6mg BHK-21/C13 DNA methylase (NSF)

● 2.2mg BHK-21/PyY DNA methylase (NSF)

For details of assay see Methods Section 4.2.

K_m values for SAM for the BHK-21/C13 and BHK-21/PyY DNA methylases was 16µM.

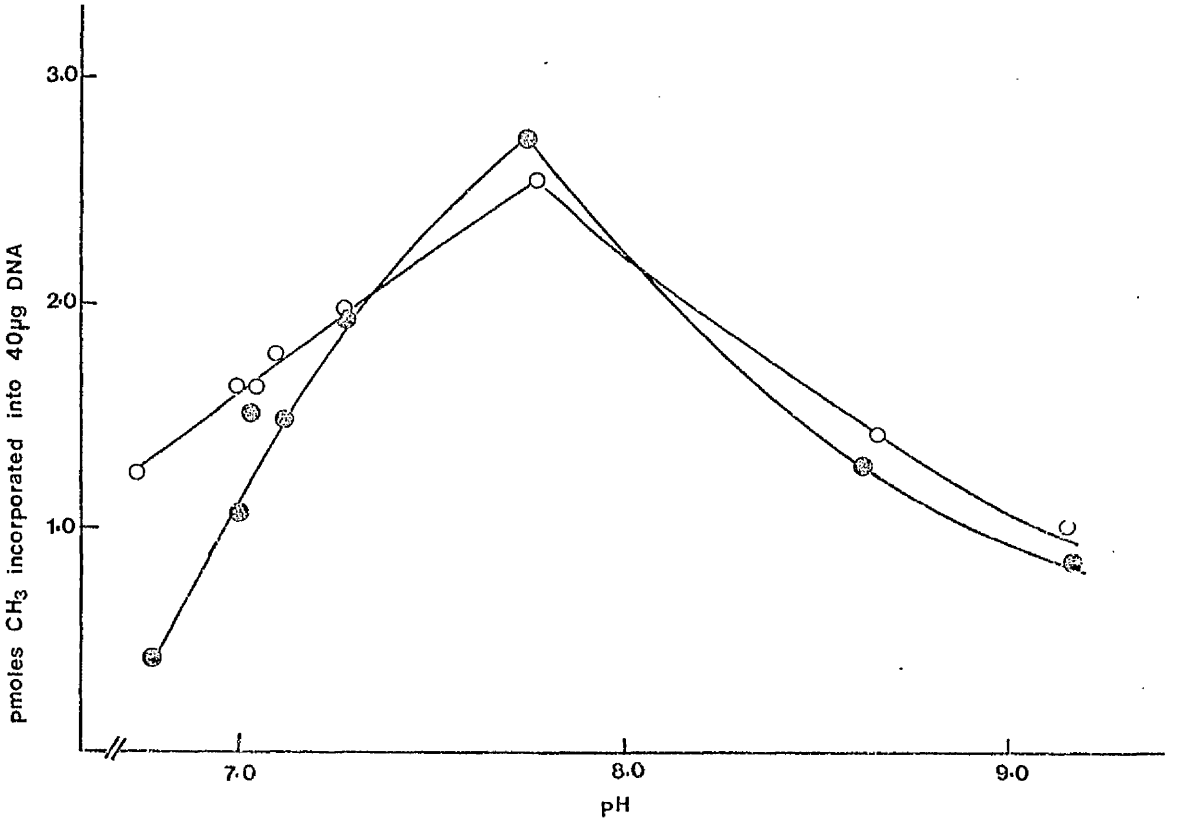


Fig 11 The Effect of pH on the methylation of E. coli DNA by the BHK-21/C13 and BHK-21/PyY DNA methylases

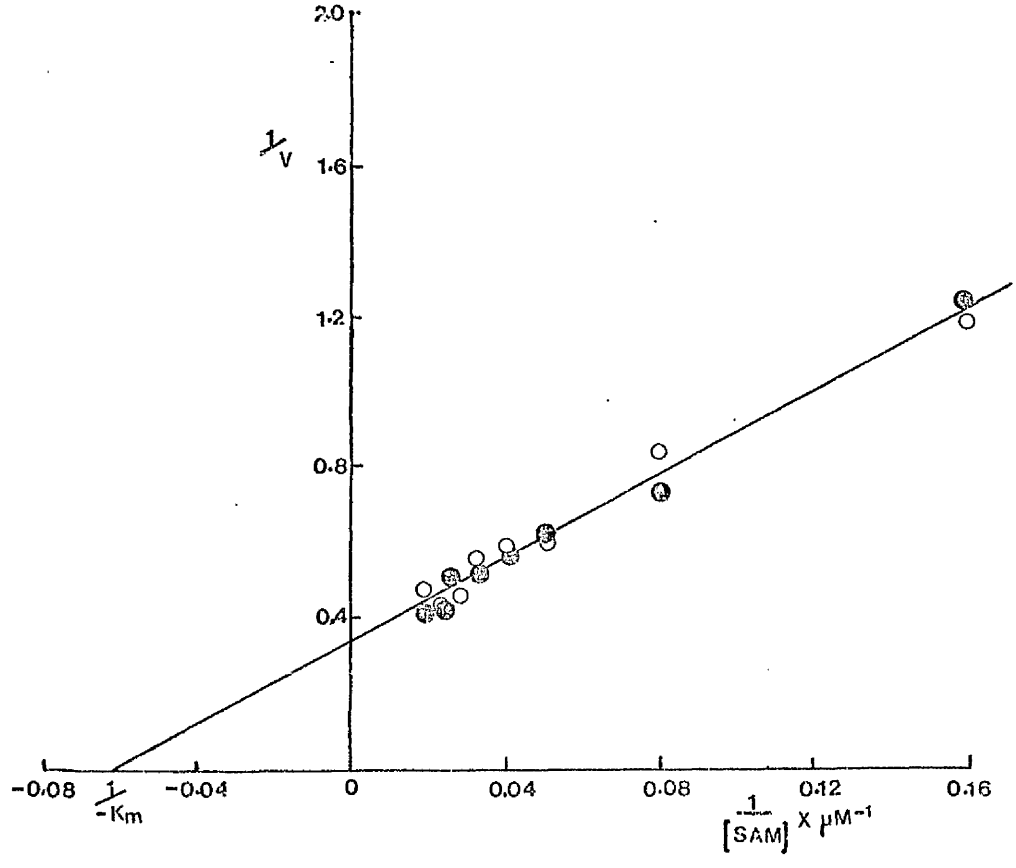


Fig 12 Lineweaver-Burk plot of substrate S-adenosyl-L-methionine

Fig 13 Effect of DNA concentration on *in vitro* methylation of *E. coli* DNA by BHK-21/C13 and BHK-21/PyY DNA methylase preparations

The standard assay mixture (140µl) contained 3.3µCi S-adenosyl-L-[methyl-^{3}H] methionine (1µCi/nmole), various concentrations of *E. coli* DNA as indicated and either O———O 3.58mg BHK-21/C13 DNA methylase preparation (NSF) or ❶———❶ 2.19mg BHK-21/PyY DNA methylase preparation (NSF). The reaction was carried out at 37°C for 1 hr.

For details of assay see Methods 4.2.

Fig 14 Time course of methylation of *E. coli* DNA by BHK-21/C13 and BHK-21/PyY DNA methylases

The methylase assay mixture (140µl) consisted of 3.3µCi S-adenosyl-L-[methyl-^{3}H] methionine (1µCi/nmole), 40ug *E. coli* DNA and O———O 130µg BHK-21/C13 DNA methylase preparation or ❶———❶ 70µg BHK-21/PyY DNA methylase preparation At the times indicated by the arrows 120µl of either BHK-21/C13 DNA methylase (1.3mg/ml) or BHK-21/PyY DNA methylase (0.7mg/ml) and 20µl of S-adenosyl-L-[methyl-^{3}H] methionine (3.3µCi, 1µCi/nmole) were added.

For details of assay see Methods 4.2.

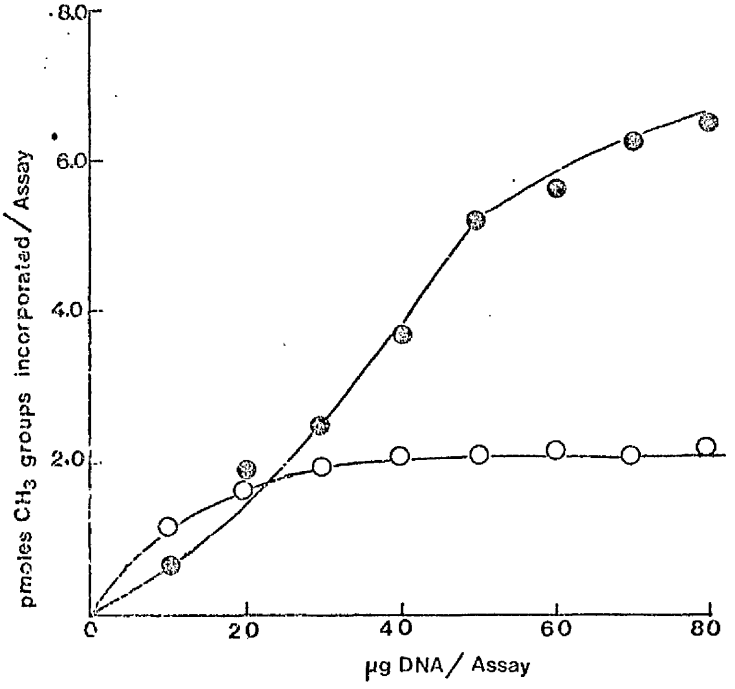


Fig 13 Effect of DNA concentration on in vitro methylation of E. coli DNA by BHK-21/C13 and BHK-21/PyY DNA methylase preparations

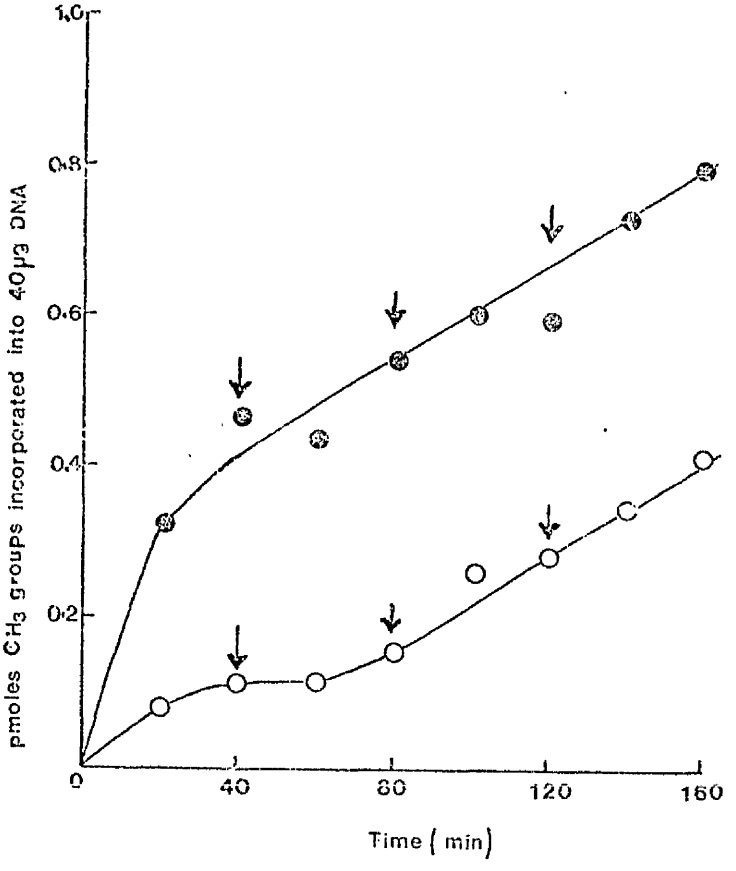
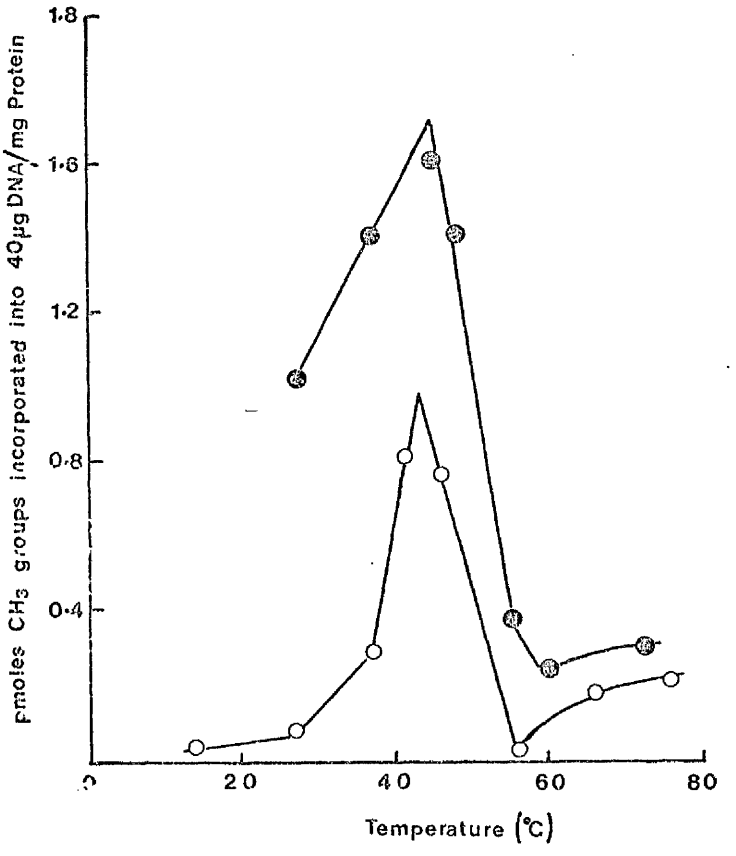


Fig 14 Time course of methylation of E. coli DNA by BHK-21/C13 and BHK-21/PyY DNA methylases

Fig 15 The Effect of temperature on the methylation of *E. coli* DNA



The standard assay mixture (140µl) contained 3.3µCi S-adenosyl-L-[methyl-^{3}H] methionine (1µCi/nmole), 40µg *E. coli* DNA and

●——● 920µg BHK-21/PyY DNA methylase (NSF)

or O——O 3.5mg BHK-21/C13 DNA methylase (NSF)

The reaction was carried out at various temperatures for 1 hr. For details regarding assay conditions see Methods Section 4.2.

Table 7 Methyl acceptance activity of various nucleic acid substrates using DNA methylase preparation from BHK-21/C13 and BHK-21/PyY cells

140µl of reaction mixture contained 40µg DNA, 3.3µCi S-adenosyl-L-[methyl-^{3}H] methionine (1µCi/nmole) and 251µg of BHK-21/C13 cell or 45µg of BHK-21/PyY cell DNA methylase. For details of assay see Methods Section 4.2. $(dA\text{-}dT)_n\text{-}(dA.dT)_n$ was dissolved in 0.015M NaCl - 0.0015M trisodium citrate (pH7.0) and concentration of the alternating copolymer used in the methylase assay was 14µg. Heat denaturation of the DNAs was done at 100°C for 10 min in buffer M [50mM-tris-HCl pH7.8, 1mM EDTA, 1mM dithiothreitol and 10% (v/v) glycerol] followed by rapid cooling. The concentration of the DNAs in the assay mixture was 40µg except phage λ DNA concentration which was 15µg.

(-) no detectable radioactivity over control sample.

3.5 Mode of action of DNA methylases from BHK-21 cells

DNA methylases prepared from BHK-21 cells seem to prefer heat denatured DNA to "native" DNA. Table 7 shows the methyl groups acceptability of various nucleic acid substrates using DNA methylase preparation from BHK-21/C13 and BHK-21/PyY cells.

Table 7 Methyl acceptance activity of various nucleic acid substrates using DNA methylase preparation from BHK-21/C13 and BHK-21/PyY cells

DNA Source	Condition	BHK-21/C13 Enzyme dpm/assay/60min	BHK-21/PyY Enzyme dpm/assay/60 min
E. coli	"native"	1176.0	2599.6
	heat denatured	13856.0	6139.8
M. lysodeikticus	"native"	7890.0	7683.6
	heat denatured	12282.0	31210.4
Phage λ	"native"	-	326.4
	heat denatured	4511.8	2630.0
Calf Thymus	"native"	816.4	677.8
	heat denatured	7980.0	4634.0
$(dA.dT)_n$ $(dA.dT)_n$	"native"	-	-
	heat denatured	-	-

Drahovsky and Morris (1971 a, b) suggested that rat liver DNA methylase forms two types of complexes with DNA which are distinguishable on the basis of their sensitivity to ionic strength. A weak complex is formed at 0°C and it is sensitive to NaCl concentration and a tight complex formed at 37°C is resistant to NaCl inhibition to a concentration of 0.2M. Other workers however have been unable to repeat this finding with the rat liver enzyme (Adams, R.L.P. private communication) nor have Turnbull and Adams, 1976 been able to show this tightly-bound DNA-enzyme complex using DNA methylase prepared from Krebs II ascites cells.

The BHK-21/C13 DNA methylase was inhibited by increasing levels of NaCl. Even if the enzyme and DNA were incubated together at 37°C for 5 min then NaCl added, there was no change in the pattern of inhibition (see Fig 16b). Thus there was no evidence of a tightly-bound DNA-enzyme complex resistant to NaCl inhibition with the BHK-21/C13 DNA methylase preparation nor in fact with the BHK-21/PyY DNA methylase preparation (Fig 16a). However it was noticed that 0.1M NaCl caused a slight increase in activity of the BHK-21/PyY DNA methylase preparation whereas the same concentration caused a decrease in the activity of the BHK-21/C13 cell DNA methylase preparation. Drahovsky and Morris (1971 a) showed for the rat liver enzyme that methylation of double stranded DNA decreased as the NaCl

Figs 16 (a) and (b)

Effect of NaCl on the methylation of *E. coli* DNA by (a) BHK-21/PyY DNA methylase and (b) BHK-21/C13 DNA methylase

The methylase assay mixture (140µl) contained 40µg *E. coli* DNA, 3.3µCi S-adenosyl-L-[methyl-^{3}H] methionine (1µCi/nmole) and 20µg BHK-21/PyY DNA methylase preparation (as in Fig 16(a)) or 14µg BHK-21/C13 DNA methylase preparation (as in Fig 16(b)). The reaction was carried out at 37°C for 1 hr. For details see Methods Section 4.2.

