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STUDIES ON DNA METHYLASE
FROM ASCITES CELLS

by

JOHN F. TURNBULL, B.Sc.

Thesis presented for the degree of
Doctor of Philosophy

University of Glasgow,
October, 1976.
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I thank Professors R.M.S. Smellie and A.R. Williamson for making available the facilities for this research, and I acknowledge receipt of a Research Studentship from the Medical Research Council.

I am particularly indebted to Dr. Roger Adams for his unfailing interest and constructive advice throughout the course of this work.

I must also thank various members of the Department of Biochemistry for helpful discussions and Miss J. Gillies for typing this thesis.
ABBREVIATIONS

These are as laid down in the Biochemical Journal Instructions to Authors (revised, 1976) with the following additions:

<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>NEM</td>
<td>N-ethyl maleimide</td>
</tr>
<tr>
<td>PAS</td>
<td>p-aminosalicylate (sodium salt)</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>SAM</td>
<td>S-adenosyl-L-methionine</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
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SUMMARY

The DNA of higher eukaryotes contains small quantities of the minor base 5-methylcytosine, formed by enzymatic transfer of methyl groups from S-adenosyl-L-methionine (SAM) to cytosine residues on polymeric DNA. The function of this methylated base is uncertain. In order to examine the mechanism and specificity of methylation, DNA methylase has been purified some 400-fold from Krebs II ascites tumour cells. This enzyme preparation has the following properties:

(1) There is no evidence for more than one enzyme species.
(2) The only product of methylation by the enzyme is 5-methylcytosine in DNA. Methylation is absolutely dependent on added substrate DNA.
(3) The enzyme methylates DNA from a variety of animal, cultured cell and bacterial sources, but at widely differing rates. Higher rates of in vitro methylation are generally observed with DNAs from rapidly dividing cells.
(4) Several experiments on the mechanism of action of the enzyme indicate that it operates by a series of repeated transient binding events at different sites in the DNA, rather than by an initial binding at a specific site followed by a linear traverse of the DNA helix.
(5) The enzyme can methylate both native and denatured calf thymus DNA; in *E. coli* DNA, however, only single-stranded regions are methylated even in a "native" DNA preparation. This could be caused by the enzyme requiring hemimethylated sites in double-stranded DNA, which are presumably absent from *E. coli* DNA.

(6) Methylation of denatured DNA by the enzyme shows a strikingly greater resistance to inhibition by salt than does methylation of native DNA. The explanation suggested for this is that native and denatured DNA are methylated by different forms of the enzyme, perhaps by a dimer and a monomer respectively. This is based on the results of gel filtration and density gradient centrifugation, which show that under high salt conditions the enzyme exists as a smaller species than at low salt. Although not precisely determined, the approximate molecular weights of these species are consistent with the larger being a dimer of the smaller, which has a molecular weight of about 160,000.
INTRODUCTION
1. THE OCCURRENCE OF METHYLATED BASES IN DNA

1.1. Historical Background

To people accustomed to the present hectic pace of biological research, it may seem incredible that more than 80 years elapsed between the first discovery of nucleic acids by Friedrich Miescher in 1868 and the elucidation by Watson and Crick of the double helical structure of DNA, which ushered in the new science of molecular biology. This long delay was not due to any lack of ability among the many investigators in the field, but rather to the inadequacy of classical chemical methods when confronted with such huge and complex structures as nucleic acids.

It is true, however, that traditional techniques of organic chemistry enabled most of the individual constituents of nucleic acids to be identified fairly rapidly; thus the purine bases were isolated from nucleic acids between 1879 and 1881, the pyrimidines over the period 1884 and 1909, and the sugar ribose in 1909 (from yeast). Because ribose (and uracil) were for a long time found only in yeast and plants, and 2-deoxyribose and thymine only in animal nucleic acids, it was erroneously believed for many years that RNA was the only nucleic acid of plants, and DNA the only nucleic acid of animal cells - a belief that was not finally dispelled until the 1940s, when advances in spectrophotometry, cytochemistry and histochemistry demonstrated the
presence of both RNA and DNA in a variety of cell types, and allowed some insight into their function.

Long before this error was realised, Johnson and Coghill (1925) had reported the occurrence of the modified base 5-methylcytosine as a constituent of the nucleic acid of tubercle bacilli. This finding seems to have attracted little interest at the time, and no further report of this unusual base appeared until the time of the great advances in nucleic acid analysis from 1948 onwards.

1.2. Identification of methylated bases in DNA

As is well known, the discovery by Chargaff and his colleagues of the molar equivalence of purines and pyrimidines in DNA (the so-called "Chargaff's rules") was crucial in giving support to the idea of the double helical structure of DNA, with A-T and G-C base pairing by hydrogen bonding. In the course of similar investigations of the base composition of DNA, Wyatt (1950, 1951) positively identified 5-methylcytosine as a minor component of DNA from mammalian, fish, plant and insect sources, though not of bacterial DNA. The proportion of 5-methylcytosine found was characteristic of the species studied, and varied from 0.008 to 0.075 mol/4 mol of nucleotide, with wheat germ DNA having a much higher proportion (0.23 mol/4 mol nucleotide).
The first methylated base to be found in a bacterial DNA was $6\text{-methylaminopurine}$ ($N^6\text{-methyl-adenine}$), identified by Dunn & Smith (1958) in *E. coli* 15 T$^-$. Since then, several groups of workers have established the presence of methylated bases in DNA of both prokaryotic and eukaryotic organisms (Doskocil & Sormova, 1965; Vanyushin et al, 1968 and 1970; Culp, Dore & Brown, 1970; Fujimoto et al, 1965). A consistent pattern has emerged from this work in that 5-methylcytosine is the only methylated base detected in animal cell DNA, while prokaryotes contain both 5-methylcytosine and $N^6\text{-methyladenine}$. One apparent exception to this rule has been reported by Cummings et al (1974), who found that DNA from the ciliate protozoan *Paramecium Aurelia* contains only $N^6\text{-methyladenine}$. No other methylated bases have been found in DNA, in contrast to the situation in transfer RNA, where a great diversity of methylated bases has been reliably identified.

1.3. **Species and Tissue Specificity of DNA Methylation**

As mentioned earlier, Wyatt in 1951 had found that the level of 5-methylcytosine in DNA depended on the species from which it was isolated. This was confirmed by Vanyushin et al (1970 and 1973) and Kappler (1971), who extended the study to show that methylation levels in DNA also varied between different tissues of the same species. The variation between different animal DNAs found by Vanyushin's group was about
fourfold (ranging from 0.5 to 2 mole per cent 5-methylcytosine), while Kappler detected differences of up to 50% between different mouse tissues.

Other interesting findings of Vanyushin's group were that the 5-methylcytosine content of salmon tissue DNA was reduced at the time of spawning, without any other noticeable change in the DNA, and also that sperm DNA generally seems to contain less 5-methylcytosine than somatic cell DNA of the same animal.

The existence of this type of tissue specificity of DNA methylation has led to suggestions that it might be involved in control of differentiation or transcription, an idea which will be discussed more fully in Section 5.

1.4. Methylation of mitochondrial DNA

The mitochondria of eukaryotic cells contain their own DNA, which has been well characterised. The first detailed study on whether mitochondrial DNA is methylated was published by Nass in 1973. Earlier investigators had reported a low level of DNA methylase activity in mitochondrial extracts of rat liver (Sheid et al, 1968), and in mitochondria of Physarum polycephalum (Evans & Evans, 1970). In various cultured cell lines, Nass found that mitochondrial DNA was methylated, but to a much smaller extent than nuclear DNA from the same cells. For example, in L cells one in
every 36 cytosine residues in nuclear DNA was found to be methylated as against one in 500 cytosines in mitochondrial DNA. This report also included evidence for the presence of a DNA methylase activity in mitochondria which did not appear to be the result of contamination with the cellular enzyme since it had different properties from the latter.
2. ENZYMATIC METHYLATION OF DNA IN BACTERIA

The discovery of methylated bases in DNA naturally raised two basic questions - how do they originate and what is their function? The first question has been quite convincingly answered, while the second has proved more difficult.

2.1. Evidence of enzymatic methylation of DNA at the polymer level

It seemed reasonable to suppose that DNA was methylated at the level of the preformed polymer, rather than by incorporation of methylated bases into DNA during synthesis, because other modification processes, namely the glucosylation of bacteriophage DNA and methylation of transfer RNA, were both known to occur at polymer level. (Kornberg et al, 1958; Fleissner & Borek, 1962). In 1963, Gold, Hurwitz & Anders demonstrated the presence of an enzyme activity in E.coli strain W which could catalyse the transfer of methyl groups from S-adenosyl methionine (SAM) to native acceptor DNA. This enzyme was subsequently purified 400-fold (Gold & Hurwitz, 1964) and shown to have 5-methylcytosine and 6-methyladenine in DNA as its only products. DNA methylase activities in bacteria were also reported by Fujimoto et al (1965) and by Oda & Marmur (1966), who purified an enzyme from B.subtilis which catalysed methylation of cytosine residues only, and could use both
native and denatured DNA as substrate, unlike the enzyme of Gold et al which required native DNA.

2.2. **Substrate specificity of bacterial DNA methylases**

A feature common to all the enzyme preparations described above was their inability to methylate normal DNA from the same organism as the enzyme; either heterologous DNA, or homologous DNA made deliberately methyl-deficient, was required. This implies that only a limited number of sites are available in the DNA to accept methyl groups and that these sites are already filled in the DNA extracted from normal cells. The fact that the enzymes are able to methylate heterologous DNA could indicate either that the enzymes from different species recognise different types of methylation sites, or that some other control mechanism specific to each species determines the number of the available sites which are methylated *in vivo*.

2.3. **Increased DNA methylation in response to bacteriophage infection**

Various reports have appeared of substantial increases in host cell DNA methylation after infection by bacteriophage. Gold et al (1964) detected a 100-fold increase in DNA methylase activity in *E.coli* within 6 min. of infection with bacteriophage T2. A rise in both the rate and extent of host cell DNA methylation, as well as in DNA methylase
activity, was observed by Fujimoto et al (1965), again in T2-infected *E. coli* cells. The significance of such a great change in host cell DNA modification after viral infection is not clear.

2.4. The restriction-modification system in bacteria

The only function which has so far been unequivocally assigned to methylated bases in DNA is in the restriction-modification systems which protect bacteria from invasion by foreign DNA, (usually viral) and which are vital tools in much of the current research involving recombinant DNA molecules. It is possible here to give only a very brief outline of the properties of such systems; excellent reviews have been published by Meselson et al (1972) and by Arber (1974).

Restriction endonucleases cleave double-stranded DNA molecules at specific sites, producing a number of fragments. It is thought that the enzymes recognise a particular sequence of base pairs on the substrate DNA. Restriction endonucleases are believed to occur in many bacterial strains as the products of genes carried either on the bacterial chromosomes or on plasmids. Bacteria do not destroy their own DNA because it is protected from cleavage by site-specific methylation of the DNA, for which another enzyme, the DNA modification methylase, is responsible. Both the endonuclease and the methylase are believed to recognise the same sequences on substrate DNA and each restriction/
modification system recognises a unique sequence, so that modification of the DNA by a particular methylase only protects it against the corresponding endonuclease. Also, both processes occur on the same DNA, so that its final fate is the outcome of a "race" between restriction and modification.

The product of methylation can be either N⁶-methyladenine or 5-methylcytosine, depending on the methylase. The recognition sites are frequently palindromic sequences such as the one shown below, for the _E.coli_ R1 system:

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The asterisks indicate sites of methylation, while the arrows show where endonuclease cleavage occurs if the site is unmethylated. (Data from Dugaiczyk et al, 1974). Because a hemimethylated site (i.e. one methylated in only one strand) is fully protected against cleavage and because methylation of such sites is rapid, the DNA is not degraded during replication.

A number of restriction and modification enzymes have been isolated and purified. One interesting example is the restriction endonuclease purified by Smith & Wilcox (1970) from _H.influenzae_ and the corresponding series of four DNA
methylases described by Roy & Smith (1973a), at least two of which were associated with different restriction-modification systems.

It should be noted that, because the sites for restriction and modification are quite rare within the bacterial chromosome, many of the methyl groups on the bacterial DNA are not in fact involved in such sites, leaving open the question of what function is served by all these other methylated bases. It has been shown (Marinus & Morris, 1973) that it is possible to obtain mutants of *E. coli* K-12 which are deficient in either 5-methylcytosine or N⁶-methyladenine and that none of these mutations is lethal, suggesting that methylation may have some useful, but not indispensable, function. These authors did find, however, that the mutants deficient in N⁶-methyladenine restricted bacteriophage DNA less well than the wild type.

This interpretation may, however, be over-simplified, because *E. coli* K-12 possesses a Class 1 restriction-modification system, which consists of a multimeric enzyme system with different subunits responsible for methylation, restriction and recognition. Inactivation of any one of these functions automatically leads to inactivation of the others, so that a mutation inactivating the methylase in such a system would not be lethal, and it is possible that Marinus & Morris have found such a mutant. Because their mutants appeared to modify DNA from phage lambda normally, the authors argue that the restriction-modification system must be fully
functional, but the possibility cannot be excluded that the
bacteria have more than one restriction-modification system
and that the mutation resides in one but not all of these
systems. It would be interesting to try to repeat these
experiments in a strain where the modification and restriction
components are coded for independently, to see if methyl
deficiency was still invariably non-lethal.
3. ENZYMES OF DNA METHYLATION IN EUKARYOTES

3.1. Work with crude nuclear extracts

While it seemed reasonable to suppose that 5-methylcytosine in mammalian DNA would be acquired by enzymatic methylation of the preformed polymer by the transfer of methyl groups from SAM in the same way as bacterial DNA (Section 2), attempts to extract such enzymes from eukaryotic cells proved fruitless for some time. It was not until 1967 that a mammalian DNA methylase activity was reported (Burdon et al., 1967). These workers found that, in mouse ascites tumour cells, DNA methylase activity was firmly bound to the chromatin fraction of the cell nucleus, unlike the tRNA methylase activity which was present in the cytoplasm. Similarly, when Sheid et al. (1968) reported the extraction of a DNA methylase from various rat tissues, the enzyme activity remained associated with a high-speed nuclear pellet after extraction of nuclei with 0.15M NaCl. This association with insoluble nuclear material probably explains why eukaryotic DNA methylases were so much harder to find than the bacterial enzymes, which can be readily solubilised from the cells. Like the bacterial enzymes, the methylase extracted by Sheid et al. from rat liver was unable to methylate homologous DNA, but it did methylate several bacterial DNAs, as well as calf thymus DNA, to some extent.

The next animal cell DNA methylase to be found was extracted by Kalousek & Morris (1968) from rat spleen nuclei. They
examined both high speed nuclear pellets as used by Sheid et al and chromatin as used by Burdon et al. The most striking property of this new enzyme was its ability to methylate its own DNA (cf bacterial enzymes, and Sheid et al, above), which might suggest that some DNA in these cells is incompletely methylated in vivo. These authors also demonstrated rigorously that the product of methylation was the deoxyribonucleoside of 5-methylcytosine in DNA, a necessary step in establishing the genuineness of a putative DNA methylase, and they demonstrated the importance of eliminating RNA and protein methylation from the assay. They found that only 13% of the radioactive methyl label incorporated into acid-insoluble material by their enzyme was resistant to both alkaline hydrolysis (which digests RNA) and deproteinisation; presumably RNA and protein methylase activities are present in the crude preparation. Assays for DNA methylase should therefore incorporate both an alkaline (or ribonuclease) digestion and a deproteinisation step.

