DNA SYNTHESIS AND METHYLATION IN NORMAL AND TRANSFORMED CELLS

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ABSTRACT

In this study, DNA methylation was examined during the eukaryotic cell cycle, and shown to occur throughout the S phase as well as during the "early" G_2 phase. However, DNA synthesis and methylation of newly synthesized DNA did not occur simultaneously, but the latter lagged behind DNA synthesis by about two hours. Once added during the S phase, the methyl groups were stably maintained in the DNA.

Various compounds which are known to affect DNA synthesis in tissue cultured cells, were tested for their ability to alter the methylation status of DNA. The effects of three DNA synthesis inhibitors, viz. hydroxyurea (HU), 1-β-D-arabinofuranosyl cytosine (ara-C) and aphidicolin were examined on a normal embryonic lung fibroblast cell line (WI-38) and its two transformed counterparts, a simian virus 40 (SV 40) transformed line (SVWI-38) and a γ-irradiation transformed cell line (CT-1). HU was shown to enhance hypermethylation of pre-existing DNA strands in the normal cells, while ara-C and aphidicolin caused hypermethylation of newly synthesized DNA strands.

The effects of various concentrations of a known inducer of gene expression, sodium butyrate, were examined on these three cell lines as well. During a 16-20 hour treatment period, at butyrate concentrations of between 5 and 20 mM, no

adverse effect on cell morphology was observed. Cell growth, in the presence of butyrate for 14 hours, showed that butyrate was more toxic on the transformed cells than on the normal cells. However, at 5 mM butyrate, DNA synthesis was inhibited by 75% in the normal cells, and was unaffected in the transformed lines. RNA synthesis was not affected in the transformed cells, whilst in the normal cell line, RNA synthesis was decreased to 76% of the control value, at sodium butyrate concentrations as low as 5 mM. Protein synthesis also was unaffected in the transformed cells and only slightly ($\pm$ 10%) inhibited in the normal cells at 20 mM butyrate. SDS polyacrylamide gel electrophoresis of proteins synthesized in the presence of 10 mM sodium butyrate, showed that most proteins were unaffected. Two high molecular weight proteins in the WI-38 cells appeared to be modified during butyrate treatment, while one protein was induced by butyrate treatment in the CT-1 cells. More importantly though, butyrate treatment also resulted in hypermethylation of DNA, as shown by MSP 1 and Hpa II restriction endonuclease digestion and high pressure liquid chromatography analysis. Butyrate appeared to specifically cause hypermethylation of pre-existing DNA strands in the WI-38 cells, while the SVWI-38 and CT-1 cells showed preferential hypermethylation of newly synthesized DNA strands. However, the hypermethylated state was only heritable if the methylation event occurred in newly synthesized DNA. Hypermethylation on pre-existing DNA was rapidly lost in the subsequent

generation. It would therefore appear that methylcytosines are only maintained in the DNA if they are generated on newly synthesized DNA.

This study has clearly shown that the heritability of DNA methylation patterns is closely linked to DNA replication.

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CONTENTS

CHAPTER 1

INTRODUCTION

The regulation of eukaryotic gene control is clearly a complex process. Numerous mechanisms have been proposed to account for gene activity. It is becoming increasingly apparent that no single mechanism is solely responsible for the control of gene expression and that activation probably depends on coordinated regulation of and by a number of factors. Understanding the mechanisms involved in gene expression is vital in understanding the process of neoplastic transformations and in doing so, to aid in cancer treatment. Gene regulation involves an interplay between the DNA and the chromosomal proteins (namely the histones, non-histone proteins, HMG proteins and the regulatory proteins). Various DNA or protein modifications have been suggested as possible mechanisms that either enhance or reduce DNA-protein interactions. Gene transcription requires the controlled unfolding of packaged, "supercoiled" DNA, and the weakening of DNA-protein interactions at selected genes. DNA is packaged around an octomeric nucleosome core particle, composed of two molecules each of the histones H2A, H2B, H3 and H4 (1). Acetylation of the histone proteins has been suggested as one of the controlling factors involved in gene regulation. Introduction of a negatively charged group at the basic amino terminal end of the histone

molecule lessens its attraction for the negatively charged phosphate backbone of the DNA, thus probably facilitating read-through by the DNA or RNA polymerases.