●——● NaCl concentration was adjusted before incubation at 37°C

▲- - -▲ DNA, SAM and enzyme were incubated at 37°C for 5 min then NaCl concentration adjusted followed by an addition incubation of 1 hr at 37°C

Fig 16 (a) Effect of NaCl on the methylation of E. coli DNA by BHK-21/PyY DNA methylase

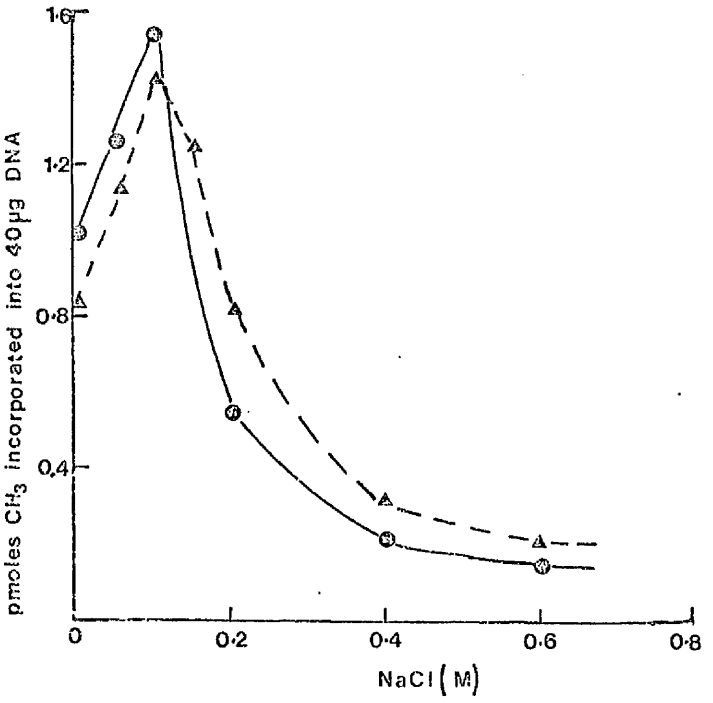
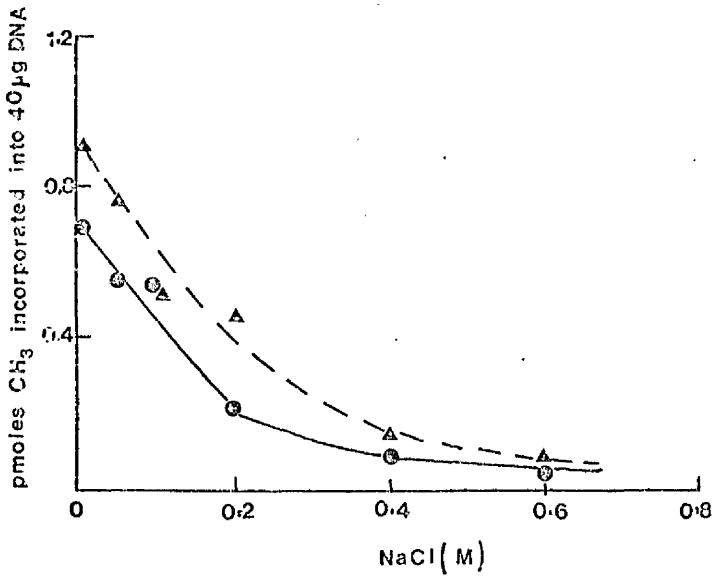


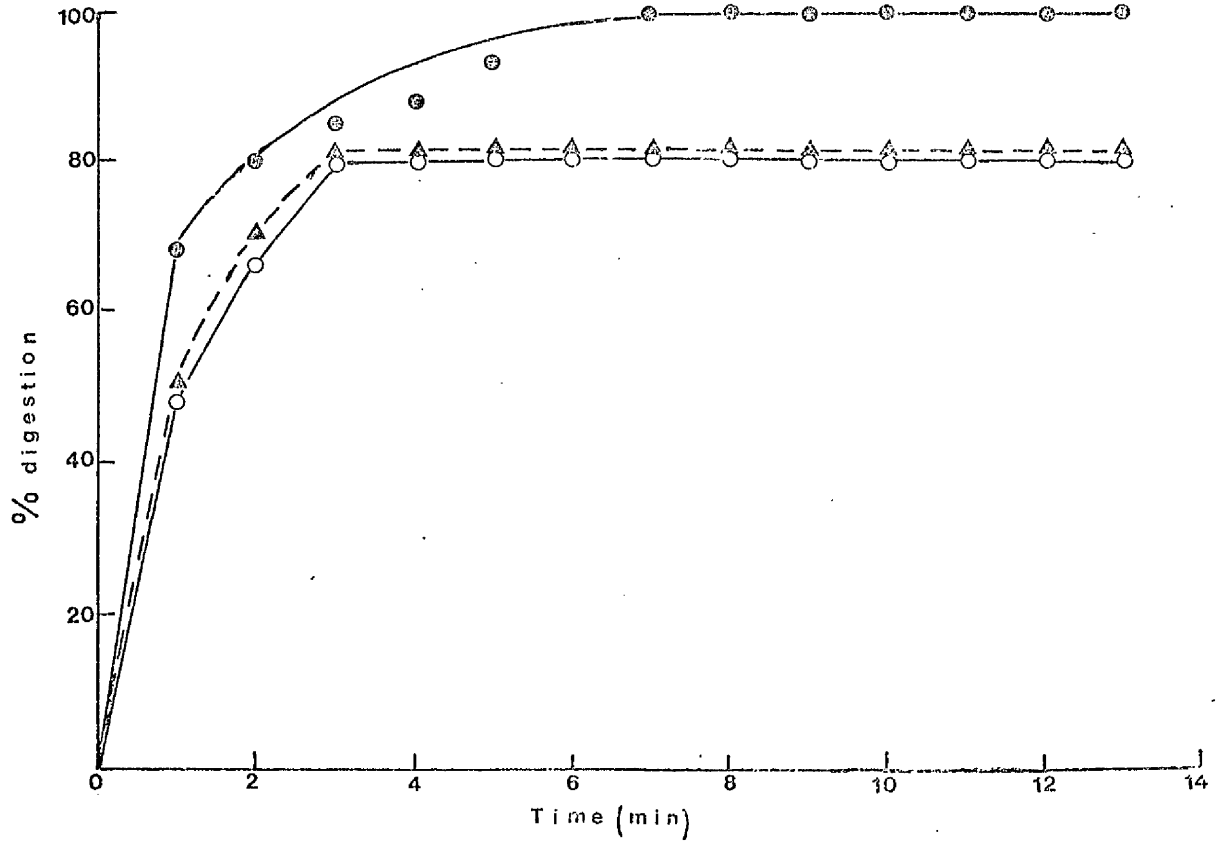
Fig 16 (b) Effect of NaCl on the methylation of E. coli DNA by BHK-21/C13 DNA methylase



concentration was increased whilst the methylation of single-stranded DNA increased although high concentrations of NaCl were inhibitory. From these studies it might be inferred that the BHK-21/Cl3 enzyme methylates double stranded DNA whilst DNA methylase from BHK-21/PyY cells methylates single-stranded DNA. However this does not appear to be the case with these hamster cell enzymes. When the enzymically methylated DNA was isolated and subject to digestion with S_1 nuclease to determine the specificity of these methylases with regard double or single-stranded methylation, the methyl groups transferred by the BHK-21/Cl3 cell DNA methylase appeared in regions of "native" E. coli DNA which were in fact susceptible to S_1 nuclease (i.e. single-stranded regions). Whilst the situation was similar using the enzyme preparation from the BHK-21/PyY cells, there was a small level (20%) of methyl groups incorporated into S_1 resistant material. This proportion was the same whether or not the BHK-21/PyY enzyme is assayed in the presence or absence of 0.1M NaCl (Fig 17). It is therefore not surprising that the BHK-21/Cl3 DNA methylase preparation only methylates phage λ DNA after heat denaturation (see Table 7).

When heat denatured E. coli DNA is offered as substrate to this BHK-21/Cl3 DNA methylase in the presence of various concentrations of NaCl it does however exhibit the characteristic feature anticipated for double and single

Fig 17 S_1 nuclease digestion of E. coli DNA methylated in vitro by BHK-21/C13 DNA methylase and BHK-21/PyY DNA methylase in the presence and absence of 0.1M NaCl



40μg [^{3}H-methyl] E. coli DNA methylated in vitro by BHK-21 DNA methylases were dissolved in 2ml of buffer (0.03M sodium acetate pH4.5, 3 x 10^{-5}M $ZnSO_4$, 0.01M NaCl), to which was added 50μg heat-denatured sonicated calf thymus DNA and 100 units/ml S_1 nuclease. The reaction was incubated for various times at 50°C, and stopped as described in Methods Section 5.1.

●——● S_1 nuclease digestion of E. coli DNA methylated in vitro by DNA methylase from BHK-21/C13 cells.

▲- - -▲ S_1 nuclease digestion of E. coli DNA methylated in vitro by BHK-21/PyY DNA methylase in the presence of 0.1M NaCl.

○——○ S_1 nuclease digestion of E. coli DNA methylated in vitro by the BHK-21/PyY DNA methylase.

stranded DNA methylase on the basis of Drahovsky and Morris's data (Drahovsky and Morris 1971 a, b).

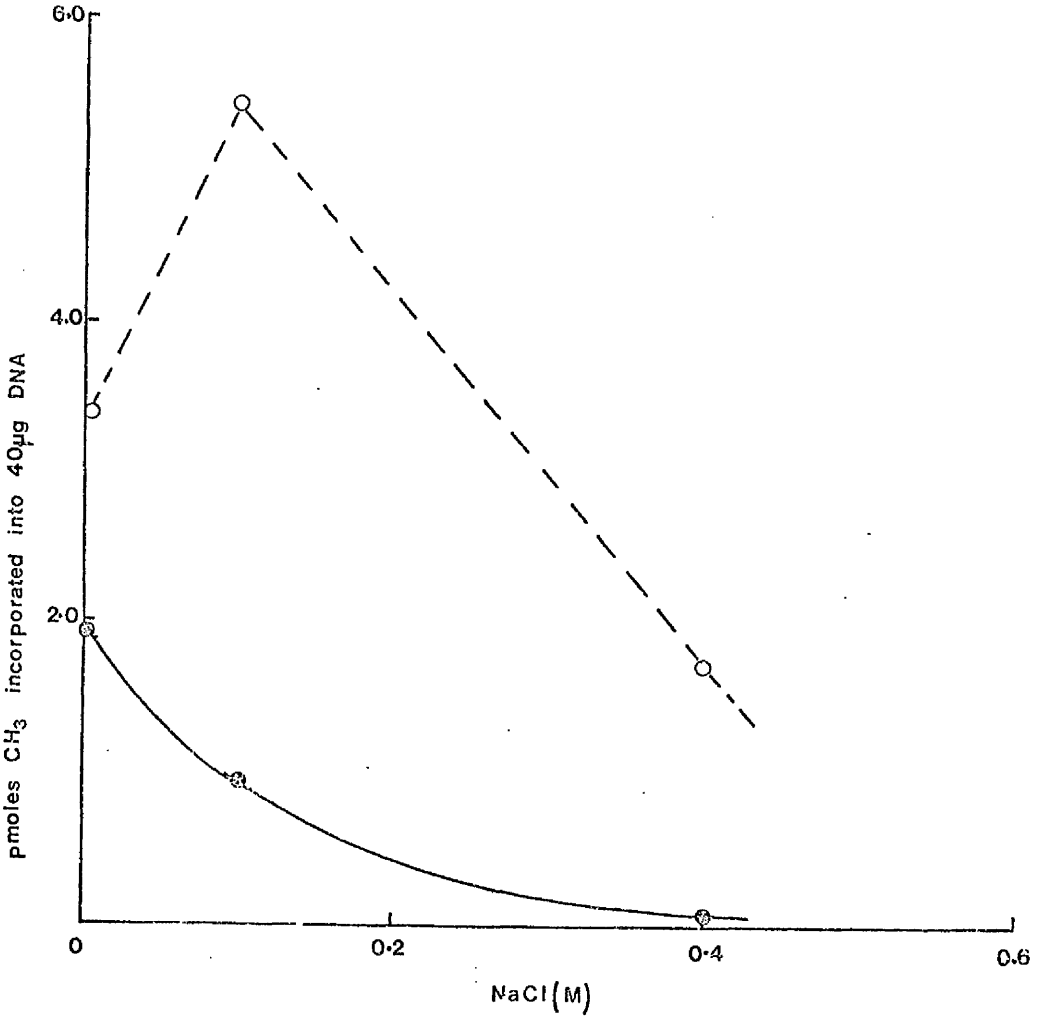
Fig 18 shows a comparison of the methylation of "native" and heat denatured E. coli DNA by DNA methylation of native and heat-denatured E. coli DNA by DNA methylase preparation from BHK-21/C13 cell. Thus at least the BHK-21/C13 enzyme shows some similarity to the rat liver enzyme. But this may be superficial in as much as the methylation of the so called "native" DNA was actually in single-stranded regions. This possibility was not explored in the report of Drahovsky and Morris (1971 a, b).

3.6 Sequence Specificity of BHK-21 DNA Methylases

3.6.1 Pyrimidine isostichs methylated by the BHK-21 DNA methylases

The question of the sequence specificity of the two BHK-21 cell enzyme preparations was examined in some detail. E. coli DNA methylated in vitro by either BHK-21/C13 DNA methylase or BHK-21/PyY DNA methylase was mixed with $^{32}PO_4$ HeLa DNA and depurinated and the resulting pyrimidine fragments were fractionated on cellulose acetate strips followed by homochromatography as described in Methods Section 5.3. Whilst both DNA methylases predominantly methylated cytosine in the sequence -RpCpR- the preparation from the BHK-21/PyY cell line showed a slightly greater

by BHK-21/C13 DNA methylase preparation in the presence of varying concentrations of NaCl



The methylase assay mixture (140µl) contained 40µg "native" or heat-denatured E. coli DNA, 3.3µCi S-adencsyl-L-[methyl-^{3}H] methionine (1µCi/nmole), and 85µg BHK-21/C13 DNA methylase. The reaction was carried out at 37°C for 1 hr. Heat denaturation of DNA was carried out at 100°C for 10 min in buffer M [50mM-tris-HCl pH7.8, 1mM EDTA, 1mM dithiothreitol and 10% (v/v) glycerol] followed by rapid cooling. For details of assay see Methods Section 4.2.

●———● methylation of native E. coli DNA

O- - -O methylation of heat-denatured E. coli DNA

ability to methylate di-, tri- and tetra- pyrimidine sequences than was the case for the preparation from the BHK-21/Cl3 cell line (Fig 19). The distribution of sequences containing the cytosines methylated by the enzyme from the BHK-21/Cl3 cells were nevertheless similar to the sequences previously found *in vivo* for the BHK-21/Cl3 cell (Browne and Burdon, 1977). Indeed these previous studies had shown the *in vivo* pattern of methylated sequences to be the same in both BHK-21 cells. Thus the methylation pattern obtained using the enzyme from the BHK-21/PyY cells was a little puzzling. However in the presence of 0.1M NaCl the BHK-21/PyY cell DNA methylase preparation produced a pattern of methylation similar to that obtained using the BHK-21/Cl3 cell enzyme (Fig 20).

3.6.2 Pattern of methylation of the S_1 nuclease resistant DNA methylated by the BHK-21/PyY DNA methylase

Unlike the BHK-21/Cl3 DNA methylase preparation, BHK-21/PyY DNA methylase has been shown to methylate single stranded and double-stranded DNA. *E. coli* DNA methylated by the BHK-21/PyY enzyme and subject to S_1 nuclease digestion showed methylation in S_1 nuclease resistant DNA (i.e. double stranded DNA) (see Fig 17). This S_1 nuclease resistant region was analysed for the pattern of methylation of the pyrimidine isostichs. Six DNA methylase reactions were pooled at the end of the incubation. The reaction was

Fig 19 The pattern of methylation of lower pyrimidine isostichs of E. coli DNA by BHK-21 DNA methylases

[^{3}H-methyl] E. coli DNA methylated in vitro by either ●———● BHK-21/C13 DNA methylase or ●- - -● BHK-21/PyY DNA methylase was mixed with $^{32}PO_4$ HeLa DNA and depurinated. The resulting oligonucleotides were fractionated and assayed for tritium content as described in Methods Section 5.3. The percentage methylation was calculated relative to the methylation of the Cp_2 fragment. The tritium content of the Cp_2 fragment was of the order of 4,000dpm. The various points represent the mean of 5 independent estimations and the bars indicate the actual deviations from the mean. Nucleotide components in parentheses consist of more than one isomere.

Fig 20 The pattern of methylation of lower pyrimidine isostichs of E. coli DNA by the BHK-21/C13 DNA methylase and BHK-21/PyY DNA methylase in the presence of 0.1M NaCl

[^{3}H-methyl] E. coli DNA methylated in vitro by ●———● BHK-21/C13 DNA methylase or ●- - -● BHK-21/PyY DNA methylase in the presence of 0.1M NaCl was mixed with $^{32}PO_4$ HeLa DNA, depurinated and fractionated. The tritium contents of the fractionated oligonucleotides were estimated as outlined in Methods Section 5.3. The tritium content of the Cp_2 fragment was of the order of 4,000dpm. The various points represent the mean of 5 independent estimations and the bars indicate actual deviations from the mean. Nucleotide components in parentheses consist of more than one isomere.