5-methylcytosine is present in plant DNA at a much higher level than in animal DNAs (Wyatt, 1951; Shapiro, 1968), and it was from a plant source that Kalousek & Morris (1969a) extracted another DNA methylase activity. This enzyme (from pea seedlings) was characterised by showing that its only product was 5-methylcytosine; it was bound to insoluble nuclear material and, like the rat spleen enzyme, could methylate homologous plant DNA.
A rather unusual DNA methylase activity has been described by Vanyushin et al (1971), in the loach embryo. During the early stages of embryogenesis, the bulk of cellular DNA is cytoplasmic rather than nuclear. Vanyushin's group found that in loach embryo the greater part of the DNA methylase activity also was located in the cytoplasmic fraction of the cells. This enzyme, like those from rat spleen and pea seedlings, could methylate endogenous DNA as well as DNA from several different sources.

3.2. **Purification of eukaryotic DNA methylases**

Crude preparations of the type described above are of no great use for quantitative work on the action of the enzyme *in vitro* (e.g. on its reaction with different substrate DNAs), because they contain varying enzyme levels and unknown amounts of SAM-cleaving activity, as well as (in some cases) endogenous DNA. Thus it would be hard to say to what extent experimental results were produced by these unknown factors. This is the main reason for trying to obtain a more highly purified preparation of DNA methylase.

Kalousek & Morris (1969b) achieved a 195-fold purification (over crude extract) of the rat spleen enzyme in a soluble form, by carrying out an ammonium sulphate precipitation of the chromatin fraction (after first removing nucleic acids by precipitation with streptomycin sulphate). The enzyme
obtained was shown to be free of deoxyribonuclease activity, but still contained a tRNA methylase activity and what was thought to be protein methylase activity. Like the crude extract (Kalousek & Morris, 1968) it did methylate endogenous spleen DNA, but at a rate 10 to 30 times less than the reaction with E. coli DNA. The product was once more characterised as 5-methylcytosine only.

Using this method, however, it was not possible to obtain DNA methylase activity from any other tissue, particularly rat liver, the source of Sheid's enzyme. The enzyme was not purified from rat liver until 1971, when Morris & Pih were able to obtain enzyme from rat liver nuclei after gentle lysis by dilution from 0.1M sucrose into tris buffer. After this modified first step, the purification followed the same procedure as before (Kalousek & Morris, 1969b). In most of its essential properties, including ability to methylate endogenous DNA, this preparation resembled the rat spleen enzyme. Interestingly, the level of enzyme activity found in regenerating liver 24 hours after partial hepatectomy was 2- to 3-fold higher than in normal liver; this increase in specific activity began about 18 hours after hepatectomy and reached its peak at 24 hours. The fact that this peak coincides roughly with the onset of DNA synthesis during regeneration is interesting in view of the evidence linking DNA synthesis fairly closely to methylation (see Section 4). This liver enzyme was used by Drahovsky & Morris for the experiments on
the mechanism of DNA methylation described in the next section.

Recently, two reports have appeared of more extensive purification of DNA methylase from HeLa cells (Roy & Weissbach, 1975) and Novikoff rat hepatoma cells (Sneider et al, 1975). The HeLa enzyme was obtained by extracting nuclei with 0.3M NaCl followed by successive chromatography on DEAE-cellulose, phosphocellulose and hydroxyapatite. Activity of the enzyme with various substrates was studied. Again, endogenous DNA could be methylated, but only to about 30% of the level of bacterial DNA from *M. luteus*. In general, the best DNA substrates were those with the highest (G+C) content. Also very highly methylated were the synthetic substrates poly (dG-dC), poly (dG-dC) (double-stranded) and poly (dG,dC) (single-stranded), and analysis of the nearest neighbours to 5-methylcytosine indicated that most of the enzymatic methylations occurred in the sequence p(G or C)p mC pG (where mC=5-methylcytosine). The recognition site for the enzyme may, of course, be much larger than this. The enzyme methylated single-stranded *M. luteus* DNA at a higher rate than double-stranded DNA.

The enzyme from Novikoff hepatoma cells was used to examine the effect of undermethylated DNA, prepared by treating synchronised Novikoff cells with ethionine to block methylation. This DNA proved to be an 85-fold greater acceptor of methyl groups than normal Novikoff cell DNA, as might be expected on the assumption that the isolated enzyme is able to methylate the sites it would methylate *in vivo*.
3.3. Mechanism of action of rat liver DNA methylase

A series of very interesting studies on the mechanism of the rat liver enzyme was carried out by Drahovsky & Morris (1971a; 1971b; 1972). As their results have a direct bearing on some of the work described in this thesis, a fairly detailed summary of their conclusions is now given.

(1) A tightly bound complex between enzyme and DNA is formed at the outset of methylation. During the reaction, the enzyme remains bound to DNA and "walks" along the DNA molecule methylating sites as it goes, like E. coli RNA polymerase. This conclusion arose from the following evidence.

(i) Methylation is inhibited by NaCl, at low concentrations, but this inhibition is not observed if enzyme and DNA are pre-incubated at 37°C before adding NaCl. This would be expected if a salt-resistant complex were at first formed and was not dissociated during the subsequent methylation (when the presence of salt would prevent reformation of the complex after it had broken up).

(ii) A complex of DNA with the methylase was isolated by gel filtration on Sepharose 4B.

(iii) In the absence of salt, the duration of the methylation reaction did not depend on the molecular weight of the DNA substrate, but if 0.2M NaCl (which would prevent formation of new
complexes) was added after 3 min incubation, the reaction rate decreased more rapidly the smaller the DNA substrate. This also is consistent with the enzyme "walking" along a single DNA molecule until it is completely methylated, whereupon the two molecules separate.

(iv) Non-methylatable T4 DNA was shown to compete with substrate DNA in the methylase reaction, suggesting that it did interact with the enzyme. The effect of this competition could be removed by pre-incubating enzyme and substrate DNA at 37°C before adding T4 DNA, while pre-incubation of enzyme and T4 DNA before adding substrate caused almost complete inhibition of methylation. This argues in favour of an initial complex formation in much the same way as the salt experiment already described.

(2) Methylation of single-stranded DNA is much more resistant to salt inhibition than methylation of double-stranded DNA. In fact, the reaction with single-stranded DNA is stimulated by NaCl at concentrations up to 0.15M.

(3) Evidence was presented for the formation of a complex between enzyme and single-stranded DNA as well as double-stranded DNA. Moreover, in a mixture of native and denatured DNA, the complex of enzyme with single-stranded DNA appeared to be preferentially formed.
(4) On the basis of these results Drahovsky & Morris suggested that methylation of native DNA was dependent on local denaturation of the DNA helix, and that this is prevented by NaCl, thereby accounting for the different effects of salt on the reaction with native and denatured DNA.

(5) The rate of methylation of different substrate DNAs could be correlated to some extent with (G + C) content, but these differences could not be accounted for by differences in the affinity of binding of enzyme to DNA.
4. STUDIES ON DNA METHYLATION IN VIVO

Among other possible functions, it has been suggested that methylation might play a vital role in DNA synthesis, perhaps by protecting newly synthesised DNA from endonucleolytic cleavage, by analogy with the restriction/modification phenomena found in bacteria. (See Section 2.4). A good deal of research has therefore centred on how methylation and synthesis of DNA in living cells are related in time scale, and also on whether the two processes are mutually dependent.

4.1. Methylation in *E. coli* 15T

Not unexpectedly the first evidence in this field came from bacteria, with the publication in 1968 of two independent reports by Lark & Billen which came to very similar conclusions. Using density labelling techniques, whereby newly synthesised DNA was labelled with bromouracil, these workers demonstrated that synthesis of DNA in the multiauxotrophic strain *E. coli* 15T could proceed when the cells were deprived of methionine (making DNA methylation impossible) while re-addition of methionine to the culture medium resulted in rapid methylation of the newly-synthesised, "undermethylated" DNA. Furthermore, this methylation occurred before any further DNA synthesis took place. Both workers also found that DNA methylation in normally growing cells occurred at or near the replication point, in the nascent DNA strand.
Thus the idea emerged that, while DNA methylation in *E. coli* 15 T<sup>-</sup> takes place more or less simultaneously with replication, the two processes are not obligatorily linked and each can continue in the absence of the other.

4.2. *Methylation of animal cell DNA in vivo*

Once it had been shown that animal cells as well as bacteria contained DNA methylating enzymes (Burdon et al, 1967; Sheid et al, 1968; Kalousek & Morris, 1968), a number of workers began investigations on the kinetics of DNA methylation in cultured animal cells. Both Burdon & Adams (1969) and Sneider & Potter (1969) concluded that the rate and extent of DNA methylation were greatly reduced in the presence of antimetabolites which block DNA synthesis. The fact that methylation of DNA fell by a smaller proportion than DNA synthesis led Burdon & Adams to suggest that there might be a time lag between synthesis and methylation, and using partially synchronised cell cultures they showed that DNA methylation apparently lagged about 1 hour behind DNA synthesis; like DNA synthesis, it was confined to a specific period in the cell cycle. This delay, together with the fact that the rat spleen DNA methylase isolated by Kalousek & Morris (1968) could methylate endogenous DNA (suggesting the presence of incompletely methylated DNA in these cells), was persuasive evidence that DNA synthesis and methylation in eukaryotes are more widely separated in time.
than they are in bacteria.

When Kappler (1970) examined methylation of DNA in a mouse adrenal cell line in the presence of antimetabolites, he obtained similar results to those of Burdon & Adams. However, if instead of following incorporation of methyl label from \(^{14}\text{C-Me}^-\) methionine into DNA he examined the conversion of \(^{14}\text{C}-\text{labelled DNA-cytosine into 5-methylcytosine, he con-}\)
cluded that methylation followed DNA synthesis by only a few minutes at most. Kappler suggested that this discrepancy might arise from an anomalous "supermethylation" of DNA at a later stage caused by the abnormal interruption of DNA syn-
thesis by the antimetabolite.

The presence of a distinct time lag between synthesis and methylation of DNA was, however, confirmed by Adams (1971) in mouse L929 cells. The length of the lag increased as S-phase went on; DNA synthesised very early in S-phase was methylated much more quickly than that produced in late S-phase. Also, when synthesis was blocked by adding amino-
pterin after 8 hours of S-phase, methylation of DNA continued for several hours. No methylation was observed in stationary cells, nor before the onset of DNA synthesis in phytohaema-
gglutinin stimulated lymphocytes, in spite of drastic changes in other areas of cellular metabolism at this time. Because of this, it seems unlikely that changes in DNA methylation are involved in controlling transcription although the poss-
sibility of demethylation was not examined.
Schneiderman & Billen (1973) showed that synchronous Chinese hamster ovary cells synthesise a rapidly reannealing DNA, rich in 5-methylcytosine, throughout S-phase, but that the rate of methylation of this DNA relative to synthesis is greatest during the early part of S phase. It appears, therefore, that the initiation region of each replicon contains highly repetitious DNA which is also highly methylated. In Physarum polycephalum, it has been shown that methylation of DNA continues for several mitotic cycles after it is synthesised (Evans et al, 1973). Also in 1973, Adams showed that methylation of DNA in newly fertilised sea urchin embryos continues for some 24 hours after synthesis. In isolated L929 cell nuclei, half the methylation occurring in vitro is on DNA made more than 1½ hours before harvesting the cells (Adams & Hogarth, 1973). (No DNA synthesis takes place in the isolated nuclei in the absence of deoxyribonucleotides). Finally, Adams (1974) demonstrated that nascent DNA segments in mouse L929 cells are not methylated, and that the lag before methylation is longer than the time required for maturation of this DNA into its high molecular weight form. It therefore seems unlikely that methylation plays a part in stabilising DNA immediately after synthesis. In this connection it is interesting that in 1971 Culp & Black had reported that, when mouse 3T3 cells were deprived of methionine, DNA synthesis continued for some hours but was eventually inhibited. They also had evidence that methyl-deficient DNA made in these circumstances was quite stable. It is not certain whether the
eventual inhibition of DNA synthesis in methionine-deprived cells is directly due to the absence of DNA methylation or to some other consequence of methionine deficiency. Interestingly, they could detect no significant difference in the relationship between DNA synthesis and methylation between normal and SV40 virus-transformed 3T3 cells. The existence of some delay between synthesis and methylation of DNA in animal cells therefore seems well established. The presence of incompletely methylated DNA could account for the ability of the eukaryotic DNA methylases to methylate endogenous DNA; this does not occur with enzymes from bacteria, whose DNA is methylated immediately on synthesis (Lark, 1968; Billen, 1968). One might expect DNA from rapidly dividing cells, much of which will be newly synthesised, to be a better substrate for methylase enzymes than DNA from slow-growing cells.

4.3 Visualisation of methylated and unmethylated regions in DNA

Molitor et al (1976) have used the technique of DNA fibre autoradiography to visualise the distribution of methylated sites in DNA methylated in vivo. Mouse L cells were grown in the presence of either $\left[\text{Me}^{-3}\text{H}\right]$ thymidine to follow replication or $\left[\text{Me}^{-3}\text{H}\right]$methionine to follow methylation, and the DNA extracted from these cells was subjected to fibre autoradiography after pronase treatment to remove protein.
After a short (15 min) pulse, the thymidine label appeared in short sections (about 8μm) arranged in tandem along the DNA double helix; the methyl label was similarly distributed. After labelling throughout S phase, however, the thymidine labelled cells yielded long continuous tracks of silver grains on autoradiography, suggesting that replication was complete, whereas in the methionine-labelled DNA unlabelled intervals of 8-20 μm in length appeared in the linear tracks. These unlabelled sections (about 10% of the total DNA) would each correspond to about 23,000 - 58,000 base pairs, and would code for 20-50 average gene products each. It is interesting to speculate whether these specific unmethylated regions could be connected in any way with the fact that sets of genes rather than individual structural genes are involved in specific transcription of the mammalian genome, especially since the proportion of DNA complementary to RNA transcripts in various animal cells (1-10% of the total genome) is of a similar order of magnitude to the proportion found to be unmethylated in these experiments.
5. POSSIBLE FUNCTIONS OF DNA METHYLATION

No definite function has yet been identified for the methylated bases in animal cell DNA, nor for the majority of methylated bases in bacterial DNA. There is no good evidence for a mammalian analogue of the bacterial restriction-modification system described in Section 2.4. There has, however, been no shortage of suggestions, of varying degrees of plausibility, as to the possible role of methylated DNA in various processes within animal cells, based on the known facts about DNA methylation in animal cells and in bacteria.

5.1. Gross structural consequences of DNA methylation

The DNA of the Xanthomonas oryzae bacteriophage XP12 is unique in that all its cytosine residues are replaced by 5-methylcytosine (33.4 mol % in total). Ehrlich et al (1975) have shown this DNA to possess several unusual properties. Its buoyant density in neutral CsCl gradients is significantly lower than the value predicted for a DNA of this (A+T) content. (The value obtained corresponds to a normal DNA with 49% (A+T) while the actual (A+T) content of XP-12 DNA is 33.2%).