Over recent years, evidence implicating a role for DNA methylation in gene control has been accumulating (2,3,4). The conversion of cytosine to 5-methylcytosine (5-mC) introduces a methyl group into an exposed position of the major groove of the DNA helix. This may affect the binding of proteins to the DNA. It is already known that binding of histones and hormone receptors is affected by changes in the major groove (5,6,7). 5-Methylcytosine is the only modified base found in mammalian DNA. Its function in gene control has become increasingly apparent, and various correlations show its importance as a "gene-silencing" mechanism. 5-Methylcytosine arises in the DNA after enzymatic conversion of cytosine to its methylated form, through the addition of a methyl group donated from S-adenosylmethionine (SAM), by a methyltransferase enzyme. Approximately 2-7% of all cytosines are methylated <u>in vivo</u>, the 5-carbon of the pyrimidine ring being the site of modification. Ninety percent or more of the 5-mC is found in the dinucleotide sequence 5'-CpG-3', and between 50 and 70% of the CG sites are methylated, depending on the species and the tissue examined (2).

Two isochizomeric restriction endonucleases, Hpa II and Msp 1, have been used extensively in studies to correlate DNA methylation with the lack of gene expression and vice

versa. Both enzymes recognise the restriction site 5'-CCGG-3', however Hpa II will not cleave this site if the internal cytosine is methylated, while Msp 1 will cleave at this site. Similarly, if the external cytosine is methylated, Msp 1 will not cut this sequence, while Hpa II will. Using these two enzymes, tissue-specific differences in methylation patterns could be shown in the chicken, rabbit and human globin gene (3,4,8,9), chicken ovalbumin and conalbumin genes (10,11), and ribosomal DNA (10). For example, a site within the β-globin gene of reticulocytes is not methylated, while in non-expressing tissue (e.g. lung-tissue) this same site is methylated (8). Furthermore, satellite or inactive DNA shows a two-fold increase in methylation compared with actively transcribing DNA (2). Thus it is generally accepted that non-expressing genes are more methylated than active genes, this correlation being particularly favoured for the 5'-flanking regions of the gene where the promotor elements are found. DNA methylation is therefore a possible controlling mechanism in gene expression. Attention has been focussed on the <u>regulation</u> of the methylation event, for factors that alter the methylation pattern could naturally affect gene expression within the cell.

Use has been made of various drugs to alter methylation patterns of DNA. The cytidine analogue, 5-aza-cytidine, has been shown to induce phenotypic conversion of mouse fibroblast C3H 10T1/2 cells to muscle cells, adipocytes and

chondrocytes (12). This is due to the inability to methylate the nitrogen atom in the 5-carbon position of the pyrimidine ring, resulting in the loss of methyl groups from the DNA. Naturally this observation has tremendous implications in carcinogenesis. In fact, the 5-mC content of most tumor cells shows decreased methylation compared with normal cells (13). Furthermore, several alkylating carcinogens have been shown to inhibit the methylation of a variety of DNA substrates in vitro (13). Many of these agents bind covalently to guanine residues in the DNA, which may occur at CG methylation sites. The occurrence of a carcinogen adduct in the vicinity of a methylation site could alter enzyme recognition and thereby inhibit DNA methylation at that site, or possibly prevent the scanning function of the enzyme so that the DNA becomes under-methylated (14). The loss of methyl groups from the DNA has been shown to be clonally inherited by subsequent generations (15).