Fig I9

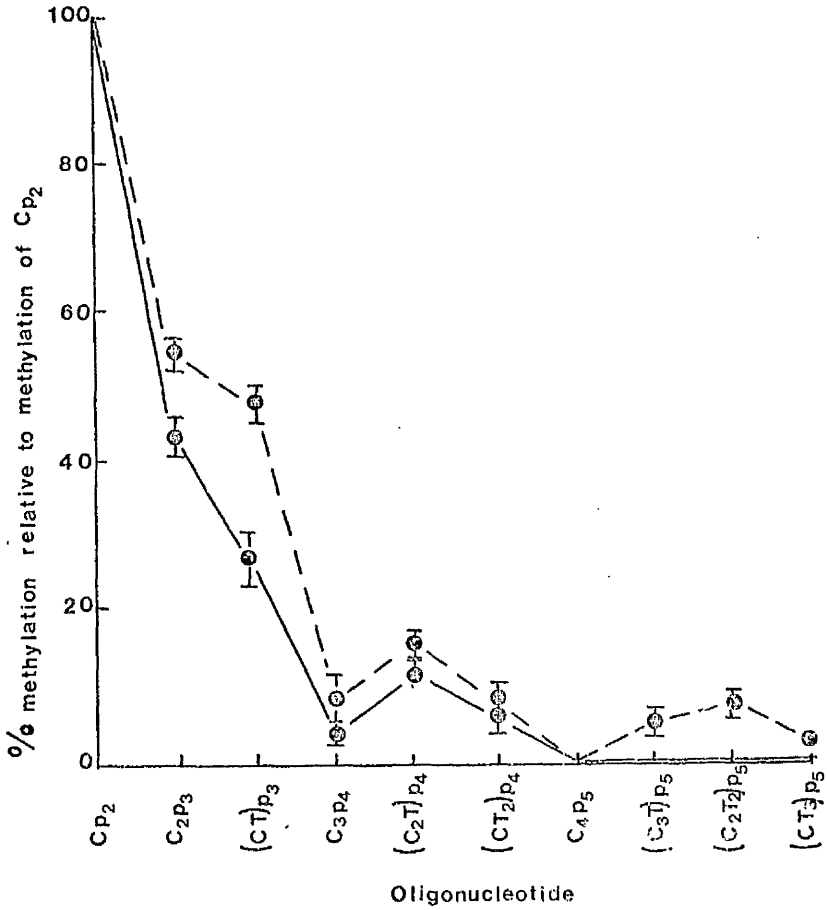
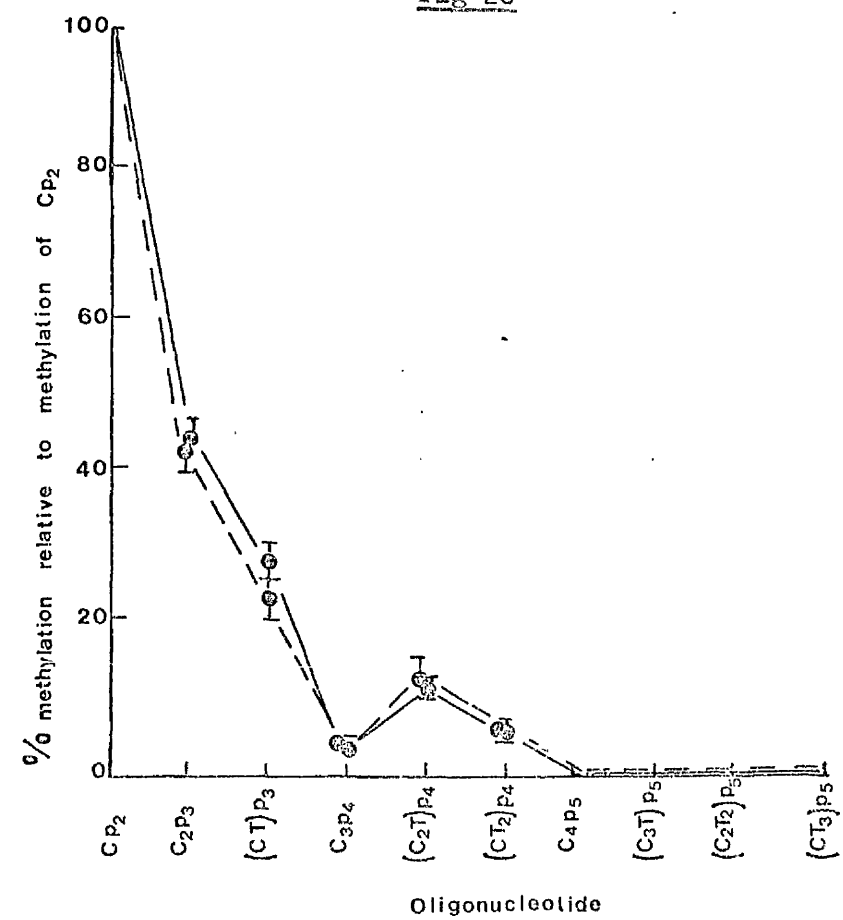

100
80
60
40
20
0
% methylation relative to methylation of C_{p_2}
C_{p_2}
C_2p_3
$(CT)p_3$
C_3p_4
$(C_2T)p_4$
$(CT_2)p_4$
C_4p_5
$(C_3T)p_5$
$(C_2T_2)p_5$
$(CT_3)p_5$
Oligonucleotide

Fig 20

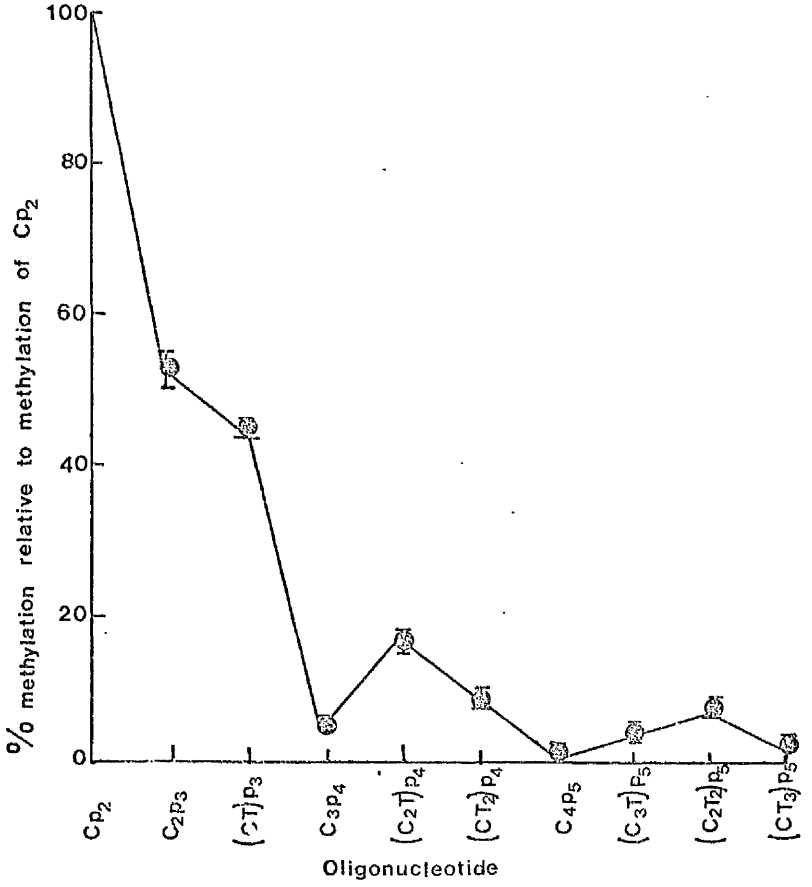

100
80
60
40
20
0
% methylation relative to methylation of C_{p_2}
C_{p_2}
C_2p_3
$(CT)p_3$
C_3p_4
$(C_2T)p_4$
$(CT_2)p_4$
C_4p_5
$(C_3T)p_5$
$(C_2T_2)p_5$
$(CT_3)p_5$
Oligonucleotide

stopped by the stopper solution and the methylated DNA recovered by alcohol precipitation after deproteinisation. The methylated DNA was subject to S_1 nuclease digestion for 3 hrs and the undigested DNA precipitated with 0.5M perchloric acid. 20µg $^{32}PO_4$ HeLa cell DNA were added and desalted by extensive dialysis. The DNAs were depurinated and fractionation as described in the Methods Section 5.3. Fig 21 shows that the pattern of methylation of the S_1 nuclease resistant DNA was not different from the pattern of methylation of the whole E. coli DNA by the BHK-21/PyY enzyme in the absence of 0.1M NaCl.

3.6.3 3' end analysis of methyl groups in dipyrimidine isostichs

In vivo studies on the methylation of DNA of Novikoff hepatoma cells have shown that with the exception of the dinucleotide (CpT) all the other pyrimidine isostichs studied had 5-methyl cytosine residues at the 3'-terminus (Sneider, 1972). In this experiment the in vitro methylated pyrimidine fragments $(CT)p_3$ and C_2p_3 obtained by cutting the spots from DEAE-cellulose plates (see Methods 5.4.1) were dephosphorylated by alkaline phosphatase treatment followed by snake venom phosphodiesterase digestion. Nearly 90% of the tritium counts were localised at the 5'-deoxycytidine monophosphate spot (see Fig 8). This

Fig 21 The pattern of methylation of S_1 nuclease resistant DNA methylated by BHK-21/PyY DNA methylase



[^{3}H-methyl] *E. coli* DNA methylated by BHK-21/PyY DNA methylase preparation was dissolved in 3ml of buffer (0.03M sodium acetate pH4.5, 3 x 10^{-5}M $ZnSO_4$, 0.01M NaCl) to which was added 100 units/ml S_1 nuclease and 50µg heat-denatured sonicated calf thymus DNA and incubated at 50°C for 3 hr. Undigested DNA was precipitated by 0.5M perchloric acid and 20µg $^{32}PO_4$ HeLa cell DNA was added and dialysed extensively. The DNAs were depurinated, fractionated and assayed for tritium content as described in Methods Section 5.3. The percentage methylation was calculated relative to the methylation of Cp_2 fragment. The tritium content of this fragment was of the order of 8,000dpm. The various points represent the mean of 4 different estimations and the bars indicate the actual deviations from the mean. Nucleotide components in parentheses consist of more than one isomere.

could only arise from digestion of the sequence TpC and not CpT. No tritium counts were present at the expected position of the deoxycytidine nucleoside. Thus both BHK-21/C13 and BHK-21/PyY DNA methylases transferred methyl groups to the 5-carbon of cytosine at the 3' end of the (CpT) fragment. It was not possible to analyse the fragment (CpC) as this turns out to be a rather poor substrate for the snake venom phosphodiesterase (see Fig 8). Longer incubation time had no effect on the digestion of either the (CpC) spot or the tripyrimidine fragment (CpCpC). However other tripyrimidine fragments were digested as judged by the pattern of their $^{32}PO_4$ label but the tritium content of these were too low for quantitative analysis (refer to Figs 19 and 20).

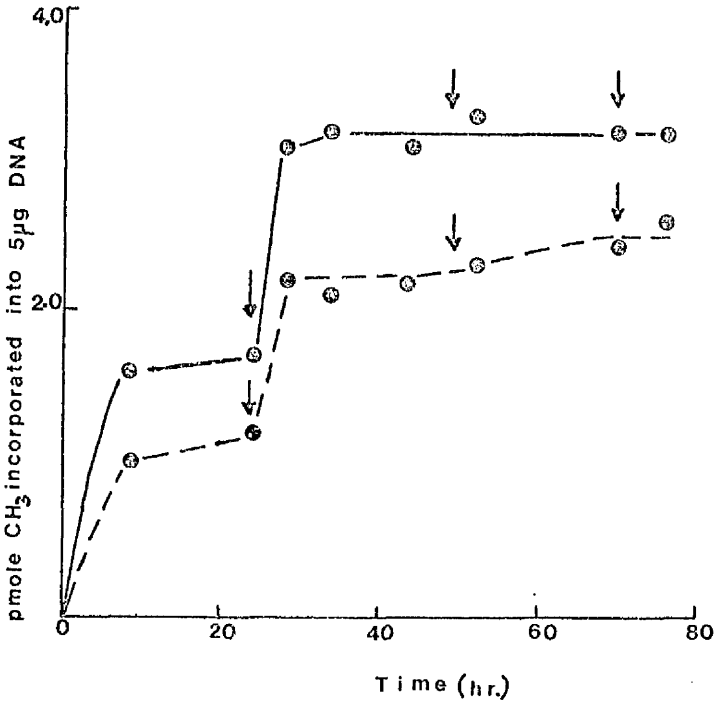
4. Organisation of the BHK-21 Cell Genome

4.1 In vitro methylation of the BHK-21 DNA

In view of the possible slight differences in sequence specification of the BHK-21/C13 and BHK-21/PyY DNA methylases, a question that arises is whether these differences can explain the quite large differences in the level of DNA methylation observed *in vivo* between these two cell lines. It had been shown (Experimental Section 1.2) that the DNA of BHK-21/C13 cells have 0.5% of their cytosines present as 5-methyl cytosine whereas the corresponding figure for BHK-21/PyY DNA is 1.0%. Are there then 0.5% cytosine

residues in the BHK-21/Cl3 DNA potential sites of methylation by the BHK-21/PyY DNA methylase? To answer this question, DNA was prepared from both cell types and offered in turn as substrates to the DNA methylase prepared from each cell type. In each case the DNA and enzyme were incubated 76 hr with addition of fresh enzyme and S-adenosyl-L-[methyl-^{3}H] methionine at 24, 48 and 70 hr in order that the maximum potential sites on the substrate DNA are modified following the technique originally used by Turnbull and Adams (1976). Fig 22 shows the course of methylation of BHK-21/Cl3 and BHK-21/PyY DNAs by the BHK-21/Cl3 DNA methylase preparation under these conditions. Table 8 indicates the maximum level of methylation attained by various combinations of DNA methylases and substrate DNA in the presence and absence of 0.1M NaCl. The final level of incorporation of methyl groups into BHK-21/Cl3 DNA and BHK-21/PyY DNA by either homologous or heterologous DNA methylases is low in all cases. Noticeably, it seems although the DNA from the BHK-21/Cl3 cell is a slightly better substrate for the BHK-21/PyY enzyme than the DNA from the BHK-21/PyY cells themselves, the level of methylation falls very short of that expected from the above arguments. In fact the differences in methyl groups accepted between the two DNAs as substrates for the BHK-21/PyY cell enzyme would account for only a further 0.018% of the BHK-21/Cl3 cytosines modified rather than the 0.5% which would theoretically be necessary to bring the level of

Fig 22 Time course of incorporation of methyl groups into BHK-21 DNAs by BHK-21/C13 DNA methylase



The reaction mixture (70μl) contained 1.65μCi of S-adenosyl-L-[methyl-^{3}H] methionine (1μCi/nmole), 5μg of either ●——● BHK-21/C13 DNA or ●- - -● BHK-21/PyY DNA, and 65μg of BHK-21/C13 DNA methylase. At the times indicated by the arrows fresh enzyme (20μl) of enzyme (1.3mg/ml) and 10μl of S-adenosyl-L-[methyl-^{3}H] methionine (1.65μCi, 1μCi/nmole) were added to the incubation mixture. The reaction was stopped as described in Methods Section 4.2.

Table 8 Methylation of BHK-21 cell DNA by homologous and heterologous DNA methylases

The reaction mixture (70µl) contained 1.65µCi of S-adenosyl-L-[methyl-^{3}H] methionine (1µCi/nmole) 5µg of BHK-21/C13 or BHK-21/PyY DNA and 65µg of BHK-21/C13 DNA methylase or 12.6µg BHK-21/PyY DNA methylase. The reaction mixture was incubated at 37°C for 76 hr with addition of fresh enzyme (20µl of BHK-21/C13 DNA methylase, 1.3mg/ml or BHK-21/PyY DNA methylase 0.63mg/ml) and 10µl of S-adenosyl-L-[methyl-^{3}H] methionine (1.65µCi/nmole, 1µCi/nmole) at 24, 48 and 72 hr. In assays containing 0.1M NaCl, the concentration of the salt was readjusted after the addition of more enzyme and S-adenosyl-L-[methyl-^{3}H] methionine. Heat denaturation of the BHK-21 DNAs was done at 100°C for 10 min in buffer M [50mM tris-Hcl pH7.8, 1mM EDTA, 1mM dithiothreitol and 10% (v/v) glycerol] followed by rapid cooling. The reaction was stopped and the radioactivity estimated as outlined in Methods Section 4.2 and 4.3.

Table 8 Methylation of BHK-21 cell DNA by homologous and heterologous DNA methylases

DNA	Enzyme	pmoles incorporation of methyl groups/5μg DNA
Native BHK-21/C13	BHK-21/C13	1.49
Denatured "	"	3.93
Native BHK-21/PyY	"	1.27
Denatured "	"	1.61
Native BHK-21/C13	BHK-21/PyY	2.70
Native BHK-21/C13 + 0.1M NaCl	"	2.27
Denatured BHK-21/C13	"	3.61
Denatured BHK-21/C13 + 0.1M NaCl	"	2.95
Native BHK-21/PyY	"	2.04
Native BHK-21/PyY + 0.1M NaCl	"	1.96
Denatured BHK-21/PyY	"	2.02
Denatured BHK-21/PyY + 0.1M NaCl	"	2.95

cytosine modification of the BHK-21/C13 cell DNA to the level encountered in the BHK-21/PyY cell. This increase would require an additional 18.3pmole of methyl groups into the BHK-21/C13 DNA. Even the presence of 0.1M NaCl did not alter drastically the level of methyl groups incorporated into these DNAs by the BHK-21/PyY cell enzyme.

4.2 Physicochemical properties of BHK-21 DNAs

Studies of the native and melting spectra of BHK-21/C13 and BHK-21/PyY DNAs following the procedure of Hirschman and Felsenfeld, 1966 have shown that these two DNAs have similar CG base pairs (54 $\pm$ 0.5%) and a number of identical physicochemical parameters. Both DNAs had Tm of 73.5°C $\pm$ 0.2°C, ΔT of 13° - 14°C and hyperchromicity of 22.5% $\pm$ 0.5% in cacodylate buffer (pH7.0) at 0.01M Na^+ concentration.

Kinetic curves for the reassociation of tritiated thymidine labelled DNAs from BHK-21/C13 and BHK-21/PyY showed no difference between the two DNAs (Figs 23 a, b). However the reassociation of the two DNAs following a label with [^{14}C-methyl] groups showed that the repetitive regions of the BHK-21/PyY DNA (cot 0.1 - 10) had twice as many methyl groups as the corresponding sequences of the BHK-21/C13 DNA.

5. Role for DNA Methylation

5.1 Bromodeoxyuridine substituted mouse L929 DNA as substrate for a BHK-21 DNA methylase

The function of DNA methylation in eukaryotes is as yet

Fig 23 A Kinetics of reassociation of BHK-21/C13 DNA

DNA from BHK-21/C13 cells labelled separately with [^{3}H] thymidine and [methyl-^{14}C] methionine were mixed and sheared by a Dawe soniprobe (12 successive cycles of sonication of 15 sec at 7 amperes followed by 45 sec cooling on ice). The sheared DNA in 0.5M NaCl, 25mM Hepes, 0.5mM EDTA, 50% (v/v) formamide (fluka) pH6.8 was heated at 65^{o}C for 10 min and incubated at 43^{o}C for various lengths of time. Cot values were computed on the basis that the reassociation of DNA in a concentration of 83µg/ml for 1 hr corresponds to a cot value of 1 (Britten and Kohne, 1968).

Fig 23 B Kinetics of reassociation of BHK-21/PyY DNA

The reassociation kinetics were carried out as outlined in legend of Fig 23 A but with [^{3}H and ^{14}C] labelled DNA from BHK-21/PyY cells.