Another anomalous property of XP-12 DNA is its melting temperature, which at 83.2°C is the highest reported for a naturally occurring DNA and would theoretically correspond to an (A+T) content of 18%.

This increase in stability of the double helical structure of DNA caused by methylation suggests a possible role for the much smaller amounts of 5-methylcytosine found in most normal DNAs. Localised regions of the DNA might contain relatively
large proportions of the minor base and thereby gain sufficient stability to regulate transcription and DNA synthesis, which are both known to involve local denaturation of the DNA. (Bick et al, 1972).

5.2. Structural consequences of DNA methylation—Fine

The products of methylation (5-methylcytosine and N6-methyladenine) will participate in Watson-Crick base pairing and should not disrupt the DNA duplex, since the methyl groups are located in the major groove of the double helix. However, it is possible that the effect of additional methyl groups will be sufficient to alter the affinity of DNA-binding proteins for their binding sites; it is known that the lac repressor in E. coli is very sensitive to slight changes in the major groove (Lin & Riggs, 1972), and also that the E. coli K restriction-modification enzyme has a high affinity for an unmethylated site but none for a methylated site (Yuan & Meselson, 1970). Therefore by affecting the binding of regulatory proteins to DNA, methylation could play a part in the control of transcription or differentiation.

5.3. Models for the role of DNA methylation in differentiation and development

Various authors have put forward models seeking to show how DNA methylation could be involved in differentiation and developmental processes in higher organisms. These models generally
start from the known properties of DNA methylation and erect on these a purely speculative theory for which there is no direct evidence. Such a high degree of speculation is probably inevitable in a field where good experimental evidence is so scanty; it is to be hoped that experiments will be designed in the future to test the validity of some of these theories. Scarano (1971) has suggested a way in which base modifications could lead to heritable changes in base sequences, an event which would have permanent regulatory consequences if it occurred in an operator region controlling the activity of adjacent structural genes. He proposes that a G-C base pair might become transformed into an A-T base pair by methylation of the C to give 5-methylcytosine, followed by deamination to thymine. A round of replication would then produce an A complementary to the G on the original strand, the net result after replication being the change from G-C pair to an A-T pair. Unfortunately, the evidence to date suggests that deamination of 5-methylcytosine to thymine at the DNA level does not occur in vivo. (Sneider, 1973).

A mechanism has been proposed by Riggs (1975) whereby DNA methylation could be involved in the phenomenon of X chromosome inactivation. This is the process by which one of the two X chromosomes in female mammals is kept permanently inactivated in order to avoid differential gene dosage between male and female animals (males, of course, have only one X chromosome). Inactivation is known to occur early in development (before implantation), and the important step consists of the activation of one and only one of the two X chromosomes in the zygote,
both of which are initially inactive. Riggs' model is based on the observed fact that bacterial modification methylases act much more rapidly on a half-methylated site than on a totally methylated one. An animal cell enzyme with this property would first methylate one strand of the unmethylated activation site on one X chromosome, leading to alteration of the methylase so that it cannot act on unmethylated sites. Since it is still very active on hemimethylated sites, the other strand of the activation site is soon methylated, and the modification will therefore be preserved during subsequent rounds of DNA replication.

An idea put forward by Holliday & Pugh (1975) resembles the Riggs model in making use of differential methylase activity on hemimethylated and unmethylated sites. Holliday & Pugh suggest that methylation may serve as a "developmental clock" by which a cell could count the number of divisions it has gone through during a particular stage of development. One form of the model proposes that control is exerted by a series of repeated sequences in the DNA which contains at one end a substrate site for a "switch" methylase enzyme which methylates one strand of the DNA. This is a signal for a second "clock" methylase which methylates both strands within the next repeated sequence. The "clock" enzyme cannot act on DNA methylated in both strands of one sequence, but after replication a half-methylated sequence will again be available as substrate. In this way an additional section of the DNA is modified at each cell division until the end is reached.
Both of these models make certain assumptions about the kind of sites recognised by mammalian DNA methylases, and these will be examined more fully in the Discussion. Other possible evidence for a role of DNA methylation in controlling DNA synthesis or transcription has been discussed in Sections 4.2 and 4.3.
6. **AIMS OF THE PRESENT WORK**

Studies on DNA methylation may be split into two broad categories: in *vivo* and in *vitro*.

The *in vivo* approach (see Section 4) has yielded much information about the timing of DNA methylation within the cell cycle, and also about the effect of changing growth conditions. This approach, however, does not readily lend itself to studies on such questions as the mechanism of action or substrate preference of the methylase enzyme. In addition, the presence in the cell of many unknown factors - e.g. SAM-cleaving activity - makes it difficult to be certain how many variables one is dealing with. For this type of work a purified enzyme is therefore desirable (although the possible effect of dissociating the enzyme from cellular control factors should not be neglected).

The primary aim of this project was to obtain DNA methylase from Krebs II ascites cells in as highly purified a form as possible and to attempt to answer the following questions:

(1) How do DNAs from different cell types differ in their ability to act as substrates for the enzyme, and can the differences be correlated with the growth condition of the cells?
(2) Does the enzyme prefer double or single-stranded DNA as substrate? This should help in deciding whether an analogy can be drawn with bacterial modification enzymes (see Section 2.4), which require double-stranded DNA.

(3) Can the enzyme act on artificially undermethylated DNA to fill the methylation sites left vacant in vivo?

(4) Does the ascites enzyme operate by the same type of mechanism as the rat liver enzyme of Drahovsky & Morris (see Section 3.3), which scans for methylation sites along the length of the DNA molecule?

(5) Can the highly site-specific nature of DNA methylation be accounted for solely by the properties of the enzyme-DNA interaction, or is it necessary to invoke additional cellular control factors absent from the purified enzyme?

(6) Do any of the data obtained with the purified enzyme bring us nearer to understanding the function of DNA methylation in mammals?
MATERIALS AND METHODS
MATERIALS

1. CHEMICALS

Most of the general chemicals used in the present work were products of British Drug Houses, Ltd., Poole, Dorset, England, and were AnalAr grade whenever possible. Sodium p-amino-salicylate and N-ethylmaleimide were also obtained from this source. Dithiothreitol and unlabelled S-adenosyl-L-methionine chloride were purchased from the Sigma Chemical Co., St. Louis, Missouri, U.S.A.

Tween 80 was obtained from Koch-Light Laboratories, Colnbrook, Bucks., England, as also were the following scintillation chemicals: hyamine hydroxide (1M in methanol), 2,5 diphenyl-oxazole (PPO), toluene and triton X-100.

p-bis (O-methylstyryl) benzene (bis-MSB) was a product of the Eastman Kodak Co. Ltd.

2. RADIOACTIVE COMPOUNDS

\[ \text{[Me}^3\text{H]} \] S-adenosyl-L-methionine and \[ ^{14}\text{C} \] deoxycytidinie were products of the Radiochemical Centre, Amersham, England.

3. BIOLOGICAL MATERIALS

3.1 Nucleic Acids

E.coli, salmon testis and calf thymus DNA were bought from Sigma Chemical Co., St. Louis, Missouri, U.S.A.
DNAs from mouse tissues (spleen, testis, kidney, pancreas, liver) were the gift of Dr. E.J. Smillie and were prepared by the method of Marmur (1961), as was DNA from stationary and S-phase L929 cells. T4 DNA was generously donated by Dr. A.D.B. Malcolm.

**Undermethylated L929 cell DNA**

This was prepared by synchronising mouse L929 cells at the beginning of S-phase using aminopterin (Adams, 1969). This block was reversed by addition of thymidine (5 x 10^{-6} M) in the presence of [14C]deoxyctydine but in the absence of methionine. DNA synthesis continues normally under these conditions for several hours, but the proportion of 5-methyl cytosine is reduced to 2.3% of the total cytosine as compared with 2.9% in the newly synthesised strand of control DNA isolated from late S phase cells. (This was shown by hydrolysis of the DNA with perchloric acid and separation of the bases as described in Section 7).

On reintroduction of methionine into the culture medium methyl groups were added to the DNA over the next 20 hours. Over this period and the succeeding 48 hours the DNA is quite stable (no labelled DNA is lost relative to controls) and is not subject to repair (no incorporation of tritiated 5-bromodeoxyuridine into light DNA occurs).
3.2. **Proteins and Enzymes**

Bovine serum albumin (BSA) was obtained from Armour Pharmaceutical Co. Ltd., Eastbourne, England. Catalase and nuclease from *N. Crassa* were products of the Boehringer Corporation, London.

3.3. **Krebs II mouse ascites tumour cells**

These were propagated by serial intraperitoneal transplantation in mice of the departmental colony and were harvested routinely after 10-14 days of growth. Cells were washed twice by suspension in ice-cold PBS (q.v.) followed by centrifugation at 800g for 5 min at 4°C. Nuclei were normally prepared immediately (see Section 5.1).

4. **CHROMATOGRAPHIC MATERIALS**

The following Whatman products were all purchased from H. Reeve Angel Ltd., London: No.1 and 3MM chromatography paper, 3MM filter circles (2.5 cm diameter), CF11 cellulose powder, DE52 diethylaminoethyl (DEAE) cellulose and P11 phosphocellulose.

Ultrogel AcA34 was obtained from LKB-Produkter, Sweden.

Sephadex G-200 was a product of Pharmacia Fine Chemicals, Uppsala, Sweden, as was Blue Dextran 2000, a high molecular weight dye for column calibration.
METHODS

1. SOLUTIONS

1.1. Buffers

1.1.a. Buffer M  This was the standard buffer used for storage, assay and purification of the enzyme. Its composition was as follows:

   50 mM tris HCl, pH 7.8
   1 mM dithiothreitol (DTT)
   1 mM EDTA
   10% (v/v) glycerol

For certain experimental procedures, NaCl at various concentrations was added to buffer M; this will be stated in the appropriate sections.

1.1.b. The following additional buffers were required in the pH experiment (Results, section 2.3):

   (i) Sodium hydrogen maleate - NaOH (0.05M), pH 5.2 and 6.0
   This was prepared by mixing 50 ml of sodium hydrogen maleate (0.2M) with 7.2 ml (for pH 5.2) and 33.0 ml (for pH 6.0) of 0.2M NaOH, and diluting to 200 ml with water. DTT, EDTA and glycerol were added at the same concentrations as in buffer M, before checking the pH and adjusting as necessary.
   (Sodium hydrogen maleate is prepared by dissolving 23.2 g maleic acid and 8 g NaOH in water at a total volume of 1 l).

   (ii) Tris HCl buffers, pH 7.1, 8.5 and 9.0
   These consisted simply of buffer M containing 50 mM tris HCl at the appropriate pH.
(iii) 0.05 M glycine-NaOH, pH 10.0  This was made by mixing 0.2 M glycine (50 ml) and 0.2M NaOH (30 ml), adding DTT, EDTA and glycerol as in buffer M and making up to 200 ml with water after checking the pH.

1.2. Phosphate Buffered Saline (PBS)
This was stored as three separate components, Solutions A, B and C, which were mixed immediately before use in the ratio 8:1:1 by volume. These solutions had the following compositions:
Solution A - NaCl, 10g/l; KCl, 0.25g/l; disodium hydrogen orthophosphate, 0.25 g/l.
Solution B - CaCl$_2$, 0.25g/l
Solution C - MgCl$_2$(hydrated), 0.25 g/l

1.3. Scintillation Fluids
(i) Toluene-PPO scintillation fluid was prepared by dissolving 2.5 diphenyloxazole (PPO) in toluene at a concentration of 5g/l (0.5% w/v).
(ii) Triton/toluene scintillation fluid consisted of 5g PPO plus 0.5g of p-bis (O-methylstyrlyl) benzene (bis-MSB), dissolved in 350 ml of triton X-100 and 650 ml of toluene.

2. ESTIMATION OF DNA AND PROTEIN
Protein was determined by the method of Lowry et al (1951), using bovine serum albumin as standard.

DNA was estimated by the method of Burton (1956), using salmon testis DNA as standard.
3. **PREPARATION OF DENATURED DNA**

Denatured DNA (E.coli and calf thymus) was prepared by heating a solution of DNA in 20 mM KCl at 100°C for 10 min, followed by rapid cooling on ice.

4. **ENZYME ASSAYS**

4.1. **DNA Methylation Assay**

The assay involves measuring incorporation of radioactivity from $\left[\text{Me}^{-3}\!\!\text{H}\right]$SAM into DNA. Stages are incorporated to remove RNA and protein, which are likely also to be methylated by the enzyme, from the assay (see Introduction, Section 3.2).

The standard assay mixture (140 µl) contained 40 µg DNA (E.coli unless otherwise stated), 3.3 µCi of $\left[\text{Me}^{-3}\!\!\text{H}\right]$SAM (1 µCi/nmole), and 100 µl of enzyme solution in buffer M (giving final concentrations of 715 µM for EDTA and dithiothreitol, 7.2% (v/v) for glycerol, 36 mM tris HCl, pH 7.8, and 23.6 µM SAM).

After incubation at 37°C for 1 hour, the reaction was stopped by adding 2 ml of a solution containing sodium dodecyl sulphate (1% w/v), EDTA (2mM), 4-aminosalicylic acid (3% w/v), n-butanol (5% v/v), NaCl (0.5 M) and salmon testis DNA (0.5 mg/ml). The last item serves as a carrier DNA in the subsequent precipitation step.

Protein was removed from the mixture by extraction with a solution of phenol (88%), m-Cresol (12%) and 8-hydroxyquinoline (0.1%). After centrifugation at 1000 g for 20 min, the upper,
aqueous layer (containing DNA) was removed, leaving protein in the interphase material.

DNA was precipitated out by mixing vigorously with two volumes of absolute ethanol. After centrifugation at 1000 g for 10 min, this precipitate was redissolved in 0.2 ml of 0.5 N NaOH. The solution was incubated for 3 hours at 37°C, to digest any RNA in the mixture.

0.1 ml portions were next spotted onto Whatman 3MM filter circles (2.5 cm diameter), washed four times in ice-cold 5% (w/v) trichloroacetic acid (10 ml per filter) and then dried using ethanol and ether. The DNA was dissolved by heating in 0.5 ml of 1M hyamine hydroxide at 60°C for 20 min. 5 ml of toluene/PPO scintillator was added to the vial and radioactivity assayed using a liquid scintillation spectrometer. DNA methylase activity is expressed either as ^3H d.p.m. incorporated per hour or as p moles of "methyl groups" incorporated per hour.

4.2. Catalase Assay

Catalase was used as a molecular weight marker in gel filtration and density gradient centrifugation.

The assay measures the breakdown of hydrogen peroxide spectrophotometrically. 100 μl of test solution was made up to 2 ml with water, and to this was added 1 ml of substrate (0.3 ml of hydrogen peroxide (100 vols) made up to 50 ml with 0.05M phosphate buffer, pH 7.0). After incubation at 37°C
for 10 min, the O.D. at 240 nm was measured against a blank solution containing no enzyme, which usually had an O.D. of 0.75 - 0.85. Solutions containing catalase activity gave much lower values.