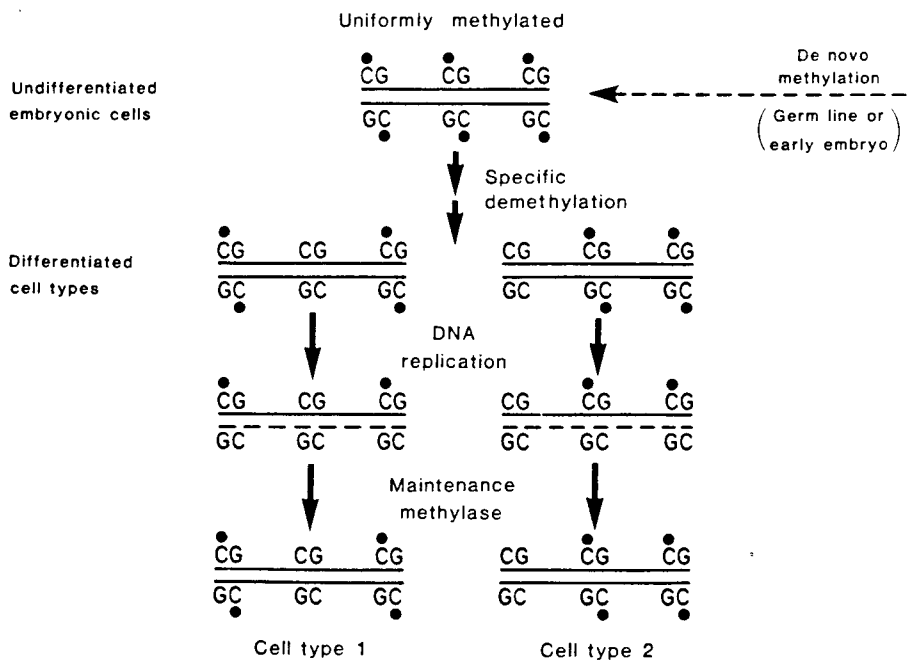
A model to account for clonal inheritance of the methylation pattern (and for which experimental proof has been provided in a number of systems (16)), was proposed by Riggs (17) and Holliday and Pugh (18). The model requires that symmetric methylation of the newly synthesized DNA strand occurs during DNA replication. Symmetric methylation is achieved by a maintenance-methylase that acts on hemi-methylated DNA only. Hemi-methylated DNA arises when one of the DNA strands of symmetrically-

methylated parental DNA becomes the template for a new round of DNA replication. Evidence for the existence of hemi-methylated DNA was provided by Jones P.A. et al (19) when treating transformed mouse embryo cells with azacytidine for 20 hours. The hemi-methylated DNA is an efficient substrate for the methylase in an in vitro methylation assay. Furthermore, duplex hemi-methylated DNA is a 20 fold better substrate for the enzyme than single stranded DNA. In this way, methylated sites remain methylated in daughter DNA strands, and unmethylated sites are not methylated. Thus clonal inheritance of the methylation pattern occurs in the newly synthesised daughter DNA.

Fig. 1.1 summarises the current model proposed for the maintenance of the DNA methylation pattern (2). In vivo results indicate that a maintenance methylase must exist. There have been several studies on mammalian DNA methylases. S-adenosylmethionine dependent DNA methylases have been purified from human Hela cells (20), rat liver (21,22), rat hepatoma (23) and mouse ascites cells (24). Each cell type (and probably each species) appears to have only one or two DNA methylases with limited specificity. Drahovsky and Morris (21) have provided evidence for the "walking-hypothesis", which is suggested as the mechanism by which hemi-methylated DNA is converted to the fully methylated form. After initial binding of the maintenance-methylase to the DNA, the methylase is thought

FIG. 1.1 Model for the maintenance of the methylation pattern

De novo methylation ensures that in the germ line or early embryo, all possible methylation sites are methylated. Specific demethylation events, thought to arise due to the inhibition of methylation by proteins during DNA replication, leads to the establishment of specific methylation patterns and thereby to cellular differentiation. Maintenance methylases ensure correct hereditability of the methylation pattern by subsequent cell generations (From Razin and Riggs, 1980 (2)).



to travel along the DNA, without dissociating from it. These authors were able to show that dissociation of the methylase from helical DNA was not possible with 0.2 M NaCl, this therefore suggesting a tight binding reaction. Furthermore, they were able to demonstrate that once the enzyme was tightly bound to the DNA, it catalysed a large number of methyl group transfers without release from the DNA. A temperature dependent local unwinding of the DNA was required before the enzyme bound tightly to the DNA.