Fig 23 A

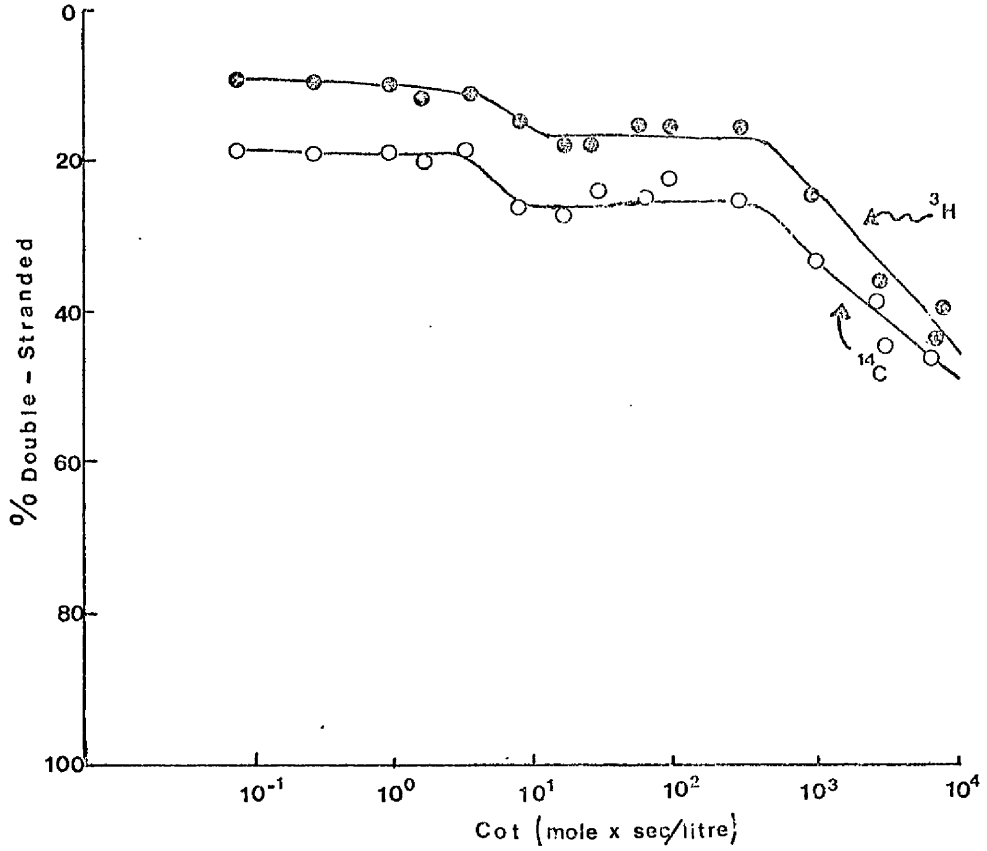
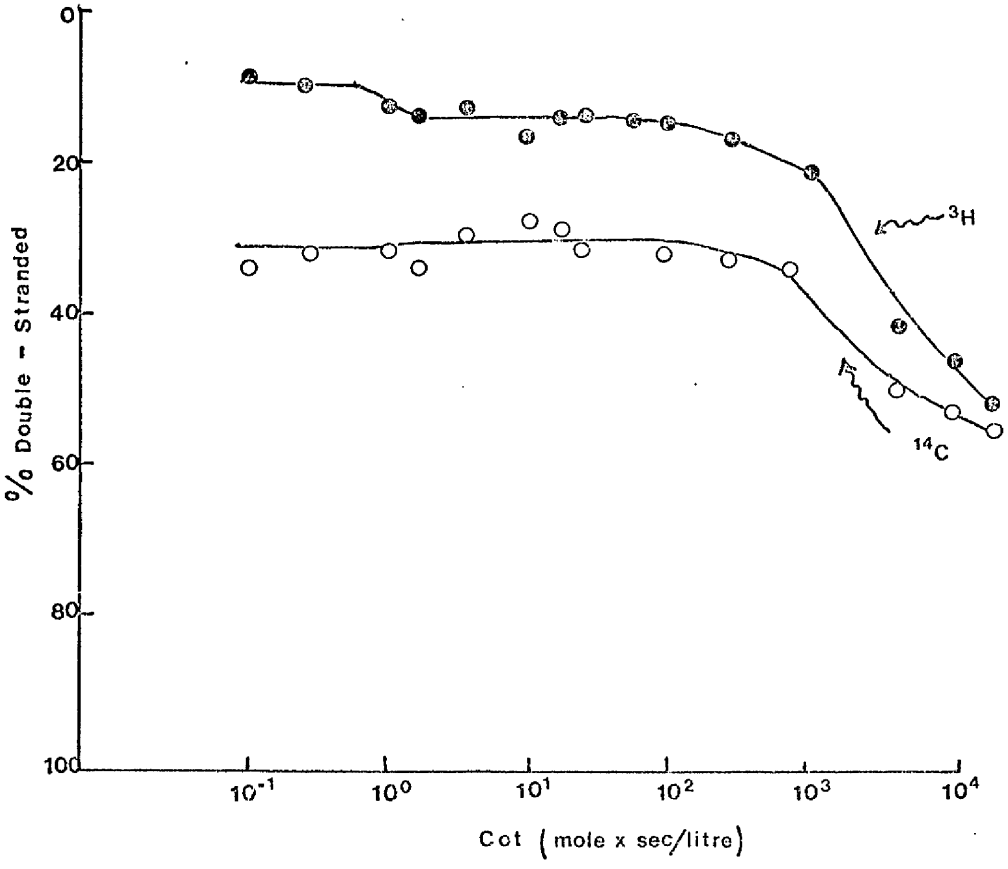


Fig 23 B



uncertain, but suggestions that methylation may affect gene expression and differentiation have been put forward. Riggs (1975) and Holliday and Pugh (1975) have published reviews and models for differentiation based on DNA methylation by sequence-specific enzymes. Bromodeoxyuridine is known to block differentiation (Bischoff and Holtzer, 1970; Weintraub et al, 1972; Walther et al, 1974; Lasher and Cahn, 1969; Coleman et al, 1970) as well as the expression of some genes of differentiated cells (Holthavsen et al, 1969; Silagi and Bruce, 1970; Stellwagen and Tomkins, 1971a, b). It was therefore hypothesised that bromodeoxyuridine substitution might affect the activity of DNA methylating enzymes and thereby affect differentiation (Singer et al, 1977). In this study this hypothesis was evaluated by assessing the in vitro methylation of mouse L929 DNA from cells grown in the presence of bromodeoxyuridine for 24 hr (one cell generation) and 3 days (3 cell generations). Control experiments were carried out using DNA from cells grown in the presence of thymidine rather than bromodeoxyuridine. It appears from the results in Table 9 that bromodeoxyuridine substitution decreases the level of methyl groups incorporated into substrate DNA at least over 1 hr in vitro. DNA from L929 cells grown for 24 hr in a medium containing thymidine appeared to be a better substrate for in vitro methylation than the DNA from cells grown for 72 hr (Table 9). However in both cases the rate of methylation was low in the presence of

Table 9 Effect of bromodeoxyuridine substitution on *in vitro* methylation of mouse L929 DNA

Source of DNA	Methyl groups incorporated into DNA (dpm/40μg DNA)
L929 cells grown in medium + thymidine for 24 hr	1289.1
L929 cells grown in medium + BrdUrd for 24 hr	280.0
L929 cells grown in medium + thymidine for 72 hr	754.3
L929 cells grown in medium + BrdUrd for 72 hr	231.0

Mouse L929 cells released from stationary phase were grown in EC_{10} medium in the presence of glycine (80μM), adenosine (60μM) and aminopterin (0.2μM) for 16 hr. The cells were transferred into fresh medium containing 10μM BrdUrd or 10μM thymidine and grown for 24 hr or 72 hr. DNA extracted from these cells were used in the methylation assay. The reaction mixture (140μl) contained 40μg DNA, 3.3μCi S-adenosyl-L-[methyl-^{3}H] methionine (1μCi/nmole) and 100μg BHK-21/PyY DNA methylase. The reaction mixture was incubated for 1 hr at 37°C. For details of assay conditions see Methods Section 4.2.

BrdUrd substitution. The mouse L929 DNAs were therefore incubated with S-adenosyl-L-[methyl-^{3}H] methionine and enzyme for 96 hr with addition of fresh enzyme and S-adenosyl-L-[methyl-^{3}H] methionine at 24, 48 and 72 hr in order to methylate the available methylation sites in the various DNAs. Figures 24a and 24b show that the apparent decrease in methylation of the BrdUrd substituted DNA noted in Table 8 may only be in fact a manifestation of a slower rate of methylation of the DNA which has arisen probably as a result of the bromouracil residues impairing the interaction of DNA methylase with the appropriate recognition sequences. However by 96 hr the overall level of cytosine residues methylated is about the same as that in the unsubstituted DNA.

NOTE: In Table 9 and Figs 24a and 24b, the DNA from the L929 cells grown in a medium containing thymidine for 72 hr is a poorer acceptor of methyl groups than the DNA from cells grown in a medium containing thymidine for 24 hr. This is because *in vivo* DNA methylation varies with the growth of the cell - the highest methylation being at the confluent stage. DNA from cells harvested at this stage serve as poorer methyl acceptors as most of their potential sites are already methylated *in vivo*.

Fig 24 a, b Time course of incorporation of methyl groups into mouse L929 DNA from cells grown in the presence of either bromodeoxyuridine or thymidine

The reaction mixture (70µl) contained 1.65µCi S-adenosyl-L-[methyl-^{3}H] methionine (1µCi/nmole), 5µg mouse L929 DNA from

(a) cells grown in the presence of either BrdUrd or thymidine for 24 hr

(b) cells grown in the presence of either BrdUrd or thymidine for 72 hr

and 50µg BHK-21/PyY DNA methylase preparation. At the times indicated by the arrows fresh enzyme (20µl, 1.1mg/ml) and 10µl S-adenosyl-L-[methyl-^{3}H] methionine (1.65µCi, 1µCi/nmole) were added. The reaction mixture was incubated at 37°C. For details of assay see Methods Section 4.2 and for the growth of cells see Legend of Table 9.

●———● L929 DNA from cells grown in the presence of thymidine

O- - -O L929 DNA from cells grown in the presence of BrdUrd.

Fig 24a Time course of incorporation of methyl groups into L929 DNA from cells grown for 24 hr in the presence of either bromodeoxyuridine or thymidine

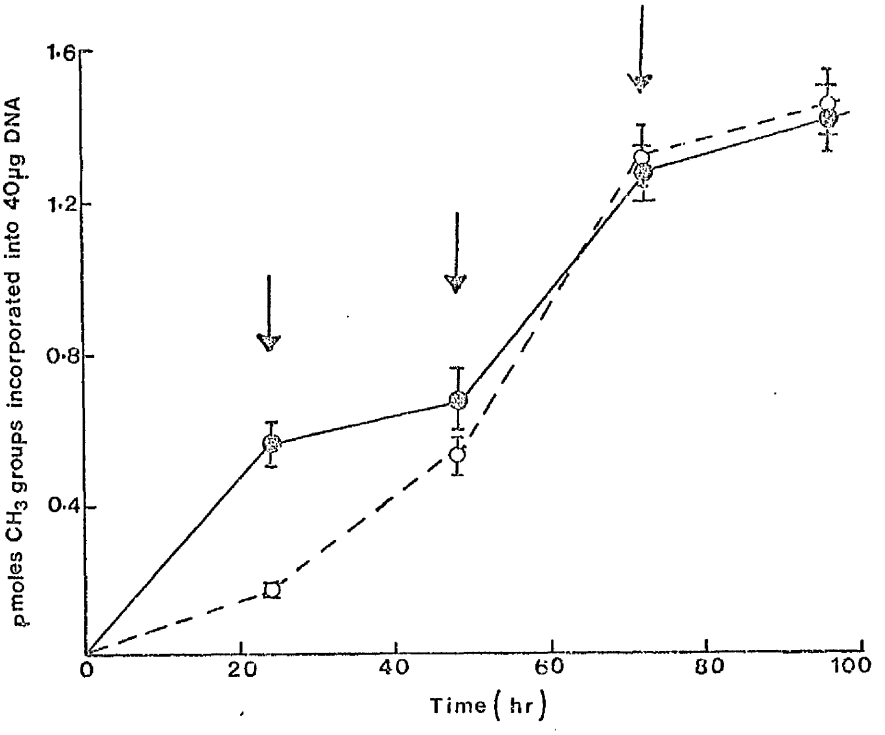
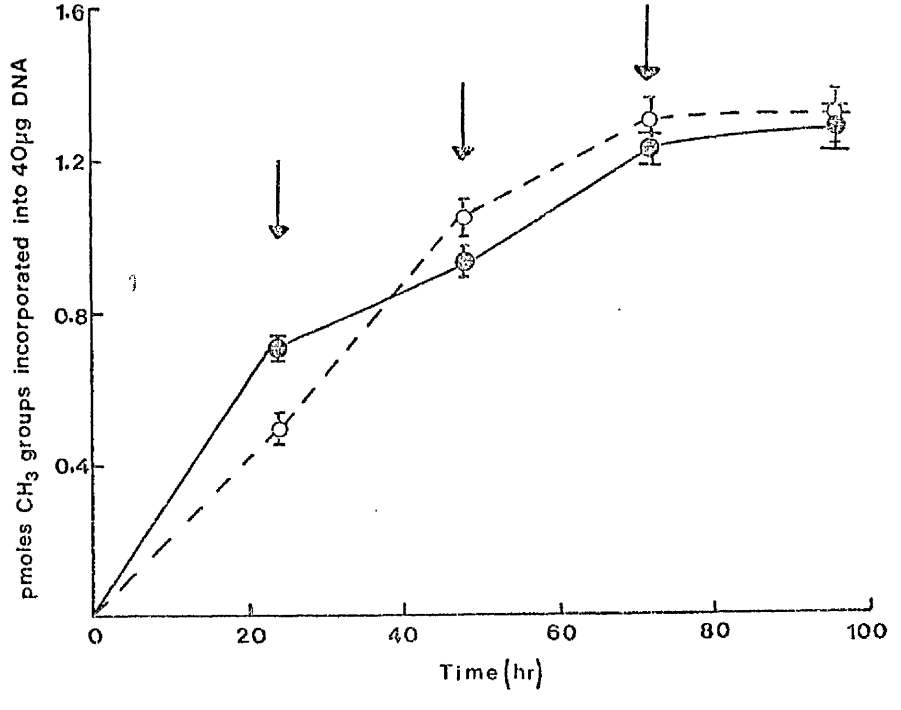


Fig 24b Time course of incorporation of methyl groups into L929 DNA from cells grown for 72 hr in the presence of either bromodeoxyuridine or thymidine



5.2 Modification-restriction Mechanism in Eukaryotes?

5.2.1 Degradation of substrate DNA by a SAM-dependent DNase

Bacterial cells contain "restriction" endonucleases which cleave DNA at specific sites but these sites can be protected from cleavage by base methylation. In this study an attempt has been made to find out whether such endonucleases exist in eukaryotes.

Nuclei from BHK-21/C13 cells were resuspended in buffer R (50mM tris-HCl pH7.8, 12.5mM EDTA, 1mM dithiothreitol, 10% glycerol). The nuclei were disrupted by making the solution 0.4M with respect to KCl centrifuged at 12,000g for 60 min and the resulting supernatant was dialysed and used as source of enzyme. Initially phage λ DNA was used as substrate and incubated at 37°C for 60 min and the products were analysed through 1.5% agarose gels (Fig 25). It was evident that concentration of SAM from 2mM to 66mM caused degradation of phage λ DNA. Further studies have shown that SAM concentration as low as 55μM can cause similar effect. This reaction is inhibited by S-adenosyl-L-homocysteine an inhibitor of DNA methylation as shown in Fig 25(i) and (j). High concentrations of nicotinamide and adenosine, other inhibitors of DNA methylation, inhibited this reaction but less so by adenosine (Fig 26). Studies on this enzyme have shown that it does not require ATP or Mg^{++} and like the bacterial type I restriction

Fig 25 Degradation of phage λ DNA by the BHK-21/C13 SAM-dependent DNase

The reaction mixture (12µl) consisted of 1µg phage λ DNA and 35µg enzyme protein and incubated at 37°C for 1 hr. For details of assay see Methods Section 4.4.
(a) Phage λ DNA (zero time) (b) Phage λ DNA (incubated for 1 hr at 37°C) (c) Phage λ DNA + enzyme (d) Phage λ DNA + 2mM SAM + enzyme (e) Phage λ DNA + 4.7mM SAM + enzyme (f) Phage λ DNA + 11.5mM SAM + enzyme (g) Phage λ DNA + 27.5mM SAM + enzyme (h) Phage λ DNA + 66.1mM SAM + enzyme (i) Phage λ DNA + 19.4mM + 3mM SAH + enzyme (j) Phage λ DNA + 46.4mM SAM + 3.3mM SAH.

*

Fig 26 Inhibition of the BHK-21/C13 SAM-dependent DNase by inhibitors of DNA methylation

The reaction mixture (20µl) consisted of 1µg Phage λ DNA and 35µg enzyme protein and incubated at 37°C for 1 hr. For details of assay see Methods Section 4.4.
(a) Phage λ DNA (b) Phage λ DNA + enzyme (c) Phage λ DNA + 1mM SAM + enzyme (d) Phage λ DNA + 1mM SAM + 10mM SAH + enzyme (e) Phage λ DNA + 10mM SAH + enzyme (f) Phage λ DNA + 1mM SAM + 20mM adenosine + enzyme (g) Phage λ DNA + 20mM adenosine + enzyme (h) Phage λ DNA + 1mM SAM + 50mM nicotinamide + enzyme (i) Phage λ DNA + 50mM nicotinamide.

*

* Unless otherwise stated all the assay mixtures were incubated at 37°C for 1 hr.

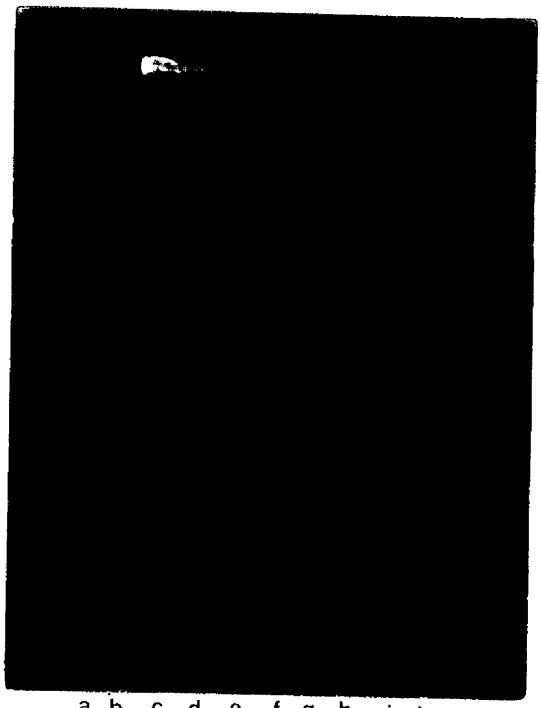


Fig 25

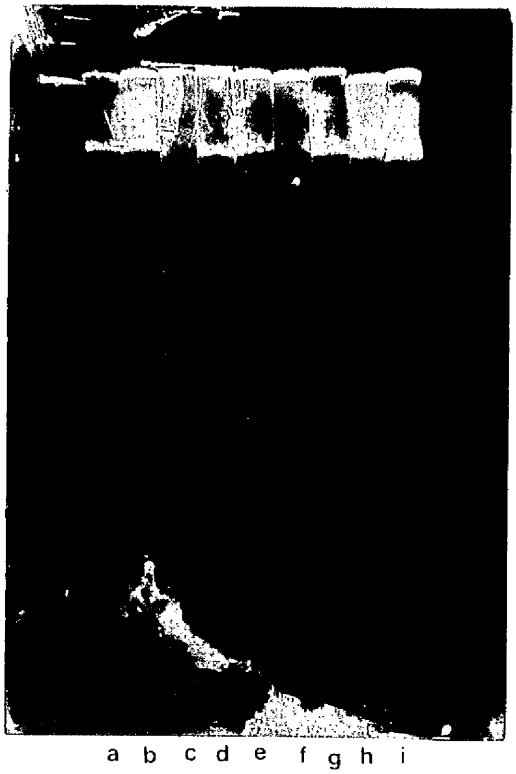


Fig 26

enzymes the products of degradation are heterogeneous and no bands are visible on the agarose gels after staining with ethidium bromide and viewing under U.V. light. It is not only phage λ DNA that is degraded but other DNAs such as T7 DNA, adenovirus type 2, Aedes albopticus and herpes simplex virus DNAs are all degraded in the presence of S-adenosly-L-methionine. It is of interest that all these DNAs serve as excellent substrates for in vitro methylation with DNA methylase preparation from BHK-21 cells. Bacteriophage ØX174 DNA (a single-stranded DNA) is not degraded (Fig 27), and the double-stranded SV40 DNA is undegraded as well, although it is converted from the circular supercoiled form I to the relaxed circular form II independent of SAM (Fig 28). Further studies have shown that this conversion of SV40 form I to form II is due to a contaminating "unwindase" activity (Burdon, R.H. private communication). However heat denatured mouse $L929_{\Lambda}^{DNA}$ isolated from cells at the mid-log phase of growth was degraded by the SAM-dependent nuclease (Fig 29).