5. EXTRACTION AND PURIFICATION OF DNA METHYLASE FROM ASCITES CELLS
(All parts of this procedure were carried out at 0-4°C)

5.1. Preparation of Nuclei
Batches of approximately $5 \times 10^9$ cells were washed twice in PBS and then allowed to swell in 5 volumes of ice-cold water before centrifugation at 800 g for 3 min. The cells were disrupted by homogenising in 1% (v/v) Tween 80 in water, using a tightly-fitting Teflon-glass homogeniser (4-5 strokes). The preparation was examined by phase-contrast microscopy for integrity of nuclei and freedom from whole cells, after which the nuclei were recovered by centrifugation at 800 g for 10 min.

5.2. Extraction of DNA methylase from nuclei
Nuclei from $5 \times 10^9$ cells were resuspended in 5 vols. of buffer M and an equal volume of buffer M containing 0.8M NaCl was slowly added with constant stirring. The supernatant obtained after centrifugation at 12,000 g for 30 min is termed the salt extract.
5.3. **Ammonium sulphate fractionation**

Solid ammonium sulphate was slowly added, with stirring, to the salt extract until the latter was 30% saturated with respect to ammonium sulphate. After centrifugation at 12,000 g for 20 min, the precipitate was discarded and the supernatant made to 60% saturation with ammonium sulphate. After further centrifugation, the precipitate was redissolved in a minimum volume of buffer M containing 0.4M NaCl.

5.4. **Gel filtration on Ultrogel AcA34**

Ultrogel AcA34 (supplied in pre-swollen form) was equilibrated according to the manufacturer's instructions and packed into a column of final dimensions 2.5 x 55 cm. The column was calibrated using Blue Dextran, catalase, $^{125}$I labelled immunoglobulin G (a generous gift of P. Singer) and haemoglobin. The redissolved ammonium sulphate precipitate (Fraction III) was applied to the column and eluted with buffer M containing 0.4 M NaCl at a flow rate of 30 ml per hour, 5 ml fractions being collected.

5.5. **Phosphocellulose chromatography**

Phosphocellulose was equilibrated with buffer M containing 0.2 M NaCl according to the maker's instructions and poured into a column of 10 ml final volume. Two column volumes of BSA (2 mg/ml) were pumped into the column, at a flow rate of 30 ml per hour, in order to saturate non-specific binding sites. The column was then washed with two column volumes of buffer M containing 0.2 m NaCl.
The fractions from the Ultrogel column containing peak enzyme activity were pooled and diluted to 0.2 M NaCl with buffer M before being pumped onto the phosphocellulose column. The column was next washed with buffer M containing 0.2 M NaCl until the O.D. at 280 nm of the eluate fell to zero. To elute the enzyme the column was washed with buffer M containing 0.5 M NaCl and 2 ml fractions were collected until the $E_{280}$ fell to zero. The fractions with peak optical densities were pooled and dialysed against two changes of buffer M (100 vols) to remove NaCl.

5.6. **DEAE-cellulose treatment**

DEAE-cellulose (supplied in pre-swollen form) was equilibrated with buffer M according to the supplier's instructions. The pooled and dialysed peak fractions from the phosphocellulose procedure were mixed with an equal volume of the DEAE-cellulose slurry. Under these conditions DNA methylase recovered in the supernatant after low speed centrifugation.

6. **OTHER FRACTIONATION PROCEDURES**

6.1. **Sephadex G-200**

Sephadex G-200 was pre-swollen and equilibrated according to the manufacturer's instructions. The gel was then packed into a column (1 cm x 50 cm) and the void volume determined using Blue Dextran. A 1 ml sample of enzyme material was applied and eluted at a flow rate of 6 ml per hour with buffer M, fractions of approximately 1 ml being collected.
6.2. DNA-cellulose

This was prepared as described by Bautz & Dunn (1971) using native calf thymus DNA. The final product was shown to have 410 μg of bound DNA per ml of packed cellulose. The procedure used in fractionation of enzyme samples is described in Results (Section 3.6).

6.3. Sucrose density gradient centrifugation

Linear 5-20% sucrose gradients (containing 20 mM tris pH 7.8) were prepared by successively layering equal volumes of 20%, 15%, 10% and 5% (w/v) buffered sucrose in a centrifuge tube. The tubes were left standing in the refrigerator overnight to allow the density gradient to form by diffusion. Enzyme samples were diluted 1:1 with 50 mM tris, pH 7.8 before applying to the gradients, because the 10% glycerol present in buffer M makes it denser than 5% sucrose. 0.2 ml of this diluted sample was layered onto the gradient and then centrifuged at 78,000 g for 14 hours at 4°C in the Beckman SW56 rotor. Gradients were harvested by upward displacement with 50% sucrose using the MSE gradient harvester, 6-drop fractions being collected.

6.4. Glycerol density gradient centrifugation

Linear 10-30% glycerol gradients were prepared in a similar way to sucrose gradients, by layering successively 30%, 20% and 10% (v/v) glycerol. Centrifugation conditions and the methods of harvesting were the same as for the sucrose gradients. (The glycerol solutions contained the other components of buffer M at the usual concentrations. Enzyme samples were again diluted with 50 mM tris to reduce their density before application).
7. IDENTIFICATION OF PRODUCT OF METHYLATION IN VITRO

E. coli DNA, enzyme and $[^3\text{H}\text{-Me}]\text{SAM}$ were incubated as in the standard DNA methylase assay (Section 4.1). The normal assay procedure was followed as far as the stage of precipitating the DNA onto 3MM filter discs using 5% TCA. After four washes in 5% TCA, the DNA was extracted into 0.5M perchloric acid at 70°C (30 min). The solution was evaporated until the perchloric acid was approximately 12N and the DNA was then hydrolysed to the bases by incubating for 1 hour at 100°C. The bases were separated by descending chromatography on Whatman No.1 paper in n-butanol-HCl-H$_2$O (65:16.7:18.3 by vol.). The position of the bases was determined using an ultraviolet lamp. The spots were shredded into scintillation fluid (toluene-PPO, see section 1.3), for estimation of radioactivity.
NOTE The experiments described in Sections 2.1 and 3.3 were performed in collaboration with Dr. R.L.P. Adams

1. **PURIFICATION OF DNA METHYLASE FROM ASCITES CELLS**

1.1. **Introduction**

A description is given in Materials and Methods of the standard procedure adopted for purifying DNA methylase from Krebs II ascites cells. The purpose of this Section is to detail the experimental steps which led to the development of this procedure and to present typical results showing the progress of the purification step by step.

1.2. **Extraction of Enzyme from Nuclei**

Treatment of ascites nuclei, prepared as described in Materials and Methods, with 0.4M NaCl followed by centrifugation at 12,000 g for 30 min leaves about 90% of the total DNA methylase activity in the supernatant. Subsequent re-extraction of the precipitate with 0.4M NaCl will liberate virtually all the remaining enzyme activity into the supernatant. This fraction is termed the salt extract.

1.3. **Ammonium Sulphate fractionation of salt extract**

Table 1 shows that on fractional precipitation of proteins from the salt extract with ammonium sulphate, 96% of total DNA methylase activity is precipitated at between 30% and 60% saturation with ammonium sulphate; since only about 60%
of total protein is precipitated in this range, a useful purification is achieved. This also provides a way of reducing the large volume of the salt extract, since the ammonium sulphate precipitate can be redissolved in a small volume of buffer M (usually less than 10 ml).

**Table 1: Ammonium Sulphate Fractionation**

<table>
<thead>
<tr>
<th>Ammonium sulphate concentration (% saturation)</th>
<th>% total DNA methylase activity precipitated</th>
<th>% total protein precipitated</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 - 30</td>
<td>3.6</td>
<td>30.2</td>
</tr>
<tr>
<td>30 - 60</td>
<td>96.4</td>
<td>60.4</td>
</tr>
<tr>
<td>60 - 100</td>
<td>0</td>
<td>9.4</td>
</tr>
</tbody>
</table>

Salt extract (100 ml) was made to 30% saturation with respect to ammonium sulphate. After centrifugation the supernatant was made to 60% saturation, and after further centrifugation to 100% saturation, with ammonium sulphate. The precipitate at each stage was dissolved in 2.5 ml of buffer M and dialysed against 2 changes of buffer M (100 vols) before assay. (100% enzyme activity corresponds to 1380 dpm per assay).

1.4. Gel Filtration

Figure 1 shows the elution profile obtained when a redissolved ammonium sulphate precipitate is subjected to gel filtration on Sephadex G-200 using buffer M as eluant. DNA methylase activity emerges as a single, well-defined peak, but since it is eluted in the excluded volume (same position as blue dextran) and coincides with the bulk of the protein in the
Gel filtration of DNA methylase on Sephadex G200.

Enzyme was extracted from nuclei and subjected to ammonium sulphate fractionation as described in Materials and Methods. After dialysing against buffer M to remove salt, a 1 ml sample of the redissolved ammonium sulphate precipitate was applied to a 30 cm x 1 cm column containing Sephadex G200. The enzyme was eluted with buffer M, 1 ml fractions being collected.

---○---DNA methylase activity (³H dpm incorporated into DNA)

---O---O---Protein concentration (µg/ml)
Protein (µg/ml)

Fraction no.

Blue Dextran

$3 \times 10^{-3}$ dpm incorporated
Figure 2

Gel filtration of DNA methylase on Ultrogel AcA34.

A redissolved 30-60% ammonium sulphate fraction (see Materials and Methods) was applied to a 2.5 x 55 cm column containing Ultrogel AcA34 and eluted with buffer M containing 0.4 M NaCl.

5 ml fractions were collected and 50 µl of each was assayed for DNA methylase activity in a total assay volume of 200 µl, to give a final salt concentration of 0.1M.

--- ○ --- ○ --- DNA methylase activity

(³H dpm incorporated into DNA)

--- ○ --- ○ --- E₂₈₀
preparation, this is clearly of little use as a purification technique. Evidently extensive aggregation of the methylase with other proteins takes place under these conditions.

This aggregation can be broken up by elution with buffer M containing 0.4M NaCl (Figure 2). (The result shown here was obtained using Ultrogel AcA34, which has similar exclusion properties to Sephadex G-200 but was chosen as the standard filtration medium for practical reasons, in that it allows higher flow rates than Sephadex while maintaining dimensional stability of the gel bed. The results obtained using Sephadex G-200 are very similar).

Under these conditions methylase activity is eluted as a broad peak roughly coinciding with a marker of catalase (molecular weight 230,000), while 280 nm-absorbing material is widely spread throughout the eluate. This Ultrogel step is included in the standard purification procedure.

1.5. Ion Exchange chromatography on DEAE-cellulose
For the initial experiment 5 ml of redissolved ammonium sulphate precipitate was pumped (after dialysis against buffer M) onto a 5 ml column of diethylaminoethyl cellulose (DEAE-cellulose) equilibrated with buffer M. The column was then washed with buffer M and 2ml fractions were collected till the material emerging had no detectable absorbance at 280 nm.
Material remaining on the column was now eluted with a linear gradient of 0-0.6M NaCl in buffer M, 2 ml fractions again being collected.

Using this procedure, all the DNA methylase activity was recovered in the initial wash, i.e. it was excluded from the cellulose (Figure 3). Since some 44% of total protein in the preparation is not eluted until a salt concentration of 0.2M, a worthwhile purification can be achieved simply by mixing enzyme with DEAE-cellulose in buffer M, centrifuging and removing the supernatant, which contains the enzyme.

1.6. Ion exchange chromatography on phosphocellulose

A 10 ml column of phosphocellulose was presaturated with BSA as described in Materials and Methods, in order to fill all the non-specific binding sites. For the initial experiment, an enzyme sample obtained by the procedure described in Section 1.5 was then pumped onto the column, which was then washed successively with buffer M, and with 0.25 M, 0.5 M, and 0.8 M NaCl in buffer M, fractions being collected in each case until the O.D. at 280 nm of the eluate fell to zero. Fractions containing peak optical densities at each salt concentration were pooled, dialysed against buffer M and assayed for DNA methylase activity.

Approximately 84% of total methylase activity was eluted from the column at 0.5 M salt, but only 14% of total protein was present in this fraction.
Figure 3

DEAE-cellulose chromatography of DNA methylase.

For details of method, see text.

--- ● --- DNA methylase activity

\(^{3}\text{H \ dpm incorporated into DNA}\)

--- ○ --- Protein (\(\mu\text{g/ml}\))

Fractions 1-5: wash with buffer M

Fractions 6-25: elution with a linear gradient of 0-0.6M NaCl in buffer M

(total volume 40 ml).
Protein (μg/ml)

Fraction no. 0 15 20 25

600 400 200 0

0 - o.6M NaCl

0 - H dpm incorporated

ξ = 10 x
Table 2: Phosphocellulose chromatography of DNA methylase

<table>
<thead>
<tr>
<th>NaCl concentration in eluant (M)</th>
<th>% total DNA methylase activity eluted</th>
<th>% total protein eluted</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>8.6</td>
<td>62.0</td>
</tr>
<tr>
<td>0.25</td>
<td>0</td>
<td>16.8</td>
</tr>
<tr>
<td>0.5</td>
<td>83.7</td>
<td>14.2</td>
</tr>
<tr>
<td>0.8</td>
<td>7.7</td>
<td>7.0</td>
</tr>
</tbody>
</table>

100% enzyme activity corresponds to 1300 dpm/assay

(In the standard purification procedure, the phosphocellulose step precedes the DEAE-cellulose treatment, in order to reduce the large volume of the Ultrogel preparation to the small volume required for treatment with DEAE-cellulose. The Ultrogel sample is diluted from 0.4M to 0.2M NaCl before application to the phosphocellulose column, from which the enzyme is eluted with 0.5M NaCl. This fraction is then dialysed against buffer M to remove all NaCl before treatment with DEAE-cellulose).

1.7. Complete Purification of DNA Methyase from Ascites Nuclei

Typical data for the purification of the enzyme are presented in Table 3. The procedure, which is described in detail in the Materials and Methods section, incorporated the various steps already documented in the most convenient order. (Fraction III,
which is omitted from this table, is the precipitate from the 30-60% saturation range of ammonium sulphate, and is not normally assayed separately before application to the Ultrogel column).

A final purification of 405-fold is achieved over the isolated nuclei, without overall loss of enzyme activity.

Table 3:  Purification Table for Ascites DNA Methylase

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein (mg)</th>
<th>Total enzyme (units)</th>
<th>Specific Activity (units/mg)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I Nuclear suspension</td>
<td>450</td>
<td>716</td>
<td>1.59</td>
<td></td>
</tr>
<tr>
<td>II Salt extract</td>
<td>360</td>
<td>1670</td>
<td>4.64</td>
<td>2.9</td>
</tr>
<tr>
<td>IV Ultrogel peak</td>
<td>55.4</td>
<td>1530</td>
<td>27.7</td>
<td>17.4</td>
</tr>
<tr>
<td>V Phosphocellulose 0.5M eluate</td>
<td>3.4</td>
<td>575</td>
<td>169</td>
<td>106</td>
</tr>
<tr>
<td>VI DEAE cellulose unbound</td>
<td>3.1</td>
<td>1990</td>
<td>643</td>
<td>405</td>
</tr>
</tbody>
</table>

1 unit of enzyme activity is defined as that amount which catalyses the incorporation of 1 p mole of methyl groups into E.coli DNA in 1 hour under standard assay conditions.
2. GENERAL PROPERTIES OF THE PURIFIED ENZYME

2.1. Characterisation of the product of in vitro methylation
As mentioned in the Introduction, some early reports of DNA methylase activity in animal cells were insufficiently rigorous in that they did not demonstrate that the only product of enzymic methylation of DNA was the deoxyribonucleotide (or deoxyribonucleoside) of 5-methylcytosine.