The DNA methylase uses S-adenosylmethionine as the methyl donor, the methyl group being added onto the 5-carbon of the pyrimidine ring of cytosine. Recently, 5-azacytidine has been used as a tool to examine the methylation event. Santi et al (25) were able to show that incorporation of small amounts of 5-azacytidine into DNA caused a dramatic decrease in the amount of DNA methylation . They therefore speculate that the mechanism of inhibition involves covalent bond formation between the methyl transferase and the cytidine molecule, or 5-azacytidine in substituted DNA. Recently the same group were able to show the formation of this covalent bond (26), where <u>in vitro</u> studies showed a stable complex formation between 5-azaC-DNA and a Hpa II methylase (a bacterial DNA-cytosine methyltransferase). Further studies have shown that this complex cannot be dissociated after as long as three days and is not affected by treatment with sodium dodecyl-sulphate (a detergent). Binding is stoichiometric giving

1 mol of 5-azaC per mol of enzyme. Furthermore, restriction digests with Msp I endonucleases (which also cleaves CCGG sequences) reduce Hpa II methylase binding by ± 40%, suggesting that the methylase binds to 5-aza-C at specific recognition sequences. Extensive treatment of 5-azaC-DNA with Msp 1 endonuclease does not completely eliminate binding, therefore the possibility that the methylase binds to 5-aza-C residues in other sequences in the DNA cannot be ruled out. Fig. 1.2A depicts the events proposed to occur during electrophilic substitution of a methyl group onto the 5-position of the pyrimidine ring (26).

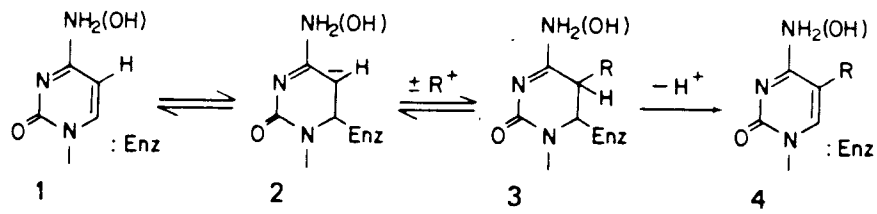
The methyltransferase initiates a nucleophilic attack on the 6-carbon of the pyrimidine ring, displacing electrons towards the 5-carbon atom, activating an otherwise inert 5-carbon for reaction with an electrophile (R^+, the methyl group of SAM). A reversible complex is formed (diagram 2 and 3 of Fig. 1.2), which after removal of the proton irreversibly results in addition of the R group to the 5-carbon, with release of the active enzyme. Because a nitrogen atom replaces the carbon atom in the 5th position of the ring in 5-aza-C, the enzyme binds irreversibly to the analogue and is trapped in an adduct complex that may be quite stable or undergo expulsion of the 6-carbon and formylation of the attacking nucleophile (27) (nitrogen has a valency of 3 and therefore does not have an additional proton). The adduct complex is shown in Fig. 1.2B. Thus

FIG. 1.2 A) Methylation of cytidine at the 5' carbon of the pyrimidine ring

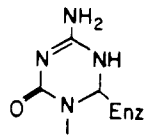
A methyltransferase initiates a nucleophilic attack on the 6-carbon atom of the pyrimidine ring of cytosine. This displaces electrons towards the 5-carbon atom making it electrophilically reactive, such that R^+, the methyl group of SAM, reacts with the 5-carbon. A reversible complex is formed (diagram 2 & 3), which after removal of a proton, irreversibly results in addition of the methyl group to the 5-carbon of cytosine. The enzyme is released during this process and is free to catalyse further methylation events.

B) Where cytosine has been replaced by 5-azacytosine, the following adduct formation results. Instead of release of the enzyme after attachment at the 6-carbon, it is irreversibly trapped, forming a covalent adduct (Santi et al (26)).

A



B



methyl group transfer is mediated via covalent complex formation between the methyltransferase and the pyrimidine base. This explains why only a small amount of 5-aza-C is required to inhibit DNA methylation so dramatically. DNA methylases are "mopped-up" by 5-aza-C substituted DNA, either as the methylase "walks" along the DNA, or possibly by homing in on specific recognition sites. The latter mode of recognition is favoured by Santi et al (26).