5.2.2. Protection of substrate DNA against degradation by methylation

DNA methylase preparation from BHK-21/PyY cells (Experimental Section 2) was used to methylate phage λ DNA before the SAM-dependent DNase assay. The DNA methylase was checked for possible nuclease activity by incubating it with phage λ DNA and SV40 DNA for 8 hr followed by analysis through 1.5% agarose gels. Phage λ DNA was not degraded after

Fig 27 Effect of the BHK-21/C13 SAM-dependent DNase on bacteriophage ØX174 DNA

The reaction mixture (12µl) consisted of 1µg bacteriophage ØX174 DNA and 35µg enzyme protein and incubated at 37°C for 1 hr. For details of assay see Methods Section 4.4.
(a) bacteriophage ØX174 DNA (zero time)
(b) bacteriophage ØX174 DNA (incubated for 1 hr at 37°C) (c) bacteriophage ØX174 DNA + enzyme
(d) bacteriophage ØX174 DNA + 2mM SAM + enzyme
(e) bacteriophage ØX174 DNA + 4.7mM SAM + enzyme
(f) bacteriophage ØX174 DNA + 11.5mM SAM + enzyme (g) bacteriophage ØX174 DNA + 27.5mM SAM + enzyme (h) bacteriophage ØX174 DNA + 66.1mM SAM + enzyme.

*

Fig 28 Effect of the BHK-21/C13 SAM-dependent DNase on SV40 DNA

The reaction mixture (12µl) consisted of SV40 DNA and 35µg enzyme protein and incubated at 37°C for 1 hr. For details of assay see Methods Section 4.4.
(a) SV40 DNA (2µg, zero time) (b) SV40 DNA (1µg, incubated for 1 hr at 37°C) (c) SV40 DNA (1µg) + enzyme (d) SV40 DNA + 4.7mM SAM + enzyme.

*

* Unless otherwise stated all the assay mixtures were incubated at 37°C for 1 hr.

Fig 27

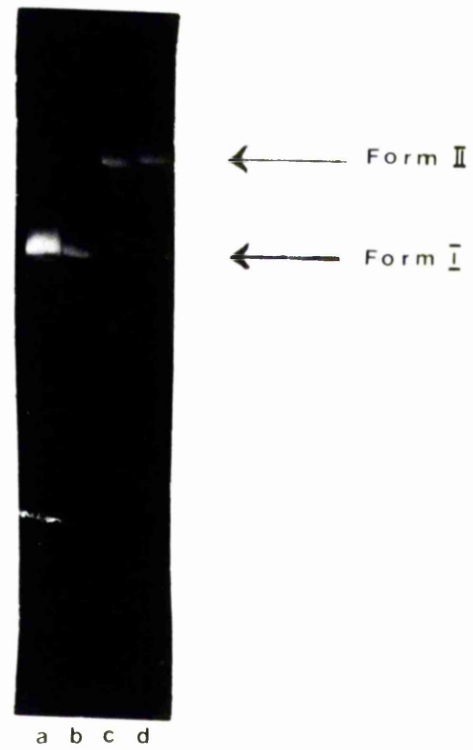
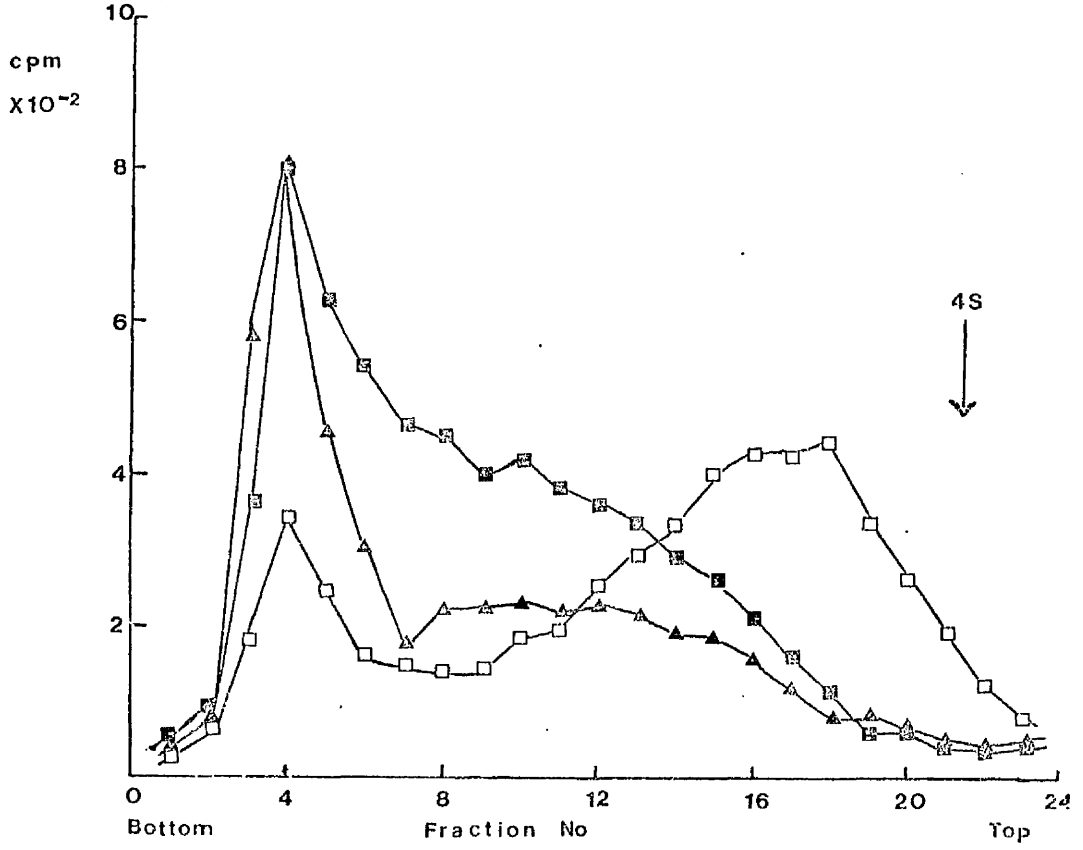


Fig 28

Fig 29 Effect of the BHK-21/C13 SAM-dependent DNase on heat-denatured mouse L929 DNA from cells harvested at mid-log phase of growth



5-20% neutral sucrose gradients of 5µg heat-denatured ^{14}C-deoxycytidine labelled mouse L929 DNA from cells harvested at the mid-log phase of growth. The DNA was incubated for 3 hr with 69µg of the BHK-21/C13 SAM-dependent nuclease (for details of assay see Methods Section 4.4). The sucrose gradients contained 60% sucrose cushion and were spun at 40,000 rpm in SW 50.1 rotor for 3 hr at 20°C

□——□ heat-denatured mouse L929 DNA

△——△ heat-denatured mouse L929 DNA incubated at 37°C with the BHK-21/C13 SAM-dependent nuclease for 3 hr

□——□ heat-denatured mouse L929 DNA incubated at 37°C with 2mN SAM and the BHK-21/C13 SAM-dependent nuclease for 3 hr.

Methylation of phage λ DNA

(a) I methyl group per 3.0×10^4 bp

(b) I methyl group per 2.1×10^4 bp

(c) I methyl group per 3.2×10^2 bp.

8 hr of incubation and though some SV40 (form I) molecules were converted to form II, no further degradation was observed. This could probably be due to the "unwindase" activity (Fig 30).

This BHK-21/PyY DNA methylase preparation was used to methylate phage λ DNA for (a) 2 hr (b) 4 hr and (c) 76 hr with addition of fresh SAM and enzyme after 24, 48 and 72 hr as described in Experimental Section 4.1. The methylated phage λ DNA contained (a) 0.050pmole of methyl groups/μg DNA (b) 0.072pmole methyl groups/μg DNA and (c) 4.54pmoles methyl groups/μg DNA. These three substrates were then offered to the SAM-dependent DNase and incubated for 1 hr at 37°C. The digested products were examined through 1.5% agarose gels. Whilst the SAM-dependent DNase degraded the unmethylated phage λ DNA, the other methylated substrates were only slightly degraded. The phage λ DNA methylated for 76 hr showed some slight degradation by the BHK-21/PyY DNA methylase but nevertheless was not degraded by the SAM-dependent DNase (Fig 31). DNAs from mouse L929 and BHK-21/C13 cells grown to stationary phase were not degraded by this enzyme (Figs 32a, b). However DNA extracted from these cells at mid-log phase (Figs 33a, b) or grown for one generation in the absence of L-methionine were degraded (Figs 34a, b). Additionally the SAM-dependent DNase degraded Xenopus laevis

Fig 30 Effect of BHK-21/PyY DNA methylase preparation on Phage λ and SV40 DNAs

10μg of Phage λ DNA or SV40 DNA were incubated in a total volume of 25μl for 8 hr at 37°C with 0.82μCi S-adenosyl-L-[methyl-^{3}H] methionine (1μCi/nmole) and 8.8μg BHK-21/PyY DNA methylase preparation. Aliquots were taken at the times indicated below and assayed through 1.5% (w/v) agarose gel. For details see Methods Section 4.4.2.
(a) 4μg Phage λ DNA at zero time (b) 2μg Phage λ DNA at 2 hr incubation (c) 2μg Phage λ DNA at 4 hr incubation (d) 2μg Phage λ DNA at 8 hr incubation (e) 2μg SV40 DNA at zero time (f) 1μg SV40 DNA at 2 hr incubation (g) 1μg SV40 DNA at 4 hr incubation (h) 1μg SV40 DNA at 8 hr incubation.

Fig 31 Protection of Phage λ DNA by methylation against degradation by the BHK-21/C13 SAM-dependent DNase

The reaction mixture (20μl) consisted of Phage λ DNA and 35μg BHK-21/C13 SAM-dependent DNase and incubated at 37°C for 1 hr. For details of assay see Methods Section 4.4.
(a) Phage λ DNA (1μg, zero time) (b) Phage λ DNA (1μg) + enzyme (c) Phage λ DNA (1μg) + 1mM SAM + enzyme (d) 2 hr Methylated Phage λ DNA (1μg, zero time) (e) 2 hr Methylated Phage λ DNA (1μg) + enzyme (f) 2 hr Methylated Phage λ DNA (1μg) + 1mM SAM + enzyme (g) 4 hr Methylated Phage λ DNA (1μg, zero time) (h) 4 hr Methylated Phage λ DNA (1μg) + enzyme (i) 4 hr Methylated Phage λ DNA (1μg) + 1mM SAM + enzyme (j) 76 hr Methylated Phage λ DNA (1.5μg, zero time) (k) 76 hr Methylated Phage λ DNA (1.5μg) + enzyme (l) 76 hr Methylated Phage λ DNA (1.5μg) + 1mM SAM + enzyme.
Unless otherwise stated all the assay mixtures were incubated at 37°C for 1 hr.

IO μg of Phage λ DNA was methylated, deproteinized and extracted as described in Section 4.2. The methylated DNA was dialysed extensively against 2 changes of IOOml of buffer M (50mM Tris - HCl pH 7.8, ImM EDTA, ImM dithiothreitol, IO% ^{v}/v glycerol)

Fig 30

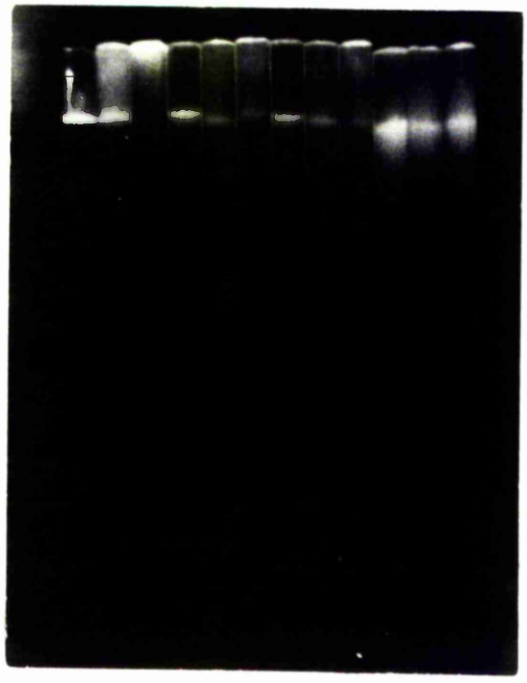


Fig 31

Fig 32a Effect of the BHK-21/C13 SAM-dependent DNase on mouse L929 DNA from cells harvested at the stationary phase of growth

5-20% neutral sucrose gradients of 5µg ^{3}H-thymidine labelled mouse L929 DNA isolated from cells harvested at the stationary phase of growth. DNA was incubated for 3 hr with 69µg of SAM-dependent nuclease (for details of assay see Methods Section 4.4). The sucrose gradients contained 60% sucrose cushion and were spun at 40,000 rpm in SW 50.1 rotor for 3 hr at 20°C.

●——● Mouse L929 DNA

■——■ Mouse L929 DNA incubated at 37°C with BHK-21/C13 SAM-dependent nuclease for 3 hr.

o——o Mouse L929 DNA incubated at 37°C with BHK-21/C13 SAM-dependent nuclease and 10mM SAM for 3 hr.

Fig 32b Effect of the BHK-21/C13 SAM-dependent DNase on BHK-21/C13 DNA from cells harvested at the stationary phase of growth

5-20% neutral sucrose gradients of 5µg ^{14}C-deoxy-cytidine labelled BHK-21/C13 DNA from cells harvested at the stationary phase of growth incubated with 69µg BHK-21/C13 SAM-dependent nuclease (same conditions as Fig 32a)

●——● BHK-21/C13 DNA

■——■ BHK-21/C13 DNA incubated at 37°C with BHK-21/C13 SAM-dependent nuclease for 3 hr.

o——o BHK-21/C13 DNA incubated at 37°C with BHK-21/C13 SAM-dependent nuclease and 10mM SAM for 3 hr.

Fig 32a

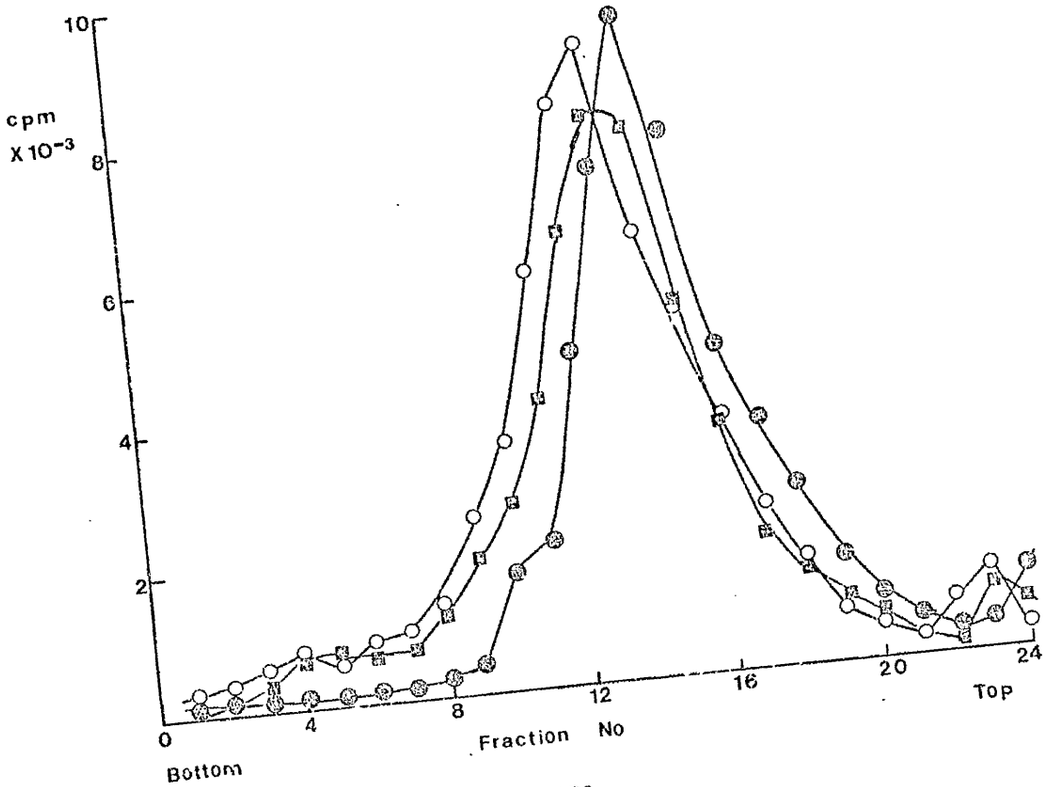


Fig 32b

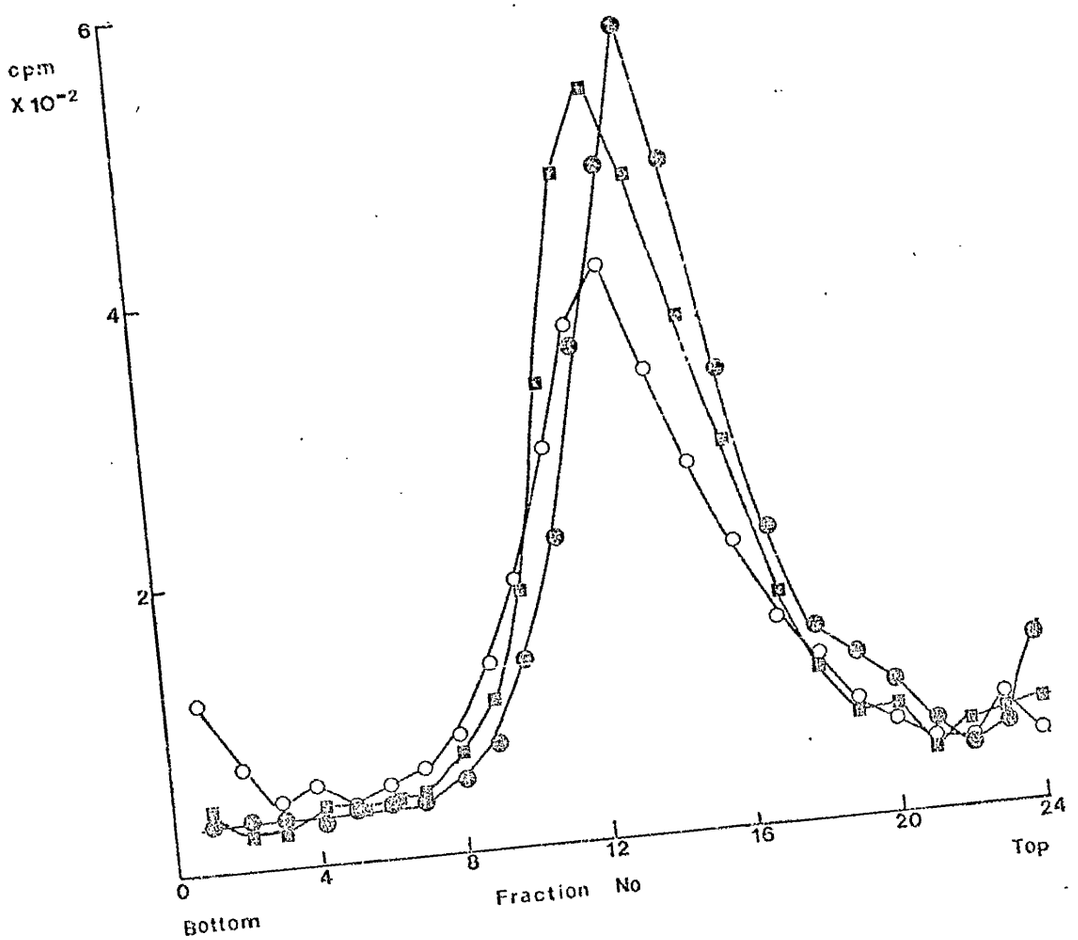


Fig 33a <u>BHK-21/C13 SAM-dependent DNase degradation of mouse L929 DNA from cells harvested at mid-log phase of growth</u>

5-20% neutral sucrose gradients of 5µg ^{14}C-deoxycytidine labelled L929 DNA from cells harvested at mid-log phase of growth. The DNA was incubated for 3 hr with 69µg of BHK-21/C13 SAM-dependent nuclease (for details of assay see Methods Section 4.4). The sucrose gradients were spun at 40,000 rpm in SW 50.1 rotor for 3 hr at 20°C.

o——o L929 DNA

▲——▲ L929 DNA incubated at 37°C with the BHK-21/C13 SAM-dependent nuclease for 3 hr.