To characterise the product of the ascites enzyme, the usual methylase assay procedure was followed as far as the alkaline digestion using E. coli DNA as the substrate. The product from this was applied to filter discs, precipitated and washed with 5% TCA in the usual manner. After drying, DNA was extracted from the filters with 0.5 N perchloric acid and then hydrolysed to the bases using 12 N perchloric acid. Chromatography in a n-butanol:HCl:H₂O system gave the result shown in Figure 4. 88% of the radioactivity migrated to a point coincident with a marker of authentic 5-methyl cytosine, with no significant peak of radioactivity in any other position.

One can be fairly certain that ribonucleotides have been eliminated by this procedure, as the alkaline digest could hydrolyse any RNA present to acid-soluble material, which should then be eliminated by washing in 5% TCA before the DNA is extracted. This experiment therefore provides good evidence that the enzyme extracted from ascites cells has the characteristics of a bona fide eukaryotic DNA methylase.
Figure 4

Identification of product of methylation by the enzyme.

Methylated DNA was extracted from filter discs and hydrolysed to bases as described in the text. Paper chromatography of this hydrolysate, together with appropriate markers, was carried out on Whatman No.1 paper for 66 h using a solvent consisting of n-butanol:HCl:H₂O in a 65:16.7:18.3 ratio by volume.
2.2. Dependence of the reaction on added DNA substrate

The effect of the concentration of *E. coli* DNA on the methylation reaction is shown in Figure 5. This demonstrates that in the absence of any added DNA no incorporation of methyl groups into DNA can be detected. The methylation observed using the purified enzyme is therefore not due to the presence of residual ascites DNA in the preparation, which might be subject to methylation by the enzyme. This result also confirms that methylation of RNA and protein has been successfully excluded from the final assay result.

The amount of *E. coli* DNA used in the standard assay (40 μg) will give very close to maximal incorporation of methyl groups in a 1 hour incubation. This level represents a great excess of DNA, as is shown by the fact that increasing the incubation time to 4 hours raises incorporation to 4.5 p moles of methyl groups per 40 μg of DNA.

2.3. pH Optimum

The reasons for determining the effect of pH on the enzyme were twofold; firstly, the existence of more than one pH optimum - or of a broad plateau of activity - might be evidence for the presence of more than one enzyme species, and secondly, if the optimum values with native and denatured DNA substrates were different this might argue in favour of a fundamental difference in the mechanism of the reaction with single and double-stranded DNA, or that different forms of the enzyme are involved in the two cases (the possible significance of this is fully discussed in Sections 3 and 4.)
Figure 5

Effect of DNA concentration on enzyme activity.

The total assay volume used in this experiment was 220 μl.
As shown in Figure 6, the enzyme shows one sharp peak of activity at a pH of about 7.9. The peak occurs in the same position with both native and denatured calf thymus DNA as substrate, though the maximal level of incorporation is higher with the former, as might be expected under the low salt conditions used (see Section 3.3). The fall in activity at high pH values does seem less pronounced with denatured DNA, but the difference is small and probably not significant.

It is interesting that the only other highly purified mammalian enzyme reported in the literature (from HeLa cells, Roy & Weissbach, 1975) showed a discrete pH optimum at 6.5. The relatively crude rat spleen enzyme of Kalousek & Morris (1969) was active over a broad pH range (7.4 to 8.6). Sneider et al (1975) reported that their purified but still very heterogeneous enzyme from Novikoff hepatoma cells gave a broad peak with native DNA, but two distinct optima with denatured DNA, while their unpurified nuclear supernatant showed a different peak. The possibility cannot be excluded that nuclei contain more than one methylase activity and that all except one are removed during the purification process, but as no firm evidence of multiple species is available from other experiments with the ascites enzyme this seems unlikely.

The enzyme molecule is not the only component of the methylation reaction which might be sensitive to pH; effects on the charged SAM molecule or on the DNA might influence binding between the three components or even the actual process of
**Figure 6**

Effect of pH on DNA methylase activity

Buffers used were as follows:

- pH 5.2 - 6.0 : 0.05M sodium hydrogen maleate
- pH 7.3 - 8.8 : 0.05M tris-HCl
- pH 9.8 : 0.05M glycine

Assays contained 40 μg calf thymus DNA

- 10 μl purified enzyme
- 90 μl of appropriate buffer
- 20 μl of $\left[^{3}\text{H}-\text{CH}_3\right]$ SAM

Samples were incubated for 5 hours.

---●---●---, native DNA

---○---○---, denatured DNA
methyl transfer. The existence of a pK value of about 6.9 might represent a critical sulphydryl group on the enzyme (see next section), while the fall in activity above pH 8 may well result from the instability of SAM under alkaline conditions.

2.4. Sensitivity of enzyme to NEM

The results described in the previous section suggest that a sulphydryl group on the enzyme may be required for its activity, so the effect on the enzyme of the sulphydryl inhibitor N-ethyl maleimide (NEM) was examined.

Addition of 5mM NEM to the standard reaction mixture caused incorporation of methyl label into *E. coli* DNA during a 1-hour incubation with fraction V enzyme to fall from 2700 dpm to a level corresponding to the blank value (less than 200 dpm). It therefore seems that a sulphydryl group is necessary for methylase activity, although it is quite possible that NEM could interact with SAM rather than the enzyme.

The effect of omitting the sulphydryl reagent dithiothreitol (DTT) from the assay buffer has not been examined with the ascites enzyme. The HeLa cell enzyme (Roy & Weissbach, 1976) did require a sulphydryl reagent for activity, and was inhibited by the sulphydryl antagonist iodoacetamide. The rat spleen (Kalousek & Morris, 1969) and rat liver (Morris & Pih, 1971) enzymes were apparently neither inhibited nor stimulated by 2-mercaptoethanol or DTT.
2.5. Time course of methylation of calf thymus DNA

Figure 7 shows the time course of methylation of both native and denatured calf thymus DNA (this was used instead of E. coli DNA for the reasons discussed in Section 3.4).

Native DNA is a better methyl acceptor than denatured DNA, as was also the case for the rat liver enzyme of Drahovsky & Morris (1971b), but not for the HeLa cell enzyme of Roy & Weissbach (1975). This experiment was conducted in the absence of salt; as will be seen in Section 3.4., salt at moderate concentrations causes methylation of denatured DNA to be favoured.

Both reactions are close to saturation levels at 10 h; however, this is not due to all the available sites on the DNA being methylated, as the addition of fresh enzyme and SAM at this time will cause the reaction to be resumed (see Turnbull & Adams, 1976). The amount of DNA used in the standard assay (40 μg) is therefore a considerable excess.

3. STUDIES ON SUBSTRATE SPECIFICITY AND MECHANISM
   OF THE ENZYME

3.1. Introduction

One approach to trying to understand the function of DNA methylation is to examine how the activity of an isolated DNA methylase is affected by the nature of the DNA substrate used. For example, one can test the idea that DNA from rapidly dividing cells will have more sites available for methylation than DNA from stationary cells, because of the
Figure 7
Time course of methylation of calf thymus DNA.

Standard assay conditions were used, i.e. $40\mu g$ DNA per assay and $100\mu l$ of Fraction V enzyme.

- Native DNA
- Denatured DNA
time lag observed between synthesis and methylation of DNA in vivo (Burdon & Adams, 1969; Adams & Hogarth, 1973). The presence or absence of methylase-susceptible sites which are left unmethylated in vivo would raise interesting questions as to how methylation is controlled in vivo. It should also be possible to demonstrate the ability of the enzyme to methylate DNA rendered artificially methyl-deficient.

Detailed studies have been published on the mechanism of action of rat liver DNA methylase (Drahovsky & Morris, 1971a and b; 1972). An important conclusion of this work was that the enzyme binds irreversibly to its substrate at the outset of the reaction and "walks" along the DNA during the reaction; this idea was followed up with the ascites enzyme in the hope of learning more about how the enzyme interacts with DNA.

3.2. Test for methylation of RNA by the enzyme

As mentioned in the Introduction, preparations of DNA methylases often contain contaminating RNA and protein methylase activities. The purified ascites enzyme was shown not to methylate RNA by the following experiment.

Standard methylase assays were set up containing 40 µg of purified ribosomal RNA from E. coli (a gift of Mr. C. MacLeod) instead of the usual DNA substrate. The assay mixtures were incubated at 37°C for 1 h or 4 h and phenol extractions carried out in the usual way. The aqueous layers from these extracts were made 5% (w/v) with respect to trichloroacetic acid to
precipitate both the RNA and any DNA that might be present. After washing 3 times with 5% TCA, the precipitates were dissolved in 0.3M NaOH and incubated overnight at 37°C to digest the RNA. The remaining acid-precipitable material (i.e. DNA) was precipitated by acidifying with TCA, and the radioactivity incorporated into RNA was estimated by liquid scintillation analysis of 0.5 ml of the supernatant in 5 ml of triton/toluene scintillation fluid.

As shown in Table 4, incorporation of methyl label into RNA does not exceed the blank value (i.e. 0 time) after a 4 h incubation (figures for parallel assays using calf thymus DNA are included for comparison).

Table 4: Action of enzyme on RNA substrate

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Incubation time (h)</th>
<th>$^3$H dpm incorporated</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA</td>
<td>0</td>
<td>1448</td>
</tr>
<tr>
<td>RNA</td>
<td>1</td>
<td>1434</td>
</tr>
<tr>
<td>RNA</td>
<td>4</td>
<td>1508</td>
</tr>
<tr>
<td>DNA</td>
<td>0</td>
<td>174</td>
</tr>
<tr>
<td>DNA</td>
<td>1</td>
<td>1399</td>
</tr>
<tr>
<td>DNA</td>
<td>4</td>
<td>1590</td>
</tr>
</tbody>
</table>

For experimental details see text (Section 3.2)
3.3. In vitro methylation of DNA from different sources

Figure 8 shows the incorporation, over a four-hour period, of methyl groups into the DNA (10 µg/assay) isolated from various mouse tissues, from cultured mouse L929 cells grown under various conditions, from \textit{E.coli} and from calf thymus. Several interesting conclusions can be drawn:

(1) The homologous ascites DNA can be methylated to quite a high level. In this respect the ascites enzyme differs from most bacterial DNA methylases which have been studied, but does resemble the rat spleen preparation of Kalousek & Morris (1968) and the enzyme recently prepared from HeLa cells (Roy & Weissbach, 1975). This fact might suggest a degree of undermethylation of the ascites DNA, which would be not unreasonable in a fast-dividing tumour cell line. The necessity of showing (Section 2.2) that the enzyme preparation does not contain homologous DNA is emphasised by this result.

(2) There is a general correlation between growth rate of the cells and the methyl accepting ability of their DNA; compare, for example, \textit{S} phase and stationary phase L929 cell DNA, or ascites cell and pancreas DNA. It is likely that this is accounted for by the relatively high proportion of newly synthesised, and presumably undermethylated, DNA in rapidly-dividing cells.
Methylation of different DNAs by the ascites enzyme.

DNA was prepared from various cells and tissues as described in Materials and Methods. Each assay contained 10μg of the appropriate DNA and 10μl of Fraction III enzyme.
(3) By far the highest rate of incorporation was shown by methyl-deficient L929 cell DNA prepared as described in Materials and Methods. Base composition analysis of this DNA showed it to be about 19% undermethylated by comparison with control DNA from late S-phase cells; presumably most of the sites left unmethylated by this process in vivo can be methylated by the enzyme in vitro. This cannot be quantitatively confirmed without carrying the reaction to completion, i.e. methylaing all the available sites.

It should be noted that none of these DNAs are fully methylated after 4 hours. Clearly a much longer incubation would be required to fill all the available methylation sites and thus to obtain a rough estimate of the degree of undermethylation of the DNA.

3.4. Differential effect of salt on methylation of single and double-stranded DNA

It has been reported (Drahovsky & Morris, 1971b) that methylation of denatured E.coli DNA by partially purified rat liver DNA methylase is much less sensitive to salt than methylation of native DNA, and that low salt concentrations actually stimulate the reaction with denatured DNA (see Introduction, Section 3.3). Drahovsky & Morris proposed that this difference was caused by the stabilising effect of salt on the DNA helix, preventing local unwinding which (they argue) is essential for methylation.
Figure 9A shows the result of a similar experiment using the ascites DNA methylase. Both native and denatured \textit{E. coli} DNA show stimulation of the reaction by NaCl at ionic strengths up to 100 mM; however, native DNA shows less incorporation at all points, has a smaller maximum stimulation by salt (78% as against 136%) and is more sensitive to salt concentrations above 100 mM.

Repeating the experiment with native and denatured calf thymus DNA instead of \textit{E. coli} DNA gave very different results (Figure 9B). This result resembles that of Drahovsky & Morris in showing a striking difference between native and denatured DNA. With native calf thymus DNA, enzyme activity is inhibited at all salt concentrations above zero, 50% inhibition being achieved at 40 mM NaCl; salt concentrations above 0.2M reduce methylase activity by more than 90%. Using denatured DNA, on the other hand, enzyme activity was stimulated by salt concentrations up to 90 mM, and 50% inhibition required a salt concentration of 175 mM. The stimulation observed with denatured calf thymus DNA was much less than with \textit{E. coli} DNA, and occurred at rather lower salt concentrations.

It has been possible to rationalise these findings in the light of some experiments by Adams (for full details see Turnbull & Adams, 1976). When the "native" \textit{E. coli} DNA used in the previous experiment was treated with nuclease from \textit{N. crassa} (which digests only single-stranded nucleic acid), about 25% of this DNA (as measured by \textit{E}_{260}) could be rendered acid-soluble (i.e. 25% of the DNA consisted of single stranded
Figure 9
Effect of NaCl on methylation of DNA.
Assays were carried out in a total volume of 0.2 ml, using 40 μg of DNA and 50 μl of enzyme Fraction V (Figures 9A and 9B) or Fraction III (Figure 9C).

Figure 9A - E.coli DNA
Figure 9B - Calf thymus DNA
Figure 9C - Native calf thymus DNA and crude enzyme (Fraction III)

<table>
<thead>
<tr>
<th>DNA</th>
<th>Enzyme</th>
<th>$^3$H dpm incorporated/hour</th>
</tr>
</thead>
<tbody>
<tr>
<td>E.coli, native</td>
<td>Fraction V</td>
<td>5400</td>
</tr>
<tr>
<td>E.coli, denatured</td>
<td>Fraction V</td>
<td>8400</td>
</tr>
<tr>
<td>Calf thymus, native</td>
<td>Fraction V</td>
<td>9000</td>
</tr>
<tr>
<td>Calf thymus, denatured</td>
<td>Fraction V</td>
<td>6700</td>
</tr>
<tr>
<td>Calf thymus, native</td>
<td>Fraction III</td>
<td>1600</td>
</tr>
</tbody>
</table>
material). Now, if the DNA was enzymatically methylated in vitro before incubation with *N. crassa* nuclease, almost 100% of the incorporated methyl label was found to be in regions susceptible to *N. crassa* nuclease; in other words, *E. coli* DNA is methylated only in single-stranded regions by the enzyme. By contrast, when native calf thymus and L929 cell DNA were used as substrates less than 10% of incorporated methyl label was found to be in single-stranded regions. Controls of fully denatured calf thymus and L929 cell DNA showed all incorporated methyl label to be susceptible to *N. crassa* nuclease digestion.