Thus the enzyme(s) involved in somatic heritability are of the "maintenance-methylase" type and specifically act upon hemi-methylated DNA, thus maintaining the symmetric methylation pattern. However, "de novo" methylases must exist that are capable of transferring methyl groups onto completely unmethylated DNA sequences (18). These enzyme(s) are responsible for determining the initial heriditable methylation pattern recognised by the "maintenance-methylases". Recent work has suggested that de novo methylases are most active in the preimplantation embryo (28,29), although there is no doubt that some de novo methylation occurs in the fully differentiated cell (13). The existence of de novo methylases has been shown in a number of systems. For example, unmethylated adenovirus DNA is methylated in some cell lines after integration into the genome (30,31). It was initially proposed that germ-line DNA is completely "methylation-free", thus providing a clean slate on which to lay down the methylation pattern during differentiation. It has

become apparent however, that the DNA of sperm and oocyte is in fact highly methylated (3,4,11,32). Thus de novo methylases probably function primarily in primordial germ-line cells, methylating all possible sites. Recently Grunwald and Drahovsky (33) isolated a DNA-cytosine-5-methyltransferase from mouse mastocytoma cells that exhibited both "maintenance" and "de novo" activities, these being monitored using hemimethylated and unmethylated DNA as substrates for the reactions respectively. Pfeifer et al (34) were also able to show that a 5-methyl-transferase purified from the soft tissue of human placenta was able to exhibit both "maintenance" and "de novo" activities.

Cellular differentiation is thought to arise due to "demethylation" events, where maintenance methylases are prevented from methylating certain genes. Recently two reports have detailed the discovery of hypomethylated stretches of satellite DNA in mouse sperm and oocyte cells (35,36). Although this appears to contradict the above model, it is suggested to arise through either suppression of de novo methylation in the primordial germ cell lineage or by direct removal of methyl groups during a stage of germ cell differentiation, which precedes the spermatogonia stage. "Demethylation" events are thought to arise by the prevention of methylation during DNA synthesis. The existence of "demethylases" <u>in vivo</u> has not been given much support, however recently a number of reports have

suggested their existence (37,38). A DNA demethylating activity has been demonstrated in nuclear extracts of murine erythroleukemia cells (37).

This brings us to the question of the role played by DNA methylation *in vivo*. As already mentioned, many examples exist (at least 40 (13)), where under-methylation is correlated with gene expression and the lack of expression with hypermethylation. It is important to determine whether the hypomethylation associated with actively transcribing genes is the controlling factor, or whether it is a consequence of gene activation. Furthermore, the correlation between under-methylation and activity is not perfect, for no change in methylation is reported in the type 1 procollagen gene, between active and inactive genes (39), while Parker et al (40) show differential methylation (SV-40 transformed fibroblasts do not express type 1 procollagen genes and have hypermethylated sites in the 3' region of the gene, while the normal fibroblasts are undermethylated at the corresponding sites). The link between undermethylation and gene activity is, however, strongest for the 5'-flanking regions of a gene, where the control sequences are found. Thirty systems that correlate under-methylation with gene expression in the 5' or 5'-flanking regions of the gene have been analysed. It has been suggested that two levels of control exist that operate independently, the first being most important for gene activity. Thus methylation of the control region

could be the primary mechanism that determines whether a gene is to be active or not, while methylation of the coding region and the introns may only provide the fine tuning. However, the absence of methyl groups in either the coding region or the control region may not be sufficient for gene expression. A good analogy is suggested by Riggs and Jones in their review on DNA methylation (13). If methylation is primarily a locking mechanism, then it should be remembered that "an unlocked door is not necessarily open".

DNA usually exists, at physiological conditions, as a right-handed double helical structure known as B-DNA (See review (41)). Under certain conditions B-DNA can however be converted to a more unstable form known as Z-DNA, a left-handed helical structure which is slimmer than B-DNA and has a larger number of base pairs per helical turn. A line joining the phosphate groups in a van der Waals diagram would show the irregularity of the backbone, thus giving it the name "Z" for zigzag DNA. Of physiological relevance is the finding that Z DNA is most favoured in stretches of DNA that have alternating purine-pyrimidine bases, due to the nucleotides of left-handed DNA alternating in syn and anti conformations (42). Not all sequences are equally favoured; DNA with alternating d(CG) sequences are the most favoured, followed by d(CA) or d(TG), while d(AT) sequences favour Z-DNA formation least of all.