□——□ L929 DNA incubated at 37°C with 10mM SAM and the BHK-21/C13 SAM-dependent nuclease for 3 hr.

Fig 33b <u>BHK-21/C13 SAM-dependent DNase degradation of BHK-21/C13 DNA from cells harvested at mid-log phase of growth</u>

Conditions used are identical to the one above. BHK-21/C13 cells were however labelled with ^{3}H-thymidine.

o——o BHK-21/C13 DNA

▲——▲ BHK-21/C13 DNA incubated at 37°C with the BHK-21/C13 SAM-dependent nuclease for 3 hr.

□——□ BHK-21/C13 DNA incubated at 37°C with 10mM SAM and the BHK-21/C13 SAM-dependent nuclease for 3 hr.

Fig 33a

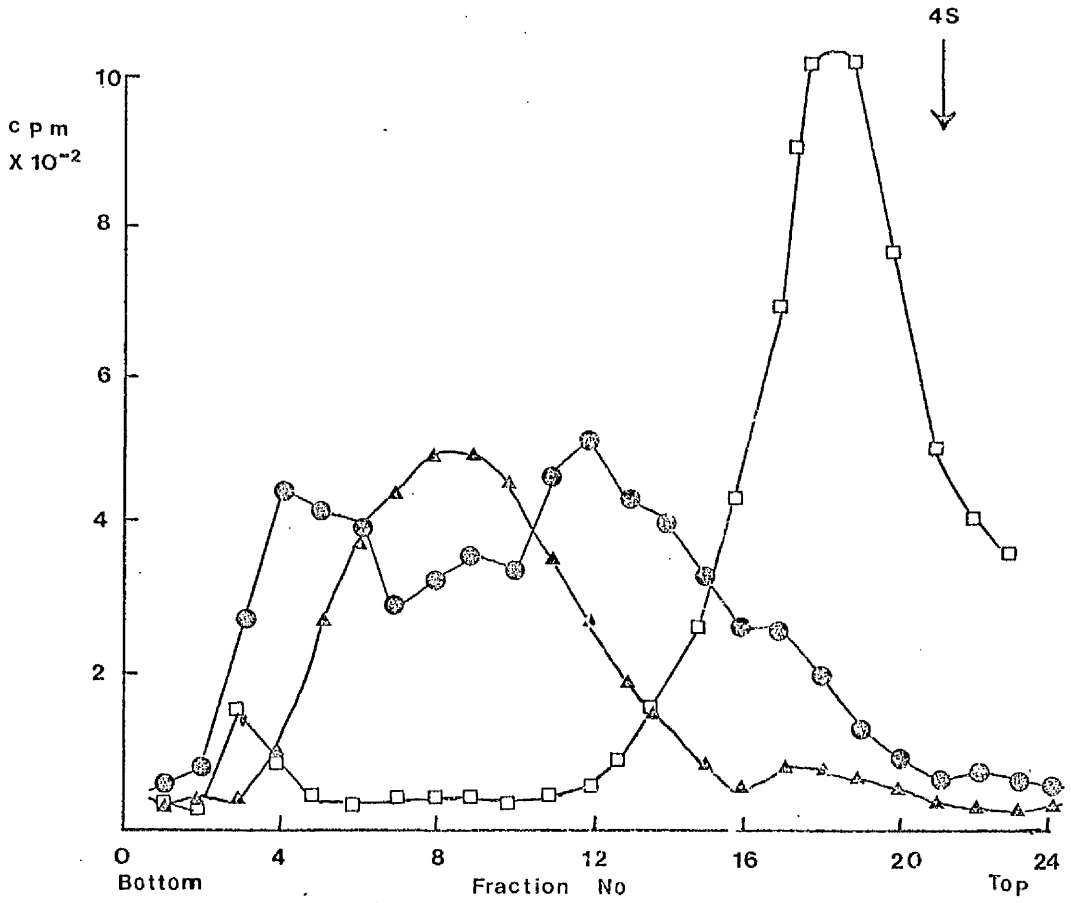


Fig 33b

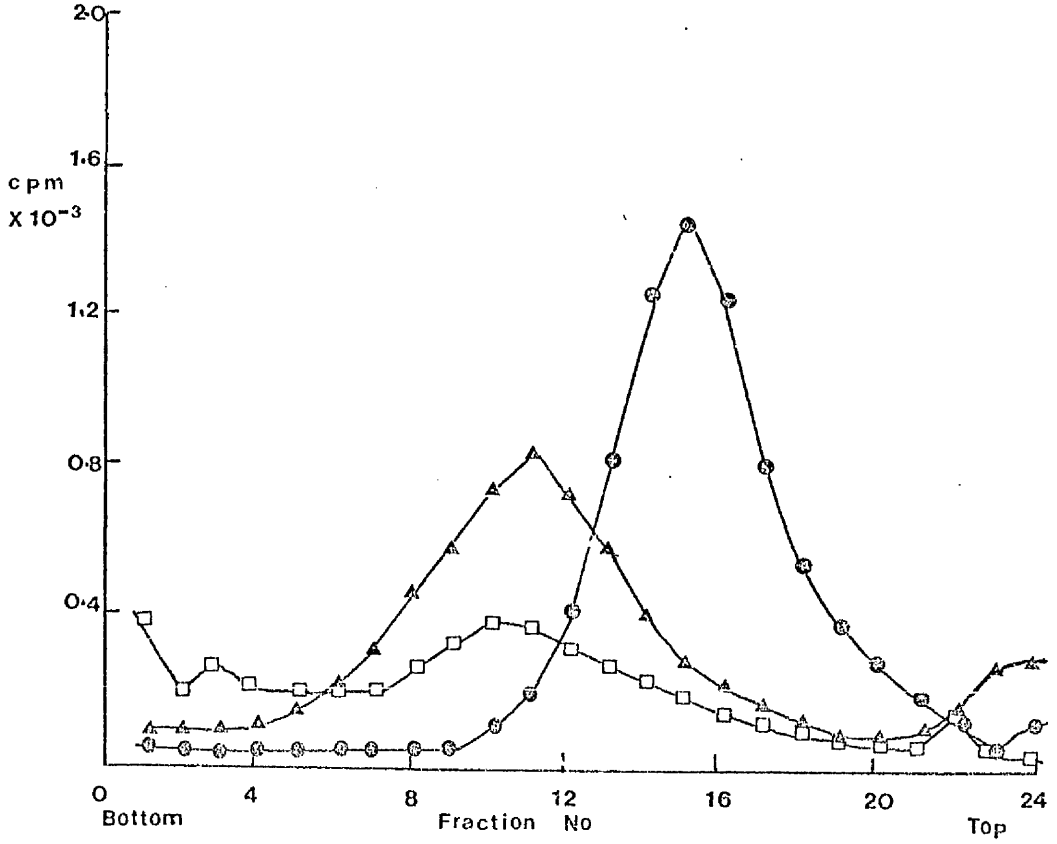


Fig 34a <u>BHK-21/C13 SAM-dependent DNase degradation of mouse L929 DNA from cells grown in a medium deprived of methionine for 24 hr.</u>

5-20% neutral sucrose gradients of 5µg ^{14}C-deoxycytidine labelled L929 DNA from cells grown in a medium deprived of methionine for 24 hr. The DNA was incubated for 3 hr with 69µg of BHK-21/C13 SAM-dependent nuclease (for details of assay see Methods Section 4.4). The sucrose gradients contained 60% sucrose cushion and were spun at 40,000 rpm in SW 50.1 rotor for 3 hr at 20°C.

o——o L929 DNA

■——□ L929 DNA incubated at 37°C with the BHK-21/C13 SAM-dependent nuclease for 3 hr.

o——o L929 DNA incubated at 37°C with 10mM SAM and the BHK-21/C13 SAM-dependent nuclease for 3 hr.

Fig 34b <u>BHK-21/C13 SAM-dependent DNase degradation of BHK-21/C13 DNA from cells grown in a medium deprived of methionine for 24 hr.</u>

Conditions used are identical to the one above. BHK-21/C13 cells were however labelled with ^{3}H-thymidine.

●——● BHK-21/C13 DNA

■——■ BHK-21/C13 DNA incubated at 37°C with BHK-21/C13 SAM-dependent nuclease for 3 hr.

o——o BHK-21/C13 DNA incubated at 37°C with 10mM and the BHK-21/C13 SAM-dependent nuclease for 3 hr.

Fig 34a

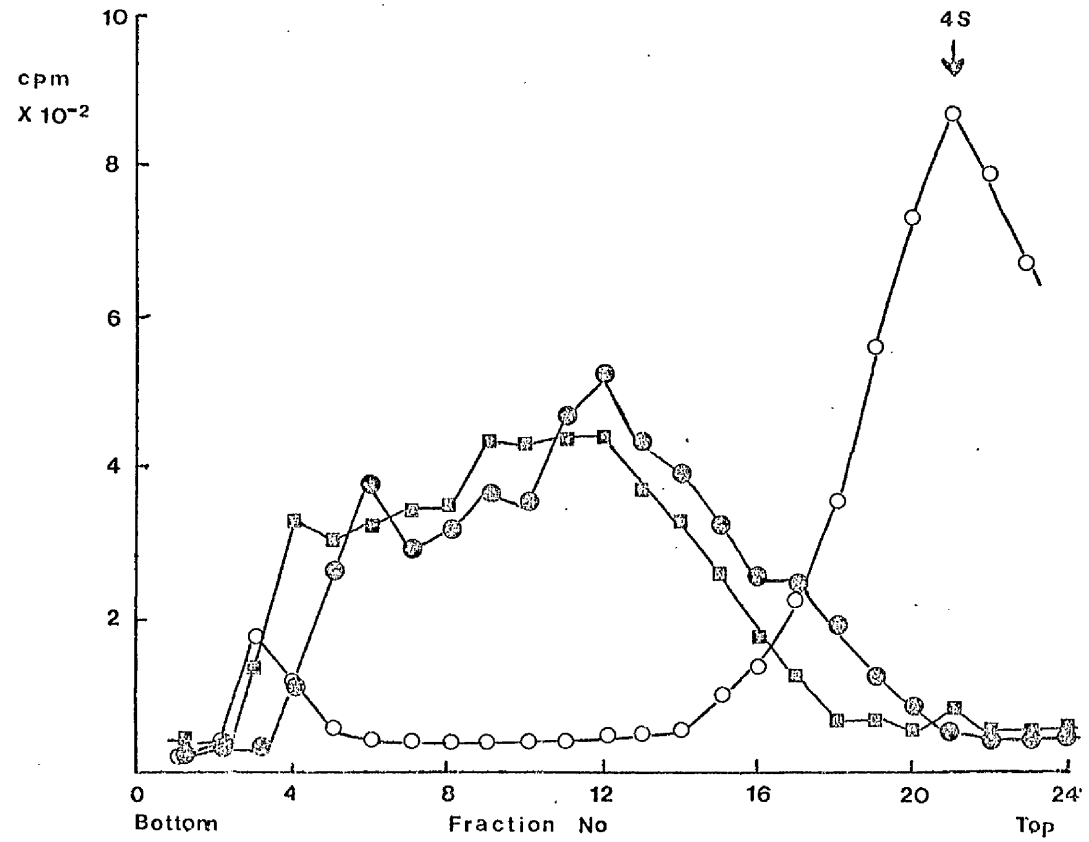
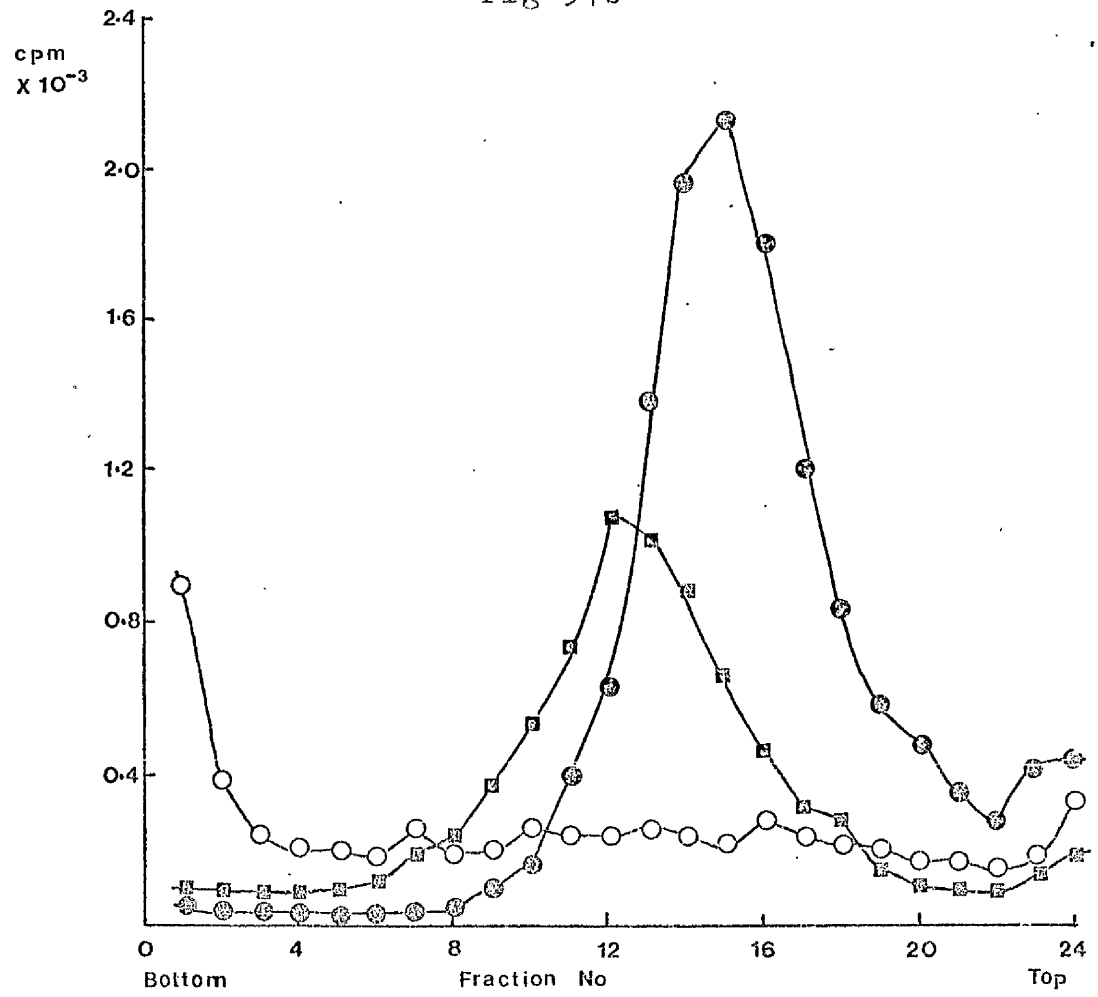


Fig 34b



and Aedes albopictus DNAs (Figs 35 and 36) even though these were extracted from cells grown to stationary phase.

Fig 35 BHK-21/C13 SAM-dependent DNase degradation of Xenopus laevis DNA from cells harvested at the stationary phase of growth

5-20% neutral sucrose gradients of 5µg ^{3}H-thymidine labelled Xenopus laevis DNA from cells harvested at the stationary phase of growth.

The DNA was incubated with 50µg BHK-21/C13 SAM-dependent nuclease (for details see Methods Section 4.4). The sucrose gradients were spun at 40,000 rpm in SW 50.1 rotor for 3 hr at 20°C.

●——● Xenopus DNA

■——■ Xenopus DNA incubated at 37°C with the BHK-21/C13 SAM-dependent nuclease for 3 hr.

o——o Xenopus DNA incubated at 37°C with 10mM SAM and the BHK-21/C13 SAM-dependent nuclease for 3 hr.

Fig 36 BHK-21/C13 SAM-dependent DNase degradation of Aedes albopictus DNA from cells harvested at the stationary phase of growth

Conditions used are identical to the one above

●——● 5µg Aedes DNA

■——■ 5µg Aedes DNA incubated at 37°C with the BHK-21/C13 SAM-dependent nuclease (50µg) for 3 hr.

o——o 5µg Aedes DNA incubated at 37°C with 10mM SAM and the BHK-21/C13 SAM-dependent nuclease (50µg) for 3 hr.