Presumably the different salt effects observed with calf thymus and *E. coli* DNA are connected with the fact that methylation of *E. coli* DNA takes place almost exclusively in single-stranded regions, whereas with calf thymus DNA the enzyme does act on double-stranded material. It is interesting to note that the salt effect observed with "native" *E. coli* DNA is qualitatively very similar to that found with denatured DNA from both *E. coli* and calf thymus, whereas the effect on truly native calf thymus DNA is entirely different.

Why the enzyme should select single-stranded sites in one type of DNA and double-stranded sites in others is obviously a very interesting question. There also remains the likelihood that the type of interaction between enzyme and DNA is fundamentally different depending on whether the DNA is native or denatured. Drahovsky & Morris have argued that salt influences the unwinding of native DNA, which is a necessary preliminary
to methylation; it is also possible that salt may affect the enzyme in such a way as to alter its substrate preference between single- and double-stranded DNA. These ideas were examined in the course of the work described later in this thesis.

3.5. The nature of the interaction between enzyme and DNA

As explained in the Introduction (Section 3.3), Drahovsky & Morris have postulated a mechanism whereby rat liver DNA methylase forms a strong DNA-enzyme complex which, once formed, is resistant to dissociation by salt or competing DNAs. One of their reasons for reaching this conclusion was that the inhibiting effect of NaCl on the methylation reaction (see above) could be greatly reduced by incubating enzyme and DNA at 37°C for a short time before adding salt, suggesting that an initial binding reaction is the salt-sensitive stage. Once this has taken place, the complex resists dissociation by NaCl. Also, since methylation proceeds normally, the complex must remain intact throughout the reaction, as reformation of new complexes would be impossible in the presence of salt.

To test this idea with the ascites enzyme, the salt concentration experiment with native calf thymus DNA described in the previous section was repeated, this time with enzyme and DNA incubated together at 37°C for 5 min before adding SAM and NaCl to the reaction mixture (Figure 9B). In this case pre-incubation had no effect on inhibition by salt. This experiment therefore does not support the idea that ascites DNA methylase forms a tight complex with DNA and remains bound throughout the reaction.
To exclude the possibility that SAM might be required for binding to take place, the experiment was repeated with SAM present from the outset. No difference was observed in the result.

One obvious difference between the present experiments and those of Drahovsky & Morris is that they used a much less pure enzyme preparation than the ascites enzyme employed here. It is therefore possible that their result depended on the presence in the crude preparation - and also in the intact cell - of accessory factors without which the enzyme cannot bind to DNA. To test this possibility, the pre-incubation experiment was repeated once more, this time using a less pure ascites preparation (dialysed Fraction III). As can be seen in Figure 9C, this produced no difference in the result, so the lack of binding to DNA by the ascites enzyme is apparently not an artefact of purification.

Similar information about the interaction of enzyme and DNA should also be provided by examining the effect of competition between a substrate DNA and another DNA which cannot be methylated by the enzyme, but may still be able to interact with it. This should have an analogous effect to that of salt on the reaction.

An experiment was carried out using calf thymus DNA (native) as substrate and DNA from bacteriophage T4 as the non-methylatable competitor. (Table 5). When both DNAs are present from the start of the incubation under conditions
where enzyme is limiting, methyl group incorporation into calf thymus DNA is reduced to about 20% of the value obtained in the absence of T4 DNA, showing that the latter is able to interact with the enzyme in some way. Pre-incubation of the enzyme with calf thymus DNA before adding T4, or with T4 before adding calf thymus, made little difference to the degree of inhibition. Now if methylation depended on an initial irreversible binding between enzyme and DNA, one would expect pre-incubation with calf thymus DNA completely to abolish the inhibitory effect of T4 DNA, while pre-incubation with T4 DNA should cause virtually complete inhibition of the reaction. (Since both DNAs are in considerable excess, as shown by the lack of stimulation upon increasing the amount of calf thymus DNA in the assay, complex formation by the first DNA in each case would leave no enzyme free to bind to the second DNA). That neither of these effects is observed argues against the formation of a tightly-bound DNA enzyme complex by ascites DNA methylase.

Table 5: Competition between calf thymus and T4 DNA as substrates for methylase

<table>
<thead>
<tr>
<th>DNA added at O min.</th>
<th>Activity (p moles CH₃ incorporated/2h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 min</td>
<td></td>
</tr>
<tr>
<td>Calf thymus</td>
<td>1.70</td>
</tr>
<tr>
<td>T4</td>
<td>0</td>
</tr>
<tr>
<td>Calf thymus + T4</td>
<td>0.35</td>
</tr>
<tr>
<td>T4</td>
<td>0.22</td>
</tr>
<tr>
<td>Calf thymus</td>
<td>0.33</td>
</tr>
<tr>
<td>Calf thymus</td>
<td>1.55</td>
</tr>
<tr>
<td>Calf thymus</td>
<td></td>
</tr>
</tbody>
</table>

Both DNAs were added to assays in aliquots of 40 µg, and 50 µl of enzyme Fraction VI was present.
3.6. Lack of interaction between enzyme and DNA-cellulose

Further circumstantial evidence for lack of binding between the enzyme and its substrate was obtained in an attempt to purify the enzyme using DNA cellulose. This material has been successfully used in the purification of a number of proteins and enzymes which bind to DNA, such as bacterial DNA polymerase (Litman, 1968), and RNA polymerase (Bautz & Dunn, 1971). On the assumption that there would be some binding between methylase and DNA, it therefore seemed reasonable to apply this technique to the purification of DNA methylase.

A 5 ml sample of enzyme (Fraction VI) was applied to a DNA-cellulose column, prepared according to the method of Bautz & Dunn. After applying the sample, the column was washed successively with buffer M containing 0.15 M, 0.4 M, 0.6 M and 0.8 M NaCl and fractions collected in each case until the OD at 280 nm was less than 0.05. Fractions possessing the highest ODs in each case were pooled and assayed for DNA methylase and protein.

As shown in Table 6, this procedure resulted in about 92% of enzyme activity being eluted from the column at 0.15 M NaCl, and none at all emerging at ionic strengths above 0.4 M. Since proteins that bind strongly to DNA are commonly eluted from DNA-cellulose columns at salt concentrations of 0.5 M or more (see, for example, Alberts et al, 1968), it would appear that any binding which does take place between the methylase DNA must be weak and easily reversible.

* The sample was made 0.13M with respect to NaCl before application to the column.
3.7. Conclusion

The experiments described in Section 3 may be summarised as follows:

(1) The rate of methylation by ascites DNA methylase in vitro depends on the source of the DNA substrate. Methylation is generally greatest with DNA from rapidly growing cells; presumably this is a reflection of the greater number of unmethylated sites in such DNA (see Introduction, Section 4.2). The very high rate at which the enzyme can react with undermethylated L929 cell DNA suggests that it is able to methylate sites which are normally methylated in vivo. It is also interesting that, because this DNA is prepared in such a way that virtually all the undermethylation is on one strand, the enzyme is presumably recognising hemimethylated sites, which are known to be favoured substrates for bacterial modification methylases (see Introduction, Sections 2.4 and 5.3).

(2) "Native" E.coli DNA - quantitatively the best substrate for the enzyme apart from denatured E.coli DNA and undermethylated L929 cell DNA - is methylated by the enzyme only in single-stranded regions while methylation of other DNAs tested took place mostly, if not exclusively, in double-stranded material. This raises questions as to what type of specificity the enzyme shows with regard to binding sites and/or methylation sites in both single and double-stranded DNA.
(3) The reaction with native calf thymus DNA is much more sensitive to salt than the reaction with denatured DNA, as Drahovsky & Morris have already reported for the rat liver enzyme. Possible reasons for this will be discussed later.

(4) The available evidence for the ascites enzyme does not support the type of mechanism suggested by Drahovsky & Morris for the rat liver methylase, whereby enzyme and DNA from a tight complex at the start of the reaction and remain bound together until methylation is complete. The results are more consistent with a loose, reversible binding going on throughout the reaction.

Table 6: Interaction of enzyme with DNA-cellulose

<table>
<thead>
<tr>
<th>NaCl concentration in eluant (M)</th>
<th>% total enzyme eluted</th>
<th>% total protein eluted</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.15</td>
<td>91.6</td>
<td>70.1</td>
</tr>
<tr>
<td>0.4</td>
<td>8.4</td>
<td>23.3</td>
</tr>
<tr>
<td>0.6</td>
<td>0</td>
<td>6.6</td>
</tr>
<tr>
<td>0.8</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

100% enzyme activity corresponds to 3700 dpm/assay.
4. SIZE AND SUBUNIT COMPOSITION OF THE ENZYME

4.1. Introduction
As discussed in Section 1.3, upon gel filtration of a crude enzyme preparation under low salt conditions, methylase activity apparently aggregates with itself or with other proteins and is eluted from the column in the excluded volume of Sephadex G-200. When eluted with buffer containing 0.4 M salt, however, the enzyme emerges in a position very close to a catalase marker, suggesting an apparent molecular weight in the region of 230,000. Presumably the aggregate found at low salt is dissociated under these conditions.

The question which immediately arises is whether this difference is caused by an effect on the enzyme molecule itself - e.g. dissociation into subunits in the presence of salt - or merely reflects gross non-specific aggregation of proteins in what is still a very impure preparation. It was therefore of interest to determine whether salt would have a similar effect on a more highly purified enzyme fraction, and also to try to gain a more accurate quantitative estimate of the enzyme's molecular weight than can be obtained from gel filtration.

4.2. Sucrose density gradient analysis
The principle of this experiment was to sediment the enzyme on sucrose gradients both in the presence and in the absence of 0.4 M salt, and then to assay each fraction for DNA methylase activity under the same salt conditions and with the same DNA
substrate (denatured calf thymus DNA at 0.1 M salt - see Section 3.3.). This should therefore show whether the presence of salt causes a change in the sedimentation behaviour of the enzyme without introducing any difference due to assay conditions. The experiment was carried out using both a relatively crude methylase preparation (redissolved ammonium sulphate pellet, Fraction III) and the usual purified enzyme (Fraction VI), for the reasons outlined in Section 4.1.

Figure 10 shows the results of this experiment. When gradients are run in 0.4 M salt, the enzyme activity sediments somewhat behind the catalase marker (molecular wt. 230,000); this result is the same for both crude extract and purified enzyme. Under low salt conditions, the apparent molecular weight of the methylase becomes greater than that of catalase, and the crude extract (Figure 10A) yields a much faster-sedimenting species than the purified preparation (Figure 10B). Another noteworthy feature is that the large, "low salt" species in both cases shows considerably lower activity than the smaller "high salt" species, in spite of both being assayed under the same conditions.

It does therefore appear that the purified enzyme as well as the crude ammonium sulphate extract is of smaller size in the presence of salt than in salt-free conditions. The fact that the high salt form from both preparations is of the same size while the low salt form is considerably larger in the case of
Figure 10

Sucrose density gradient analysis of DNA methylase

0.2 ml of enzyme (diluted 1:1 with 50 mM tris, pH 7.8) was applied to a 5-20% (w/v) sucrose gradient, prepared as described in Materials and Methods. The gradient was centrifuged at 78,000 g for 14 hours in the Beckman SW56 rotor. 6-drop fractions were collected, starting from the top of the gradient.

10A Centrifugation of ammonium sulphate fraction

10B Centrifugation of purified enzyme (Fraction VI)

--- O --- O --- No salt present in gradient
--- O --- O --- 0.4 M NaCl present
the crude preparation than in the pure preparation could suggest that in the crude extract the methylase aggregates with heterogeneous proteins which are removed during the purification procedure. In the purified enzyme, the aggregate is certainly smaller and is also much less likely to be heterogeneous, because two stages of the purification (Ultrogel and phosphocellulose) have involved salt conditions at least as severe as those used in the gradients. The aggregate would therefore dissociate, and other proteins involved in it would require to have very similar properties to DNA methylase in order to co-purify with the enzyme throughout all stages of the process. It therefore seems likely - though not proven - that the large species found at low salt with the purified enzyme could be a dimer of the smaller species (molecular weight about 160,000) found at high salt.

The discrepancy in the estimated size of the smaller species between the gradient result (160,000) and the Ultrogel result (230,000) probably arises from the fact that many proteins, especially those deviating significantly from a spherical shape, are known to run anomalously fast on gel filtration. Less easy to explain is the much lower activity shown by the large "low salt" species. One might suggest that one of the proteins bound to the enzyme is inhibitory, but whatever the initial gradient conditions, all the enzyme assays were carried out at 0.1 M salt, at which level enzyme activity with denatured calf thymus DNA is stimulated (see Section 3.3.). Both "high salt"
and "low salt" forms of the enzyme would therefore be expected to show the same activity when assayed at 0.1 M salt. To check the possibility that this low activity was caused by some property of the gradient (e.g. presence of sucrose, absence of glycerol), it was decided to repeat the analysis using glycerol density gradients (glycerol is a normal component of the assay buffer).

4.3. Glycerol density gradient analysis
As shown in Figure 11A, the methylase activity profile obtained by running the purified enzyme on a 10-30% glycerol density gradient was essentially the same as that found on sucrose gradients: in low salt, the enzyme activity sediments rather faster than catalase, while in high salt it runs more slowly than catalase. Once again the larger species obtained at low salt had much lower activity than the smaller species obtained at 0.4 M salt, so that this phenomenon does not appear to be an artefact introduced by the gradient material although the true explanation for it remains obscure.

4.4. Glycerol density gradients in the presence of SAM
One further possibility might be that the presence of SAM is required before the enzyme can assume its active form (whether this involves association or dissociation), so that the forms isolated from density gradients or columns might not represent the truly active form of the enzyme.
Glycerol density gradient analysis of DNA methylase.

0.2 ml of purified enzyme (diluted 1:1, with 50 mM tris, pH 7.8) was applied to a 10-30% glycerol gradient, prepared as described in Materials and Methods. The gradient was centrifuged at 78,000 g for 14 hours in the Beckman SW56 rotor at 4°C. 6-drop fractions were collected, starting from the top of the gradient.

11A  Gradient run with no additions

11B  Gradient run in presence of 70 μM SAM

--○---○--- No salt present

---●---○  0.4 M NaCl present
To test this idea, the glycerol gradient was repeated with unlabelled SAM (70 µM) present in the gradient. The amount of labelled SAM used in the enzyme assays was adjusted so that the final concentration and specific activity of SAM were the same as in the standard assays.

Figure 11B shows that the position of methylase activity in the gradient was unaffected by this procedure, as was the difference in activity between large and small species. SAM therefore does not play a part in determining the structure of the enzyme.