Fig 35

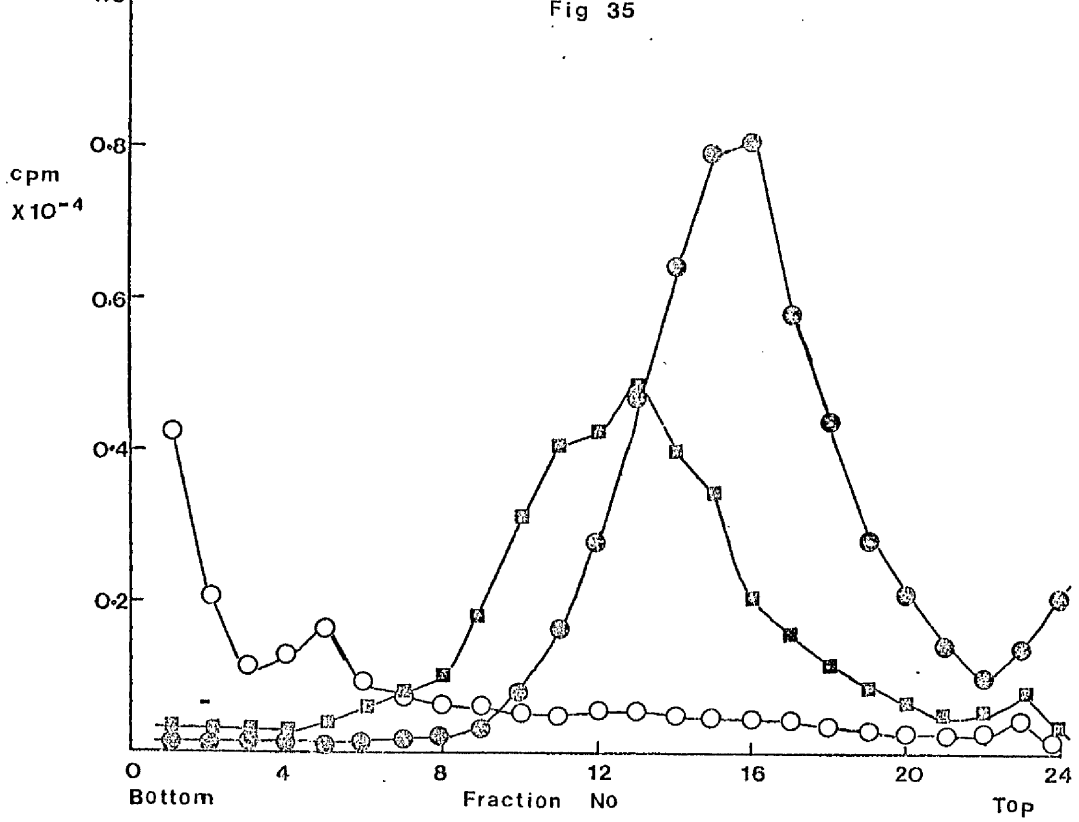
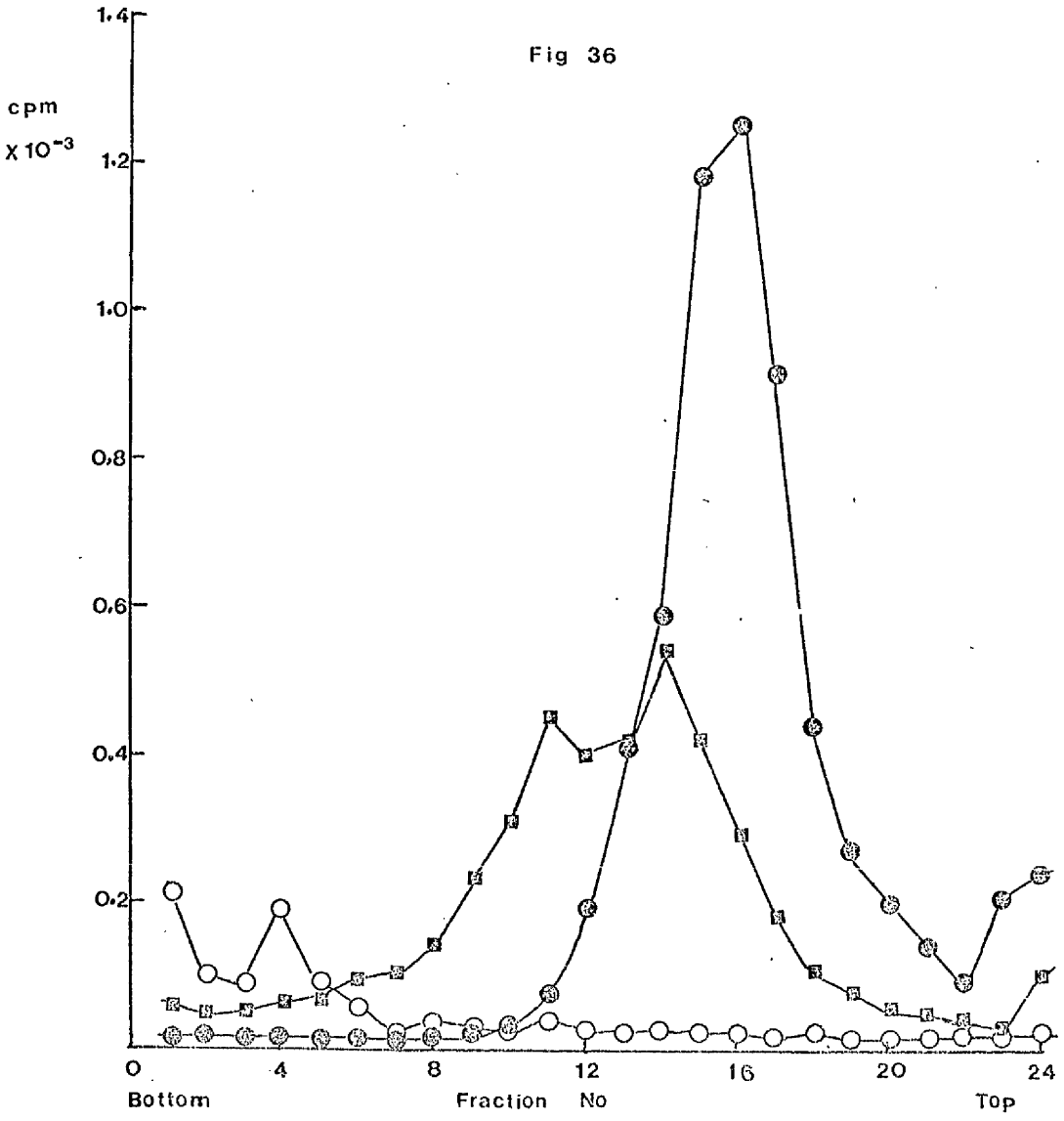

cpm
X 10⁻⁴
0·8
0·6
0·4
0·2
0
4
8
12
16
20
24
Bottom
Fraction No
Top

Fig 36


cpm
X 10⁻³
1·4
1·2
1·0
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0·6
0·4
0·2
0
4
8
12
16
20
24
Bottom
Fraction No
Top

DISCUSSION

1. THE LEVEL OF DNA METHYLATION IN BHK-21 CELLS

1.1 Is it due to a lag between DNA synthesis and methylation?

It has been shown in the Experimental Section that polyoma virus transformed BHK-21/PyY cell DNA has a higher level of 5-methyl cytosine residues than can be found in the DNA of the non-transformed BHK-21/C13 cells. Although this report had been made previously by a number of workers (Rubery and Newton, 1973; Nass, 1974; Browne and Burdon, 1977) no specific reason for this difference in DNA methylation has been given. This difference could simply be due to variation in the lag between DNA synthesis and methylation in the two cell lines. Studies on *in vivo* methylation of DNA in cultured cells lines have shown the presence of a distinct lag between synthesis of new DNA and its methylation (Burdon and Adams, 1969; Adams, 1971). This lag even varies in the S-phase of the cell cycle during which time the bulk of methylation is found (Burdon and Adams, 1969). DNA made early in the S-phase is more rapidly methylated than DNA made in late S-phase (Adams, 1971). The level of methylation may also vary during the different stages of growth of the cell. When mouse L929 DNAs isolated from cells

at early log or stationary phase of growth were incubated with Krebs II ascites DNA methylase and S-adenosyl-L-methionine for an extended length of time until no more methyl groups were incorporated into the DNAs, the DNA from the early log phase cells accepted 4 times as many methyl groups as the DNA from the stationary phase cells (Turnbull and Adams, 1976). Thus DNA from growing cells appears to be undermethylated when compared with DNA from stationary phase cells.

In mammals cytosine residues are preferentially methylated when in the doublet CpG (Doskočil and Šorm, 1962; Grippo et al, 1968). But not all CpG residues are methylated in vivo. In the study of the ribosomal DNA of Xenopus laevis blood cells using restriction enzymes, it has been shown that 1 out of every 100 CpG doublets is not methylated and in addition two regions, one in the coding region for the 28S RNA and the other in the non-transcribed spacer region, are not methylated (Bird and Southern, 1978). The biological significance of these non-methylated sites is as yet uncertain. In this study BHK-21/C13 cell DNA has been shown to have 0.5% of its cytosine residues methylated and the corresponding figure for the BHK-21/PyY cell DNA is 1.0% whereas nearest neighbour base frequency analysis indicate that 2.4% of all cytosine residues in the

BHK-21 cell genome occur in the CpG dinucleotide (Russell, 1978). This implies that many CpG dinucleotides in the DNA of these cells are in fact not methylated. The restriction enzyme Hpa II which recognises the sequence

$$5'\,C\overset{\downarrow}{-}C-G-G\,3'$$
$$3'\,G-G-C\underset{\uparrow}{-}C\,5'$$

and cleaves as shown above if the CpG doublets are not methylated, has been used to study the BHK-21 cell genome and the results reveal that non-methylated CpG doublets exist in the DNA (Browne et al, 1978). Differences in the level of DNA methylation in the BHK-21/Cl3 and BHK-21/PyY cell genomes may possibly arise as a result of variation in the frequency of these non-methylated CpG doublets.

1.2 Is it due to the presence of a virus-coded DNA methylase?

Polyoma virus DNA does not contain any methylated bases (Kaye and Winocour, 1967) and can code for limited number of proteins (Crawford and Black, 1964). When BHK-21/Cl3 fibroblasts are transformed to BHK-21/PyY cells they change their morphology, become less contact inhibited and produce tumour in susceptible animals (Stoker, 1962). These cells become more metabolically active and possess certain antigens not present in the host cell line (Marin and Macpherson, 1969). A

possibility is that the increase in genome modification observed in the transformed BHK-21/PyY cells is due to the presence of a new DNA methylase either coded by the virus genome or induced by the presence of the viral genome. Studies involving substrate DNA methylation with nuclear extracts of BHK-21/PyY and BHK-21/C13 cells show identical properties such as the Km for S-adenosyl-L-methionine, pH and temperature optima (see Experimental Section 3.3). Though most of the properties were identical there were slight differences in the sequence specificities of these two enzymes (Experimental Section 3.6). If these differences are due to an altered property of the DNA methylase in the BHK-21/PyY cell line arisen as a result of the polyoma virus transformation of these cells, then this enzyme should be able to raise the level of incorporation of methyl groups into the BHK-21/C13 DNA to that encountered in the BHK-21/PyY DNA. From Table 8, it is apparent that the level of additional methyl groups incorporated into the BHK-21/C13 DNA by the BHK-21/PyY DNA methylase falls very short of the value calculated from the difference in the in vivo level of DNA methylation in the two cell lines. From these data, it does not appear that a specific or novel DNA methylase is present in the BHK-21/PyY cell line which could explain the different 5-methyl cytosine level.

1.3 Is it due to alterationsin the organisation of the BHK-21 cell genome?

The differences in DNA methylation of the two BHK-21 cells on the other hand could be due to karyotypic alterations arising as a result of successive sub-culturing of these cells. It is a well known phenomenon that established cell lines commonly have tetraploid, polyploid or aneuploid karyotypes apparently due to non-disjunctive cell division which seems to confer selective advantage to them to grow in culture (Hayflick and Moorhead, 1961; Todaro and Green, 1963).

Alternatively the difference in the level of DNA methylation might have arisen as a result of reiteration of certain sequences in the BHK-21/PyY cell genome already containing 5-methyl cytosine residues. It has been shown in the Experimental Section that the highly repetitive region of the BHK-21/PyY cell DNA is twice as methylated as the corresponding region in the BHK-21/C13 DNA (Experimental Figs 23a and b).

The size of the haploid genome in eukaryotes is very much larger than could reasonably be expected if it consisted entirely of single copies of genes (King and Jukes, 1969; Ohno, 1971; Ohta and Kimura, 1971; O'Brien, 1973). In fact large DNA complements of eukaryotes consists of a diversity of sequences some of which are reiterated hundreds or thousands of times. Some genes particularly

those specifying the immunoglobulin light chain (Delovitch and Baglioni, 1973), histones (Kedes and Birnstiel, 1971; Weinberg et al, 1972) and ribosomal RNA (Birnstiel et al, 1969) are reiterated tens, hundreds or thousands of times. However fibrion genes (Suzuki et al, 1972) and globin genes (Bishop et al, 1972) do not appear to be reiterated. It is possible that some sequences containing 5-methyl cytosine residues serve a particular function and have been reiterated a few times, in the BHK-21/PyY cell line.

The mechanism of DNA reiteration is controversial. Whilst some workers believe the involvement of RNA transcripts and an RNA-dependent-DNA polymerase (reverse transcriptase) (Crippa and Tocchini-Valentini, 1971; Mahdavi and Crippa, 1972; Brown and Tocchini-Valentini, 1972). Others believe it is achieved by a conventional process of DNA replication (rolling circle mechanism) (Bird et al, 1973) or by an extraordinary DNA replication termed saltatory replication (Britten and Davidson, 1971). It has also been postulated that reiteration of DNA may arise as a result of random unequal cross-over between sister chromatids during meiosis or any of the many germ line mitoses (Smith, 1976). The role of reiteration of certain genes is equally uncertain. The selective reiteration of rDNA in the amphibian oocytes (Birnstiel et al, 1969) obviously prompted the idea that gene amplification might be the answer to the question

of differential gene activity as a means for the selective synthesis of specific proteins in the process of cytodifferentiation. However there is a significant redundancy of genes in the case of histones which in the sea urchin embryo are detected about 400 copies per haploid genome (Kedes and Birnstiel, 1971). It is therefore possible that the reiteration of sequences containing 5-methyl cytosine residues in the BHK-21/PyY DNA may be an example of such redundancy or possibly these sequences may be involved in as yet unknown function in the BHK-21/PyY cells.

2. MODE OF ACTION OF THE BHK-21 DNA METHYLASES

2.1 Effect of NaCl on DNA methylation

NaCl can affect the methylation of DNA in different ways such as:-

(a) the activity of the enzyme itself

(b) cause conformational changes in the DNA substrate

(c) cause both conformational changes in the DNA and affect the activity of the enzyme.

Since the methylation of double-stranded DNA by the BHK-21/C13 DNA methylase is inhibited by NaCl but the methylation of heat-denatured DNA is in fact stimulated at a concentration of 0.1M NaCl (Fig 18), it is likely that NaCl may not affect the DNA methylase *per se* but more likely affect the configuration of the DNA.

The configuration of single-stranded DNA is well known to be influenced by ionic strength. Unfolded single-stranded DNA molecules are able to form a compact ionic-strength-dependent structure with decreased contour length and high sedimentation coefficient (Studier, 1969; Bujard, 1970) but this conformational change is not due to the formation of hydrogen-bonded base-pairs (Bujard, 1970). Conversely salt exerts a stabilising influence on the helical configuration of DNA (Dove and Davidson, 1962). It seems likely that at 0.1M NaCl concentration single-stranded DNA may assume a structure which is a preferred configuration for methylation. Hence the increased level of DNA methylation of heat-denatured DNA observed with the BHK-21/C13 DNA methylase (Fig 18) in the presence of NaCl could account for the recognition of this structural change. It has been postulated that some type of transition from helical to single-stranded-like structure is a pre-requisite for the methylation of helical DNA (Drahovský and Morris, 1971b). NaCl by stabilising the helix, may prevent this transition from occurring and this might explain the inhibitory effect NaCl has on the methylation of "native" DNA by the BHK-21/C13 DNA methylase (Figs 16b and 18).

Methylation of "native" DNA by the BHK-21/PyY DNA methylase however appears to be stimulated by NaCl (Fig 16a). This difference in the methylation of "native" DNA by the BHK-21/C13 DNA methylase and BHK-21/PyY DNA methylase

may be due to the affinity of binding of the two enzymes to single-stranded regions in the "native" DNA. Drahovský and Morris 1971b showed with rat liver DNA methylase that when single-stranded and helical DNA are mixed with enzyme at 0°C before incubation at 37°C if the single-stranded DNA is in excess, all the available enzyme molecules bind tightly to it. When the temperature is raised to allow methylation to proceed, no significant quantity of enzyme remains free to bind to double-stranded DNA and consequently only single-stranded regions are methylated. From the effect increased DNA concentration has on methylation by BHK-21/C13 and BHK-21/PyY DNA methylases (Fig 13) it appears that the BHK-21/C13 enzyme has a higher affinity for substrate DNA than the BHK-21/PyY enzyme. It may therefore be that the addition of BHK-21/C13 DNA methylase to "native" DNA results in most of the enzyme molecules being bound to single-stranded regions in the DNA at 0°C and subsequent incubation at 37°C therefore leads to methylation of single-stranded DNA (In the DNA methylase assay, DNA, S-adenosyl-L-methionine and enzyme are mixed at 0°C before incubation at 37°C). This is comparable to the observation that S_1 nuclease digestion after methylation with the BHK-21/C13 DNA methylase showed virtually all the methyl groups in S_1 nuclease sensitive regions (Fig 17). Since the affinity of the BHK-21/PyY DNA methylase for substrate DNA appeared to be lower than the affinity of the BHK-21/C13 DNA methylase, it may be that not all the

enzyme molecules are tightly bound to single-stranded regions at 0°C. Hence incubation at 37°C results in the methylation of additional sites. Indeed S_1 nuclease digestion of the products of methylation by this enzyme shows that additional double-stranded DNA is methylated (Fig 17). The increased level of methylation shown by the BHK-21/PyY DNA methylase could be a result of the methylation of additional single-stranded regions in the "native" DNA that adopt the preferred methylation conformation in the presence of 0.1M NaCl. The increased methylation of denatured DNA and the decreased methylation of native DNA in the presence of NaCl had been reported by Turnbull and Adams, 1976 using calf thymus DNA and Krebs II ascites DNA methylase preparation. Whilst the above consideration may be true in explaining differences between the methylation of substrate DNA by the two BHK-21 DNA methylase preparations, it is equally likely that these differences may simply show varying effects NaCl has on these two DNA methylase preparations.

2.2 Methylation of single and double-stranded DNA

The methylation of *E. coli* DNA was investigated by Turnbull and Adams, 1976 using DNA methylase preparation from Krebs II ascites cells and they showed that virtually all the methyl groups were incorporated into regions of the substrate DNA sensitive to the action of the deoxyribonuclease *N. crassa*. This deoxyribonuclease is

specific for single-stranded DNA and they showed that the DNA they used contained 25% single-stranded regions based on the release of A_{260} material (Turnbull and Adams, 1976).

The methylation of E. coli DNA by the BHK-21 DNA methylases and analyses of the regions of DNA methylated by S_1 nuclease digestion showed that whilst the incorporation of methyl groups into E. coli DNA by both DNA methylases appeared in S_1 nuclease sensitive regions (i.e. single-stranded regions) an additional 20% of the methyl groups introduced by the BHK-21/PyY DNA methylase were located in S_1 nuclease insensitive regions (Fig 17). The additional methylation of double-stranded regions could be explained in terms of the binding of enzyme to DNA (see Discussion Section 2.1) or it could be that the BHK-21 DNA methylase preparations contain a protein contaminant which separates the DNA duplex before methylation and that the BHK-21/PyY enzyme methylates certain sequences in the E. coli DNA which reassociate during the S_1 nuclease reaction. In fact in vivo studies have shown that highly repetitive regions of the BHK-21/PyY cell DNA are twice as methylated as the corresponding regions of the BHK-21/C13 cell DNA (Figs 23a and b) and probably the DNA methylases in these cells may be methylating different sequences. But E. coli DNA does not appear to contain repetitive regions (Britten and Kohne, 1968) hence the BHK-21/PyY DNA methylase may

simply methylate certain sequences which reassociate faster than those methylated by the BHK-21/C13 enzyme.

This latter reasoning can not be used to explain the failure of the BHK-21/C13 enzyme to methylate native phage λ DNA though the BHK-21/PyY enzyme methylates this substrate. It appears therefore that whilst the BHK-21 DNA methylase preparations are by no means pure and protein contaminants may be present the idea of separation of the DNA duplex by a protein contaminant may probably not be enough in itself to explain the methylation of native and denatured DNA by the BHK-21 DNA methylase preparations.

2.3 Recognition sequence of DNA methylases

Drahovský and Morris, 1971a, b have proposed that DNA methylases may bind to DNA and move along the DNA helix scanning for methylation sites. Once a site is reached the enzyme undergoes a conformational change thus exposing the site for the transfer of methyl group from S-adenosyl-L-methionine and after the appropriate cytosine is methylated the enzyme returns to its previous state and continues the scan. The binding of DNA methylase to DNA substrate has not been observed by either Turnbull and Adams, 1976 using Krebs II ascites enzyme or by the experiments with the BHK-21 DNA methylases (Figs 16a and b).

In mammals cytosine residues are preferentially methylated

when in the doublet CpG (Doskočil and Šorm, 1962; Grippo et al, 1968). Simian virus 40 DNA has got 54 CpG dinucleotides amongst its 10,448 nucleotides (Fiers, 1978) and these are not methylated by either the Krebs II ascites DNA methylase preparation (Turnbull and Adams, 1976) or by the BHK-21 DNA methylases (results not in Experimental Section). It has therefore been suggested in this laboratory that DNA methylases may recognise a specific sequence in the DNA substrate before methylating a cytosine residue with a guanine at its 3'-end elsewhere in the DNA. Support for this idea comes from studies which show that the single-stranded polymer $(dC\text{-}dG)_{10}$ is not methylated by the BHK-21 DNA methylases whilst 3 cytosine residues are methylated per genome of the bacteriophage ØX174 after a prolonged incubation with the BHK-21/PyY DNA methylase (results not included in Experimental Section).

A number of enzymes such as the type I restriction enzymes (Murray et al, 1973; Horiuchi and Zinder, 1972) and a type II restriction enzyme R.Hga I (Sugisaki, 1978) act by recognising a sequence in the DNA but the enzyme's activity is manifested elsewhere along the DNA chain. This mode of action of restriction enzymes has been invoked to explain the action of RNA polymerase by Blattner et al, 1972. DNA methylase may also require recognition sequences and on the basis of the HS β-satellite from the Kangaroo rat Dipodomys ordii (Fry et al, 1973) it has

been suggested by Browne, 1976 that the recognition sequence may be A-C-A-C-(N_n)-$\overset{*}{C}$-G ($\overset{*}{C}$ - methylated cytosine). But computer analysis of the number of CpG doublets preceded by this sequence in the 5S DNA of Xenopus laevis oocytes and SV40 DNA can not be reconciled with either the in vivo or in vitro level of methylation of these DNAs (Burdon, R.H. 1978 private communication).

2.4 Biological significance of single-stranded DNA methylation

A number of biological processes involve the occurrence of single-stranded DNA in vivo. In DNA replication various experiments carried out are generally consistent with the idea of discontinuous synthesis of at least one strand of DNA (Smith et al, 1970; Okazaki et al, 1970; Geider and Hoffman-Berling, 1971; Strätling and Knippers, 1971 and Coming and Mattoccia, 1970). This generates single-stranded regions which are later joined together to give a continuous strand. But whilst lots of single-stranded DNA occur in vivo during replication, Adams, 1974 has shown that short chain "Okazaki pieces" synthesised during DNA replication are not methylated and this finding confirmed an earlier report that in the replication of Lilium DNA, methylation does not occur until after the completion of nascent DNA chains (Hotta and Hetch, 1971).

Single-stranded regions in DNA have been identified in different developmental stages of the sea urchin (Case et al, 1974) in ageing cells (Chetsanga et al, 1975) in regenerating rat liver DNA (Hoffman and Collins, 1976) and in the nucleolus of ascites tumour cells (Amalric et al, 1973). While it is likely that these single-stranded regions may have arisen as a result of the isolation procedure, the base composition, localisation and hybridisation studies (Amalric et al, 1973; Groner et al, 1975) suggest that there may be a class of single-stranded DNA which may constitute part of the cellular genome and may be involved in transcription (Gron et al, 1975). Whilst newly synthesised DNA is not methylated (Adams, 1974) it is possible that the BHK-21 DNA methylases which appear to prefer single-stranded DNA may methylate this class of single-stranded DNA if they carry the appropriate recognition sites and possibly protect them against nuclease activity.

3. ROLE FOR DNA METHYLATION

3.1 Role of DNA methylation in cellular differentiation

The level of 5-methyl cytosine residues in DNA is known to show tissue specific variation. For example in various organs of developing chick embryo the level of 5-methyl cytosine ranged from 3.6 ± 0.04 mole % in the

chorioallantoic membrane to 4.19±0.02 mole % in the brain (Kappler, 1971). It has also been shown that DNA methylation is highest in the brain and liver DNA while the DNAs from kidney, spleen and muscle of the chick have low 5-methyl cytosine content (Vanyushin et al, 1970; 1973). Sperm DNA contains a lower 5-methyl cytosine content than somatic cell DNA (Vanyushin et al, 1970; Turnbull and Adams, 1976). These variations may reflect a role of DNA methylation in cellular differentiation or alternatively such differences might only reflect a variable delay between DNA synthesis and its methylation.

Bromodeoxyuridine substitution into DNA is known to affect the expression of some genes of differentiated cells (Bischoff and Hotlzer, 1970; Weintraub et al, 1972; Walther et al, 1974; Lashner and Cahn, 1969; Coleman et al, 1970; Holthavsen et al, 1969; Silagi and Bruce, 1970; Stellwagen and Tomkins, 1971a, b) and it had been postulated that this effect may take place by changing the pattern of DNA methylation (Singer et al, 1977). However this seems unlikely in view of the results in Figs 24a and b which show that in vitro incorporation of methyl groups into DNA is not affected by prior substitution of bromouracil residues into the DNA. This finding agrees with the results of Singer et al, 1977 which show that the level of in vivo DNA methylation was unaffected even when the level of

BrdUrd substitution into newly made rat hepatoma DNA was 95%. Thus it seems the effect BrdUrd substitution has on the expression of specific genes does not alter the pattern of DNA methylation. It does not appear that DNA methylation is required for the transcription of certain genes. The amplified extra-chromosomal DNA coding for the ribosomal DNA in Xenopus oocytes which is actively transcribed is not methylated whereas the corresponding somatic rDNA is highly methylated (Dawid et al, 1970). Also the 5S DNA from Xenopus laevis oocytes methylated at almost every CpG doublet (Miller et al, 1978) was transcribed faithfully when injected into the nucleus of Xenopus oocytes (Brown and Gurdon, 1977) and the cloned 5S DNA which is not methylated when treated similarly was also transcribed with high-fidelity (Brown and Gurdon, private communication). Extensive methylation of the injected DNA seems improbable since it has been observed that oocytes have a low level of DNA methylase activity (Burdon, R.H. private communication). It appears therefore that methylation of DNA is not required for transcription.

However other workers have reported that DNA methylation may play a role in the regulation of gene expression. Volpe and Eremenko, 1974 have shown that labelled DNA from HeLa cells hybridised to hnRNA and the hybrid had an appreciable number of 5-methyl cytosine residues but hybrids formed between polysomal messenger RNA

and DNA had no significant 5-methyl cytosine residues. They therefore concluded that the highly methylated region was near the promotor 5' end of the DNA transcriptional unit and may correspondto the region of regulatory genes. Christman et al, 1977 had reported that when Friend erythroleukemia cells were induced to differentiate with dimethyl sulphoxide (DMSO) or ethionine the DNA from these cells were found to be hypomethylated (i.e. they served as excellent acceptors of methyl groups in in vitro methylation than the DNA from untreated cells). This provides circumstantial evidence that DNA methylation may play a role in the regulation of gene expression.

3.2 Cleavage-modification system

3.2.1 Selective cleavage of DNA

Most bacterial cells contain on the one hand restriction enzymes which recognise specific sets of nucleotides within DNA and make a double-stranded cleavage if these sites are unmethylated and on the other hand methylases which recognise the same sets of nucleotide sequence and methylate them thus preventing the action of the restriction enzymes (Arber and Dussoix, 1962; Dussoix and Arber, 1962; Arber, 1974). While it is debatable whether such enzymes occur in eukaryotic cells a number of phenomena occurring in eukaryotic organisms may require cleavage-modification enzymes.

For example the selective uniparental (maternal) inheritance of chloroplasts DNA may require an endonucleolytic enzyme that cleaves the paternal DNA but inactive towards the possibly modified maternal DNA (Sager, 1972; Sager and Kitchin, 1975). The selective loss of human chromosomes in human-mouse cell fusion experiments (Weiss and Green, 1967; Pontecorvo, 1971; Ruddle, 1972) may involve a modification enzyme which recognises specific sites on the mouse DNA and modifies them whilst another enzyme cleaves the unmodified human chromosomes (Sager and Kitchin, 1975). Cleavage enzymes might be expected to occur in Tetrahymena for the excision of ribosomal DNA (Gall, 1974) in Stylonichia (Prescett and Murti, 1973) and the hypotrichous ciliates (Wesley, 1975) to account for the drastic change from micronucleus to macronucleus.

In the Experimental Section 5.2, nuclear extracts of BHK-21/C13 cells have been shown to possess a DNase activity which degrades substrate DNA but inactive towards DNA protected by prior methylation by the BHK-21 cell DNA methylase. This enzyme requires S-adenosyl-L-methionine as cofactor but does not require Mg^{2+} or ATP. Apart from BHK-21/C13 nuclei, this enzyme is also found in macronuclei of Tetrahymena pyriformis G.L (Villadsen and Burdon, 1978) and Xenopus laevis oocytes (Stage 10) (Burdon, R.H., 1978 private communication). Unlike bacterial restriction enzymes which do not cleave DNA

methylated on only one strand at the recognition site (Meselson and Yuan, 1968; Vovis et al, 1972) the DNase from nuclear preparation of BHK-21/C13 cells degrades such DNAs.

The mode of action of the SAM-dependent nuclease is as yet unknown. Simian virus 40 DNA is not methylated by mammalian DNA methylases and this DNA is not degraded by the SAM-dependent nuclease. The single-stranded bacteriophage ØX174 DNA has got only one methylated base in its 5,375 nucleotide genome (Razin et al, 1975; Friedman and Razin, 1976) and after 76 hr of incubation with the BHK-21/PyY DNA methylase only 3 more cytosine residues are methylated. It is perhaps conceivable, because of its limited sites for methylation, that this DNA has no sites for the SAM-dependent nuclease. However heat-denatured L929 DNA isolated from cells at the mid-log phase of growth is degraded by this nuclease (Fig 29). Mouse L929 and BHK-21/C13 DNAs from cells harvested at the mid-log phase of growth or from cells grown in a medium deprived of methionine for one generation are degraded (Figs 33a, b and Figs 34a, b). As has already been discussed (Discussion Section 1.1) DNA from growing cells may be less methylated than DNA from cells at the stationary phase of growth. It therefore appears that the availability of unmethylated cytosine residues may be an important pre-requisite for the nuclease activity since DNA from stationary phase cells

(Mouse L929 or BHK-21/C13 cells) are not degraded (Figs 32a, b). Like restriction enzymes, the SAM-dependent nuclease does not appear to degrade DNA previously methylated by the cell's DNA methylase and probably other DNA methylases with similar sequence specificity to the BHK-21 DNA methylase may be able to protect DNA from degradation by the BHK-21/C13 SAM-dependent nuclease. But DNA from cells whose DNA methylase has a different sequence specificity may be degraded, for example Aedes and Xenopus DNAs though harvested from stationary phase of growth are degraded (Figs 35 and 36). The BHK-21/C13 SAM-dependent nuclease is inhibited by S-adenosyl-L-homocysteine, a potent inhibitor of DNA methylase and the corresponding enzyme from Tetrahymena is additionally inhibited by 5-methyl cytosine and 6-methyl adenine compounds, the products of DNA methylation while cytosine stimulates its activity (Villadsen and Burdon, 1976). It therefore appears that S-adenosyl-L-methionine may not just act as a cofactor but may be consumed in the degradation reaction.

3.2.2 The role of SAM in restriction

It is not yet clear the role S-adenosyl-L-methionine plays in the bacterial restriction reaction involving the EcoB, K and P systems. One possibility is that it may be a source of free energy or may just play a steric

role perhaps as an allosteric effector of the restriction enzymes (Meselson et al, 1972). It is however known that the Eco RI and Eco RII and Haemophilus endonucleases have no requirement for the addition of S-adenosyl-L-methionine while for the action of the Eco P restriction enzyme it is helpful but not essential (Meselson and Yuan, 1968). None of these observations lead to firm conclusions regarding the role of SAM in restriction but it seems simpler to reconcile them with a steric role than with direct chemical participation in the endonucleolytic mechanism itself.

The intracellular concentration of SAM in BHK-21 cells has not yet been determined but this concentration is given as 50μM in rat liver cells and it seems this is rigorously maintained in these cells (Eloranta, 1977). In most of the in vitro assays of the SAM-dependent nuclease, concentrations of SAM used were between 2mM to 10mM, though 55μM could bring about similar effect over an extended time of incubation. It is difficult to show unequivocally the role of SAM in this nuclease activity. It does however appear that SAM is not used to methylate the substrate DNA. Preliminary studies have shown that the SAM-dependent nuclease could be isolated from DNA methylase activity of BHK-21/C13 cells by DEAE-cellulose Chromatography. Whilst the SAM-dependent nuclease appears in the flow-through material the DNA methylase is weakly bound to DEAE-cellulose and it is eluted with low salt.

3.2.3 Nature of fragments generated by restriction enzymes

The first restriction enzymes Eco B and Eco K were detected by their ability to degrade unmodified DNA as compared to modified DNA which was resistant to their action. Degradation was detected by testing biological activity (Linn and Arber, 1968) or by measuring changes in sedimentation velocity (Meselson and Yuan, 1968). Both procedures are time consuming but simpler assay procedures take advantage of the endonucleolytic nature of restriction enzymes and measure changes in viscosity as a result of degradation (Smith and Wilcox, 1970; Middleton et al, 1972). Alternative procedures based on filter binding of the lac repressor-lac operator complex (Fanning et al, 1976) or the binding of circular phage λ DNA to nitrocellulose filters have been described (Reiser and Yuan, 1975). Assays of this sort are the ones currently available for the class I-restriction enzymes because of the absence of specific degradation products.

The fragments generated by class II restriction enzymes are discrete and these may be fractionated by agarose gel electrophoresis and stained with ethidium bromide and visualised by their fluorescence upon U.V. irradiation of the gel (Aaiji and Borst, 1972; Sharp et al, 1973)

It can be seen from the agarose gels in Fig 25 of phage λ DNA digested by the BHK-21 SAM-dependent nuclease that

no bands are visible and that the absence of low molecular weight peaks in the sucrose gradient profiles of Aedes and Xenopus DNA (Figs 35 and 36) digested by the SAM-dependent nuclease for 3 hr points to the possibility of this enzyme producing acid soluble fragments or probably other nucleases may be present and causing further degradation of the DNA. The latter possibility may be the case as the gradient profiles show that incubation of DNA with enzyme alone leads to the loss of radioactive label in the DNA. Though mouse L929 DNA was degraded to fragments of about 4S after incubation for 3 hr, no further experiments were carried out to show whether this represented a limit digest. It may therefore be said that this "4S DNA" is further degraded to give acid soluble material or alternatively it could be that this enzyme may recognise sequences in mouse L929 DNA which simply occur less frequently than they do in BHK-21/C13 DNA or phage λ DNA. Since methylation of DNA by the BHK-21 cell DNA methylase protects it from degradation by the BHK-21/C13 SAM-dependent nuclease and since in DNA methylation most of the methyl groups are preferentially in CpG doublets (Doskočil and Šorm, 1962; Grippo et al, 1960) one assumption is that this nuclease may recognise and cleave unmodified CpG doublets.

Assuming then that the BHK-21/C13 SAM-dependent nuclease recognises the sequence NpCpGpN, this tetranucleotide should occur once every 256 base pairs. However taking into account the low CpG dinucleotide frequency of

vertebrate DNA (Swartz et al, 1962; Russell et al, 1976) and the 40% C+G base content of for example Xenopus laevis DNA (Dawid, 1965), this tetranucleotide should occur once every 256 x 4 x 1.25 base pairs long. If these sites are randomly distributed then after digestion of the Xenopus DNA with the SAM-dependent nuclease, fragments of at least 8×10^5 daltons should have been released or even longer fragments if this sequence is non-randomly distributed and taking into account that some of the CpG doublets are already modified in vivo. Of all the DNAs incubated with the SAM-dependent nuclease only mouse L929 DNA gave fragments of 4S as determined by velocity centrifugation. As has already been stated this may not be a limit digest and possibly further incubation would have led to the release of acid soluble material. It could also be that the nuclease recognises certain sequences in mouse DNA which occur more frequently in the other DNAs used in the assay. Taken together, it does not however appear that unmodified CpG doublets are the sequences recognised and cleaved by the SAM-dependent nuclease. A number of alternative explanations could be given to explain the action of the enzyme but so long as the SAM-dependent nuclease remained unpurified these might fall short of its actual mode of action in the face of other contaminating nucleases present in the nuclear extract.

3.2.4 The biological significance of cleavage-modification systems

Despite evidence (Bron *et al*, 1975) suggesting the notion that bacterial restriction enzymes operate *in vivo* as part of a restriction-modification system, the possibility exists that they may have some other function, for instance they may function in site-specific recombination (Roberts, 1976). Recently the Eco RI restriction enzyme has been shown to be involved in site-specific recombination (Chang and Cohen, 1977) and a site-specific single-stranded endonuclease from the eukaryote *Chlamydomonas* may be involved in recombination to explain the non-reciprocal recombination events that occur in the vegetative cells (Burton *et al*, 1977). The BHK-21/C13 SAM-dependent nuclease degrades its own DNA only when under-methylated. This may reflect the recognition of specific sites and if this is the case it is likely that this enzyme may be involved in site-specific recombination rather than restriction-modification mechanism. As already discussed the BHK-21/C13 SAM-dependent nuclease differs from bacterial restriction enzymes in its substrate specificity and cofactor requirements. Furthermore a number of experiments designed to show the occurrence of restriction phenomenon in eukaryotic cells have so far shown no such mechanism. For example when portions of the λ bacteriophage DNA were inserted into SV40 genome from which portions of the late genes have been removed and then propagated in eukaryotic

cells with the aid of helper viruses, it was observed that the λ segments retained their accustomed cleavage sites for restriction enzymes (Nussbaum et al, 1976).

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