4.5. Conclusion

The results of both gel filtration and density gradient centrifugation indicate that the active form of ascites DNA methylase has a considerably higher molecular weight (over 300,000) under low salt conditions than under high salt conditions, where the apparent molecular weight is about 160,000. The high molecular weight form obtained from a crude enzyme preparation appears larger than that obtained from a highly purified preparation, suggesting a greater degree of non-specific aggregation in the former case. The latter species is more likely to consist of an association of the small enzyme species into dimers, although this has not been conclusively proven by these experiments. The most obvious simple approach to this problem, gel electrophoresis, suffers from the disadvantage that enzyme activity could not be recovered from the gel and it would therefore be impossible to tell which band(s) on the gel represented the enzyme.
It is also not practicable to examine the effect of interaction with DNA on the enzyme, since no firm binding between enzyme and DNA could be demonstrated (Sections 3.4 and 3.5). Formation of a stable DNA-enzyme complex would provide a useful probe to separate the enzyme from other proteins in the preparation.
DISCUSSION
1. **GENERAL**

1.1. **Introduction**

Using the procedure described in Materials and Methods, a relatively pure DNA methylase can be obtained from Krebs II ascites cells. Some general properties of the enzyme have been studied, mainly in order to establish its credentials as a genuine DNA methylase. (Results, Section 2). Other work described in this thesis falls into three main categories - substrate specificity, mechanism of action and analysis of subunit composition. From these results, some tentative conclusions will be drawn about the control of DNA methylation. Possible reasons for the difference between the mechanism of the ascites enzyme and that of the rat liver enzyme (Drahovsky & Morris, 1971a and b) will be discussed. A model will also be suggested for the differing interactions of the enzyme with native and denatured DNA, based on the size and subunit data.

1.2. **Characteristics of the purified enzyme**

The extent of purification achieved using the standard procedure is of much the same order as that reported by Roy & Weissbach (1975) for the HeLa cell enzyme. The purified ascites enzyme does not methylate RNA to a significant extent, although purely indirect evidence suggests that it does include some protein methylase activity (omission of the phenol/m-cresol extraction from the standard assay procedure results in
a considerable increase in the incorporation of methyl groups into acid-insoluble material). The only methylated nucleotide produced by the enzyme is 5-methylcytosine in DNA, and no methylation can be detected if no substrate DNA is present in the incubation. These two results confirm the genuineness of the DNA methylase activity and show that the methylation observed is not due to DNA present in the enzyme preparation.

There is no compelling evidence at any stage of the purification procedure for more than one species of the enzyme (although aggregation occurs at low ionic strength). The presence of a single, sharp pH optimum also indicates a unique enzyme species. The HeLa cell (Roy & Weissbach, 1975) and Novikoff hepatoma cell enzymes (Sneider et al, 1975) also appear to consist of a unique species, in contrast with the series of DNA methylases found in the H. influenzae restriction-modification system. As will be explained in Section 5.2, the presence of only one methylase species has implications for the control of DNA methylation in eukaryotic cells.

2. **THE SIGNIFICANCE OF THE SUBSTRATE SPECIFICITY OF THE ENZYME**

2.1. **Methylation of Heterologous DNAs**

It seems safe to assume that the extent to which a given DNA can be methylated *in vitro* by an isolated DNA methylase will provide a measure of how many methylatable sites are left
unmodified \textit{in vivo} - though the reason for this lack of methylation may remain an open question. It is therefore interesting that \textit{E. coli} DNA was the best methyl acceptor among those DNAs tested with the ascites enzyme (apart from the artificially undermethylated L929 cell DNA). Bacterial DNAs have been found in the past to be very good substrates for mammalian DNA methylases (Drahovsky & Morris, 1972; Roy & Weissbach, 1975; Sneider et al, 1975), and this has been correlated with both the high G+C content and low 5-methylcytosine content which are common in bacterial DNA. However, Drahovsky & Morris found that the correlation of \textit{in vitro} methylation with G+C content did not always hold. Clearly, bacterial DNA methylases do not recognise the same type of sites as animal cell enzymes - for one thing, they methylate adenosine as well as cytosine, unlike the eukaryotic methylases. It is known that the methylases of bacterial restriction-modification systems recognise highly specific sites of several base pairs in length. It could be that the site recognised by mammalian enzymes is simpler and therefore occurs more frequently in the bacterial DNA than the restriction-modification sites. (See Section 2.2 for further discussion).

When DNAs from a variety of animal tissues were tested as substrates, it did appear that those isolated from rapidly-growing cells are better substrates than those from slow-growing cells. If the experiment described in Section 3.3 of Results were continued until all the DNAs were fully saturated
with methyl groups, it should be possible to obtain at least a rough estimate of the degree of undermethylation of these DNAs by expressing the saturation level of methyl incorporation in vitro as a fraction of the overall level of 5-methylcytosine in the DNA, as reported in the literature. Such an experiment has been described by Turnbull & Adams (1976). It was found that the undermethylated mouse L929 cell DNA would accept methyl groups on 9.1% of its methylatable cytosines (calculated from the 5-methylcytosine content of normal L929 cell DNA). Assuming that all these methyl groups were incorporated on one strand (because of the method of preparation of the DNA), this figure agrees quite well with the overall methyl deficiency in the DNA calculated by base analysis, which came to 19%. This gives reason for confidence that the ascites enzyme does act upon sites which would be methylated in vivo.

While the different methyl accepting abilities of various DNAs can be plausibly explained by the existence of a time lag between synthesis and methylation, which would cause more sites to be unmethylated in DNA from rapidly dividing cells, it is perhaps equally plausible to suggest that the DNAs have a varying content of specific, unmethylated sites which may serve as recognition signals for the binding of regulatory proteins. Masking of methylation sites by these proteins would prevent methylation in the intact cell, but the sites would of course be available in isolated DNA as used in the in vitro experiments.

*This figure represents the methyl deficiency in the daughter strand of the DNA synthesised during methionine deprivation.*
This interpretation would also explain the methylation of homologous DNA, a common property of mammalian DNA methylases but not of the bacterial enzymes. Further implications of this suggested control mechanism will be considered later (Section 5.2).

2.2. Methylation of double and single-stranded DNA

Bacterial modification methylases, which are in many ways the best understood DNA methylation systems, are notable for three basic properties; they act only on double-stranded DNA, they recognise highly specific sites in the DNA, and they act very slowly on a totally unmethylated site but much more rapidly on a hemi-methylated site (i.e. methylated in one strand). It is instructive to find out if any of these properties are shared by animal cell DNA methylases, particularly since some proposals for the function of DNA methylation in animals depend on the enzymes exhibiting some or all of these properties (see Introduction, Section 5.3).

It seems to be generally true that denatured DNA can act as a substrate for isolated mammalian DNA methylases (Drahovsky & Morris, 1971b; Roy & Weissbach, 1975; Sneider et al., 1975), and the ascites enzyme is no exception to this finding. In common with the rat liver enzyme of Drahovsky & Morris, the interactions of the ascites methylase with native and denatured DNA seem to be quite different, as judged by their response to salt inhibition; possible reasons for this will be discussed later (Section 3).
A much more unusual feature of the ascites enzyme is the fact that it methylates *E. coli* DNA almost exclusively in single-stranded regions, whereas the eukaryotic native DNAs tested are methylated in double-stranded regions. The consequences of this are that "native" *E. coli* DNA (which in the preparation used contains some 15-25% single-stranded material) behaves in the same way as fully denatured DNA in its response to salt, and that fully denatured *E. coli* DNA is a rather better substrate for the enzyme than "native" DNA. Treatment of the "native" DNA with *N. crassa* nuclease to remove the single-stranded regions results in a drastic reduction of its methyl-accepting ability. (Turnbull & Adams, 1976). Native calf thymus DNA, on the other hand, is a better substrate (under low salt conditions) than is denatured calf thymus DNA. Evidently, therefore, the methylation of native DNA by the enzyme requires recognition of a specific double-stranded site which does not exist to a significant extent in native *E. coli* DNA. What is the likely nature of this site?

It has been found that in animal cell DNA the distribution of methyl groups is non-random; 5-methylcytosine is found predominantly in the nucleotide doublet CpG (Doskočil & Žorm, 1961 and 1962; Grippo et al, 1968). More recent studies confirm this finding and suggest that the sequences adjacent to the methylated CpG doublets are random (Browne et al, in the press). Now it will be recalled that undermethylated mouse *L929* cell DNA proved to be an extremely good substrate for the
ascites enzyme. (Results, Section 3.3). Because of the way in which it is prepared, this DNA should be undermethylated in only one strand. Enzymatic methylation of this DNA in vitro would therefore occur at hemimethylated sites of this type:

\[
\begin{align*}
5' & - - - \text{CpG} - - - 3' \\
3' & - - - \text{GpmC} - - - 5'
\end{align*}
\]

(mC = 5-methylcytosine)

It seems reasonable to postulate that this hemimethylated site is required for recognition by animal cell enzymes. This is plausible since methylation in animal cells normally takes place on recently synthesised DNA, which of necessity would contain hemimethylated sites. Also, the fact that CpG in double-stranded DNA forms a self-complementary sequence would ensure the passing on of an unaltered methylation pattern from one generation to the next. By analogy with bacterial modification methylases, it is probable that a totally unmethylated site will not be methylated by the enzyme (or at most very slowly). Thus if most of the CpG sites in native E. coli DNA were unmethylated in both strands this could account for the very low methylation of this DNA by the enzyme. It has been argued in the previous Section that the sites recognised by bacterial DNA methylases are almost certainly different from those recognised by the animal cell enzymes, so any sites of the latter type occurring in bacterial DNA will not have been methylated in vivo.

If this is the explanation, then why does the enzyme methylate
single-stranded E. coli DNA (or any other single-stranded DNA)? It will be argued in Section 4 that some of the data presented in this thesis are most easily interpreted by proposing that methylation of native and denatured DNA are carried out by different forms of the enzyme, one of which might be specific for the hemimethylated self-complementary CpG sites in native DNA, while the other acts upon CpG in single-stranded DNA.

3. MODEL FOR INTERACTION OF ENZYME WITH NATIVE AND DENATURED DNA

A common property of both the ascites DNA methylase and the rat liver enzyme (Drahovsky & Morris, 1971b) is that, while the enzymes will methylate both native and denatured DNA, the effect of salt on the two reactions is sharply different. In the absence of salt, double-stranded DNA is methylated at a higher rate than single-stranded by both enzymes. As the salt concentration is increased, however, the rate of methylation of double-stranded DNA by the ascites enzyme falls off sharply and is reduced by 50% at a salt concentration of only 40 mM, whereas the methylation of single-stranded DNA is stimulated at salt concentrations up to 100 mM (with maximum stimulation at 70 mM NaCl) and at higher salt concentrations falls off relatively slowly. (The results obtained by Drahovsky & Morris with the rat liver enzyme were similar).
In seeking an explanation for this effect the first question to be answered is whether the action of salt is to bring about some change in the enzyme molecule, or whether it affects the configuration of the DNA. Drahovsky & Morris concluded that the binding of the rat liver enzyme to native DNA required some local unwinding of the DNA helix, which is inhibited by salt, and obviously is not necessary with denatured DNA. Their reasoning can be summarised as follows. They had shown that an essential step in methylation was the formation of a tightly-bound DNA-enzyme complex which did not dissociate till the reaction was complete. Since salt does not inhibit the methylation of single-stranded DNA, the binding of enzyme to single-stranded DNA also is presumably unaffected by salt. Evidence for local unwinding of native DNA in the process of binding to the enzyme comes from the observation that, in an incubation mixture containing both native and denatured DNA, the enzyme binds preferentially to denatured DNA, and also from the fact that binding of enzyme to native DNA is strongly temperature-dependent while binding to denatured DNA is not. This is consistent with, but does not prove, the idea of local unwinding of DNA during methylation, though it should be remembered that the enzyme may require to unwind DNA at the methylation site, but not at the initial binding site.

The essential starting point for this hypothesis is the finding that the enzyme binds irreversibly to DNA at the outset of the reaction. As has already been discussed (Results, Sections 3.5 and 3.6), there is no evidence for the formation of such a complex by the purified ascites enzyme. There is therefore
no pressing reason to believe that the action of salt is exerted on the DNA helix. In addition, there is other evidence to suggest that increasing salt concentration produces a major physical change in the ascites enzyme molecule.

The results of gel filtration, as well as of sucrose and glycerol density gradient centrifugation, show that at high salt concentrations the apparent size of the enzyme is decreased. This would reflect the dissociation of an aggregate, either between subunits of the enzyme or between the enzyme and other proteins. As mentioned in Results (Section 4.5), the former is more likely to be the case, at least with the purified enzyme, because various stages of the purification process are likely to have broken up any non-specific aggregation that may have existed.

Since the approximate molecular weights (from density gradient analysis) of the large and small species are just over 300,000 and about 160,000 respectively, it seems reasonable to postulate that the enzyme exists at low salt as a dimer which dissociates at higher salt concentrations into subunits of molecular weight about 160,000 which still possess methylase activity. This could explain the different pattern of salt inhibition with native and denatured DNA, according to the following model. The dimeric form of the enzyme which exists under low salt conditions has a strong preference for native DNA as substrate. On the addition of salt (even at relatively low concentrations) the enzyme dissociates into the smaller subunits, which show greater activity with denatured DNA as substrate. This would explain
why moderate salt concentrations have a positive stimulatory effect on the reaction with denatured DNA, while at the same time causing drastic inhibition of the methylation of native DNA (at 70 mM NaCl, methylation of denatured DNA is maximally stimulated, whereas methylation of native DNA is reduced by almost 70%). Higher salt concentrations (0.1-0.4 M) inhibit both reactions, presumably by affecting the interactions between enzyme, SAM and DNA (note that, while density gradients were run at either 0 or 0.4 M NaCl, all assays were carried out with denatured calf thymus DNA at 0.1 M NaCl). Since some degree of methylation of both types of DNA does take place, at all but the most extreme salt concentrations, it seems probable that an equilibrium exists at all times between the monomer and the dimer, and that the position of this equilibrium is altered by the presence of NaCl. This explanation is favoured over the situation where one or other form of the enzyme is totally inhibited by the presence or absence of salt.

The unusual results obtained with E. coli DNA are interesting in the context of this model. Methylation of "native" E. coli DNA by the enzyme occurs almost exclusively in single-stranded regions, which constitute up to 25% of the total DNA. This "native" DNA shows the same pattern of salt stimulation as fully denatured E. coli DNA (see Figure 9), but the degree of stimulation with the "native" DNA is quantitatively rather less. This is consistent with the model, since total denaturation of the DNA would serve to make available more single-stranded DNA for methylation. In addition, it has been suggested in Section 2.2
that native E.coli DNA is not methylated by the enzyme because the latter requires a hemimethylated site in native DNA, and sites of this kind are absent from E.coli DNA. The fact that the enzyme readily methylates single-stranded DNA from both E.coli and other sources does not at first sight fit in with the requirement for a hemimethylated site, but this is easily rationalised if different forms of the enzyme are responsible for methylating native and denatured DNA.

The foregoing discussion has implicitly assumed that the two subunits are identical, but it is just as likely that they are non-identical. One of them could be a "recognition" subunit, which could recognise only double-stranded hemimethylated sites, while the other, "methylation" subunit would methylate single CpG doublets only when the "recognition" subunit had bound to a hemimethylated site. The effect of separating the subunits would be that the "methylation" subunit would methylate only CpG sites in single-stranded DNA. There is a precedent for this theory in that type 1 restriction/modification systems are known to be multimeric proteins with separate subunits for the recognition, modification and restriction functions.

4. MECHANISM OF METHYLATION OF NATIVE DNA

The only detailed studies on the mechanism of action of a DNA methylase have been those of Drahovsky & Morris using the partially purified rat liver enzyme (Drahovsky & Morris, 1971a and 1971b; 1972). In the first of these papers, evidence (summarised in Introduction, Section 3.3) was presented that a
necessary first step in methylation is the temperature-dependent formation of a tightly-bound complex between enzyme and DNA, and that methylation proceeds by means of the enzyme molecule "walking" along the DNA, scanning for methylation sites as it goes, and not leaving the DNA until methylation is complete. This behaviour is comparable with that of other enzymes which interact with nucleic acids and show temperature-dependent formation of complexes with their templates, e.g. *E. coli* RNA polymerase holoenzyme (Hinkle & Chamberlin, 1970; Zillig et al, 1970) and Qβ RNA polymerase (Silverman & August, 1970).

Similar investigations with the purified ascites enzyme have failed to provide any evidence for the formation of a tightly bound DNA-enzyme complex. Pre-incubation of enzyme and DNA did not abolish the inhibition of the reaction by salt, in contrast with the results of Drahovsky & Morris. In recognition of the fact that the enzyme preparation used by these authors was much less highly purified than the ascites enzyme used in the present work, the experiment was repeated using a relatively crude ascites methylase preparation (Fraction III) to check for the presence of possible binding factors which are removed on further purification. No difference was observed. In addition, pre-incubation of enzyme with DNA did not diminish the effect of competition for binding between substrate DNA and non-methylatable T4 DNA. Both these results indicate that a stable complex has not been formed between enzyme and DNA during the 5 minutes of pre-incubation. Also, no interaction could be shown between
the enzyme and DNA-cellulose (although this is less definite evidence, since the experiment was carried out at 4°C and the binding, if any, might be temperature-dependent).

Obviously some form of binding between enzyme and DNA has to take place to make methylation possible, but from these results the binding must be weak and transient. It would appear that the enzyme binds loosely and reversibly to DNA and that methylation occurs only when it is bound to a methylatable site. Such a binding is likely to be quite random, rather than occurring only at specific sites, for the following reason. It will be argued later (Section 5.2) that the methylation site recognised by mammalian DNA methylases is likely to be no more complex than a self-complementary CpG doublet, and that whether or not specific CpG sites are methylated is determined solely by regulatory factors (presumably proteins) within the cell. If this is so, then a highly specific interaction of the enzyme with a particular sequence of DNA is surely redundant.

What are the merits and demerits of the two models for the mechanism of DNA methylation? Drahovsky & Morris have argued that the "walking" model, by providing an orderly scan of the DNA helix, would be less prone to errors and to missing methylation sites than a more random type of interaction. This may be true, but it is difficult to reconcile such a model with the
actual situation in vivo, where DNA is intimately associated with chromosomal proteins which are bound to obstruct the orderly movement of an enzyme molecule along the DNA. Repeated random enzyme-DNA binding events on those sections of the DNA which are unmasked by chromosomal proteins would seem well adapted to this situation, particularly in view of the results of Molitor et al (1976), which suggest that unmethylated sites may occur in definite clusters at intervals on the mammalian genome. Such clusters could represent definite regions of the chromosome which are obscured by proteins during the methylation process.

The "walking" model introduces several other complications which are absent from the "on-off" mechanism. Firstly there is the question of how the enzyme specifically recognises one end of a DNA molecule. This is unlikely to be dictated by a specific base sequence, since the methylation of sonicated DNA of varying molecular weights still shows kinetics which are consistent with the enzyme traversing the whole length of the molecule (Drahovsky & Morris, 1971a). A further question which emerges is how energy is obtained to drive the scanning process. RNA polymerases, which are thought to move along their DNA templates, obtain energy by hydrolysis of pyrophosphate liberated during polymerisation. Since SAM is a high energy compound, with a free energy of hydrolysis roughly comparable with that of ATP, the hydrolysis of SAM during the methylation reaction could be a potential energy source for translocation of the enzyme, although the small number of methylation sites in the DNA (theoretically about one every 100 base pairs) would make the overall energy yield quite small. Alternatively, if one
molecule of SAM were hydrolysed for every base pair traversed, giving an energy yield of the same order as polymerisation, the process would be somewhat wasteful of SAM.

It can be argued against the "on-off" hypothesis that it does not make the most economical use of enzyme, since the avoidance of errors and omissions would require a fairly high enzyme:DNA ratio.

Whichever of these two models is favoured, it must be remembered that all these experiments were carried out using naked DNA and isolated enzyme, and that the characteristics of the reaction in the intact cell may be very different, bearing in mind the highly organised nature of the mammalian chromosome. Nevertheless the knowledge that the ascites enzyme does not possess a strong affinity for particular sites in DNA is useful in formulating ideas about how methylation is controlled, and does seem to agree with a type of mechanism whereby the location of methylated sites is determined by cellular regulatory processes rather than by interaction of the enzyme with specific sequences in DNA.

5. REGULATION AND FUNCTION OF DNA METHYLATION IN ANIMAL CELLS

5.1. Reasons for suggesting a regulatory role
There are a number of largely indirect reasons for thinking that DNA methylation in eukaryotes serves a useful function,
rather than being the result of chance or an evolutionary throwback. If methylation were unnecessary for survival, what would be the evolutionary advantage of conserving the necessary enzyme system? In any case, methylation of bacterial DNA is known to play a vital part in the restriction-modification system (though this appears to require only a small proportion of all the methylated bases present), so it seems reasonable to look for an equally important, though not necessarily similar, function in animals. The wide variation in methylation levels between different cell types, and the close link between synthesis and methylation of DNA, point to the possibility that DNA methylation plays some part in cellular processes, perhaps in transcriptional control. At the level of DNA structure, the introduction of methyl groups affects the geometry of the major groove of the double helix, and this could upset the binding of regulatory proteins, by analogy with the E. coli lac repressor (Lin & Riggs, 1972). There is also evidence that methylation of nucleotides in the 5-position has a stabilising effect on the helix (Pietrzykowska & Shugar, 1966) presumably because of some hydrophobic interaction, and this could affect processes such as replication and transcription which are known to involve some degree of unwinding of the helix.

5.2. How is methylation controlled?

Since only a very small proportion of cytosine residues in animal cell DNA are methylated, the process must be highly site-specific. From the point of view of deciding what function is served by DNA methylation, it would be useful to know what
properties distinguish methylated sites from non-methylated ones.

As already mentioned in Section 2.2, the distribution of methyl groups in animal cell DNA is quite specific; 5-methylcytosine is found predominantly in the sequence CpGp after hydrolysis of calf thymus DNA with spleen DNase II (Doskocil and Sorm, 1961 and 1962). Some recent comparative work has extended this conclusion to other species (Browne et al, in the press). Several different cultured cell lines were grown in the presence of \[^{3}H\text{-CH}_3\] methionine. The isolated DNA was depurinated and the distribution of the incorporated methyl label in pyrimidine tracts was determined by fingerprinting. The results showed a remarkable similarity between all the animal cell DNAs studied; about 50% of methyl label occurs in the monopyrimidine fraction, with the rest divided among the remaining pyrimidine isopliths in the distribution which would be expected if the sites of methylation were no more specific than CpG. Furthermore, samples of E.coli and calf thymus DNA methylated in vitro using the purified ascites enzyme also showed essentially the same pattern. (For the reasons set out in Section 2.2, it is likely that the site actually recognised by the ascites enzyme is a hemimethylated self-complementary CpG sequence, rather than a totally unmethylated CpG sequence in each strand of the DNA.)

It therefore seems that CpG is the sequence in which methylation is localised in most mammalian DNAs. Yet there must be some
additional, more subtle control operating in vivo, since DNAs isolated from animal cells can generally be methylated further by isolated enzymes from either the same or different species, indicating that not all CpGs are methylated in vivo. The data of Browne et al appear to exclude the possibility that enzymes from different cell types recognise different sequences in the immediate vicinity of the methylation sites, so this leaves three main possibilities to be considered for the control mechanism:

(1) Each cell type contains a family of methylases, each recognising different sequences close to, but not immediately adjacent to, the methylation site. Methylation is controlled by switching on and off either the synthesis or the activity of these enzymes. This theory is unattractive mainly because, in the case of the ascites enzyme, there is no evidence for multiple enzyme species at any stage of the purification procedure. The possibility does exist that by using a specific type of DNA in the enzyme assay one is selecting for one particular enzyme species, but in view of the ability of the enzyme to act on a variety of DNAs this can probably be disregarded.

(2) Methylation depends on recognition, not just of a hemi-methylated CpG doublet, but of another sequence several base pairs away from the CpG as well. While each cell type has only one enzyme, the nature of the additional
recognition sequence varies from one cell type to another. No direct evidence is presently at hand either to confirm or to refute such a model. To answer this question one would require a DNA in which all the CpGs in one strand were known to be unmethylated. A small amount of this DNA could then be methylated to saturation levels using the isolated enzyme and the measured saturation level of methyl incorporation compared with the estimated total CpG content of the DNA. This would show whether or not all the CpG doublets in the DNA were being methylated. If not all CpG doublets were methylated, then this would indicate that an additional sequence is recognised by the methylase, since other possible cellular control factors are absent. Moreover, the proportion by which actual methylation fell short of the theoretical total would provide a rough statistical estimate of the length of the sequence involved. The difficulty with this experiment lies in obtaining a DNA which is totally unmethylated in one strand; for instance, the methyl deficiency in the undermethylated L929 cell DNA already described falls far short of the required level.

(3) All eukaryotic DNA methylases recognise the same simple site, and the number of sites methylated in vivo is determined by regulatory factors (presumably proteins) in the cell. The removal of these factors by isolating
DNA from the cells allows hitherto unmethylated sites to be recognised by either homologous or heterologous enzymes \textit{in vitro}. This explanation is adequate if the enzyme can recognise wholly unmethylated sites as well as the hemimethylated sites with which it is usually presented after replication \textit{in vivo}. If, however, it can recognise only hemimethylated sites - as suggested in Section 2.2 - then sites kept totally unmethylated \textit{in vivo} would still not be methylated by the enzyme \textit{in vitro}, and the different rates of methylation of different DNAs must be purely a reflection of the time lag between synthesis and methylation of DNA \textit{in vivo}. Another consequence of this situation would be that the methylation of a particular totally unmethylated site would be determined once and for all - perhaps during differentiation - and after the initial event this methylation would be preserved automatically through successive rounds of replication. Thus cellular control factors would be responsible only for the initial methylation event and not for day-to-day post-replication methylation in the cell. A suggestion will be made in Section 5.3 as to how such a process might operate, and could play a part in control of differentiation.

Some tentative evidence has been reported that DNA methylation can be affected by cellular factors other than DNA and the enzyme (Burdon & Douglas, 1974). A chromatin-like preparation, prepared by extracting nuclei of Krebs II ascites cells in low-salt buffer, catalysed incorporation of methyl groups into DNA from SAM. Addition to this
preparation of either nuclear extract, cytoplasm or cytosol fractions (none of which contain detectable DNA methylase activity) causes methylation to be stimulated. The nature of the stimulatory factors and the mechanism of the process would have to be much better characterised before any definite functional significance could be assigned to them.

5.3. Model for involvement of DNA methylation in control of differentiation

The kinetic experiments described in the Introduction (Section 4) seem to rule out any function of DNA methylation connected with stabilising newly synthesised DNA. It is also difficult to envisage a way in which a complex process such as DNA methylation could be involved in short-term control of ordinary cellular processes. Yet the orderly way in which DNA methylation follows replication suggests that conservation of methylated sites is necessary to the cell in some way.

As mentioned in the previous section, it seems likely that the initial methylation of a given site is an irreversible event, and any change in gene expression which might be brought about by methylation would therefore be permanent. Thus the theories of Riggs (1975) and Holliday & Pugh (1975) both propose DNA methylation as determining a permanent change in the cell's functions; in the former case, X chromosome inactivation, in the latter case, a molecular "clock" which records how many cell divisions have taken place (see Introduction, Section 5.3).
All suggestions in the field of control of differentiation must inevitably be speculative, but the following model takes account of most of the known properties of mammalian DNA methylation as discussed in this thesis.

It has been observed that 5-methylcytosine content is lower in sperm than in somatic cell DNA (Vanyushin et al, 1970), and also that levels of in vitro DNA methylation are 100 times higher in nuclei from sea urchin embryos at the early blastula stage than at the late gastrula stage (Scarano & Tosi, 1976). As differentiation proceeds, therefore, more methyl groups are apparently introduced into DNA. The action of methylation might therefore be in some way to switch off a gene so that it can no longer be transcribed. Suppose that in the germ cells most genes - or at least control regions thereof - are unmethylated. (This might be achieved by specific demethylation of the parental DNA during spermatogenesis and oogenesis). As differentiation proceeds, those genes not required in the function of the differentiated cell will be progressively methylated and therefore switched off. The day-to-day function of the DNA methylase will now be to maintain methylation of these genes as the cell divides, an easy operation if methylation occurs in CpG sequences.

How is this initial methylation event triggered off? If the
methylase recognises unmethylated double-stranded sites as well as hemimethylated sites, methylation could proceed simply by removal of a masking protein from the site in response to some sort of signal. It is likely, however, that the methylase recognises only hemimethylated sites, for the reasons discussed earlier. On this assumption, the initial methylation must depend on the production of some signal (perhaps a protein) which makes it possible for the enzyme to methylate a wholly unmethylated site. This signal - hereafter referred to as "helper" - would be produced only transiently at a specific time and would recognise a specific sequence in the DNA. Its mode of operation might be to separate the strands of the DNA at the methylation site, so that the methylase could recognise the site. It is only necessary to methylate one strand in the initial step - after this, methylation will proceed by the normal enzymatic process and would be conserved from one generation to another. Obviously, since control of transcription would involve whole genes, or more likely groups of genes, this process is likely to occur at a large number of sites at one time. It is interesting that Molitor et al (1976) found, by fibre autoradiography of DNA that chromosomal DNA seemed to be unmethylated in distinct regions spaced out throughout the genome, interspersed with larger methylated regions. Also, since these unmethylated sections formed about 10% of the total length of the chromosomal DNA and this is of the same order as the proportion of the mammalian genome which is complementary to RNA transcripts, it might be reasonable to suggest that those groups of genes which are transcribed are unmethylated.
What is the advantage of using methylation to keep genes switched off, rather than produce a specific binding protein, which would remain permanently attached to the DNA, instead of the "helper"? The most obvious advantage is one of economy. Large amounts of the many different DNA-binding proteins would have to be produced at each cell division, whereas each "helper" would be produced once only and the modification of the DNA preserved thereafter by the action of the methylase.

6. **POSSIBLE FUTURE RESEARCH**

The idea discussed above, that DNA methylation plays a part in control of differentiation and development, should be well worth following up. One way to approach the question is to choose a suitable system - such as *Xenopus* - and to test systematically for DNA methylase activity both in the oocyte and in the embryo at various stages of development. (According to the above model, enzyme levels should increase as development proceeds). If no activity can be detected, it is necessary to try to find out whether this is due to inhibition by cellular control factors, or to the lack of some factor (e.g. "helper" described above) which stimulates activity. Also, the specificity of the methylases at various stages should be examined, to see if they can methylate double-stranded, under-methylated DNAs at a greater rate than the enzyme from the differentiated ascites cells. If the isolated embryonic enzymes showed very different behaviour from the ascites enzyme, this might argue in favour of a different enzyme being responsible
for initial methylation during differentiation, rather than an alteration in the specificity of the normal methylase by some kind of control factor, as outlined in the previous Section.
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