In physiological solutions, the B and Z forms of DNA exist in equilibrium. This equilibrium is shifted towards the more stable B form of DNA, because electrostatic repulsion between the negatively charged phosphate groups of Z-DNA make it more unstable (these groups on opposite strands are closer together in the Z form than in B-DNA). However, under physiological conditions, Z-DNA can be stabilised by a number of factors. Negative supercoiling of the DNA is one mechanism (43), but most important is the introduction of methyl groups at the 5-carbon of cytidine (44). Behe and Felsenfeld (45) were able to show that poly (dG/d^mC) required 3 orders of magnitude less Mg^{2+} to stabilise Z-DNA formation, than unmethylated poly (dG-dC). Increased stability is thought to be due to the introduction of methyl groups into a hydrophobic pocket created by the imidazole group of guanine of the next base pair, which would otherwise be filled by hydrophilic water molecules (46).

Since Z-DNA formation is dependent upon alternating purine-pyrimidine bases, the occurrence of these segments in genomic DNA is important. The 5' flanking regions of a number of genes (47,48) have been shown to contain such sequences. For example, the circular genome of the simian DNA tumor virus (SV-40) contains three segments of DNA that has 8 base pairs of alternating purines and pyrimidines within a region that regulates transcription and

replication (49). More precisely, the Z-DNA segments form part of the transcriptional enhancer element of the viral early promotor (50,51,52). Their presence in promotor regions of genes indicates that they may function in a regulatory capacity. Because the conversion of cytosine to 5-mC stabilises Z-DNA formation, it is possible to envisage a more definitive role for the occurrence of these modified bases in CpG stretches of eukaryotic DNA. Possibly 5-mC is the trigger that converts stretches of B-DNA to Z-DNA. In this form, the DNA is inaccessible to DNA and/or RNA polymerases (53). Removing the methyl group destabilises the Z form, and conversion of these areas back to the B form of DNA could produce a local increase in negative supercoiling, which could alter local chromatin structure and facilitate the entry of RNA polymerases onto the DNA molecule.

It can thus be deduced that any compound that either stimulates or inhibits DNA methylation,could affect gene expression. Chapter 2 examines the methylation event itself. If, as suggested by this study, DNA replication is required for the maintenance of altered methylation patterns, it is important to determine when during the cell cycle, bulk methylation of newly replicated DNA occurs. The question addressed in this study was whether methylation is restricted to the S-phase or whether some delayed methylation occured in the G2 phase of the cell cycle. Inhibitors of DNA synthesis have been shown to

cause hypermethylation of DNA. Boehm and Drahovsky (54) have shown that 1-β-D-arabinofuranosylcytosine (which has been used to treat acute myelogenous leukemia (55)), caused hypermethylation of a tumor cell line. In this study, the effects of three inhibitors of DNA synthesis were examined in normal and transformed cells, in order to investigate whether similarly, hypermethylation was induced in our cells. Chapter 3 investigates the effects of ara-C, hydroxyurea and aphidicolin on DNA synthesis and methylation in normal and transformed cells.

Sodium butyrate is a known inducer of gene expression in a number of cells (56,57). Various effects of this compound were investigated on normal and transformed cells in culture. In particular, it was of interest to determine whether perturbations of the methylation pattern occurred during treatment. Furthermore, in this study, butyrate was used as a tool to investigate the methylation event itself. Chapter 4 details the numerous effects of butyrate treatment on normal and transformed cells, and also how DNA synthesis is required for the maintenance of the altered methylation state.

It is hoped that this study will provide a deeper understanding into the role played by DNA methylation in gene expression.

CHAPTER 2

DNA METHYLATION DURING THE S AND EARLY G2 PHASES OF THE CELL CYCLE

2.1. INTRODUCTION

Genomic methylation patterns of eukaryotic cells are clonally inherited via semiconservative replication, by maintenance methylases that specifically act on hemimethylated DNA (2,3,58,59). Controversy does, however, exist as to the precise timing of the methylation event(s). Methylation must occur after daughter strand synthesis, but the methylation event could occur within seconds or minutes after synthesis, or up to just before the daughter strand itself acts as a template in a further round of replication. Precisely when the methylation event occurs may have an important role in a regulatory capacity. It has been proposed to be the manner in which eukaryotic cells regulate DNA synthesis at replication forks, so that each DNA segment of the cell is replicated only once in each cell cycle (60). For this reason a number of studies have investigated cell-cycle linked methylation events.

Growing eukaryotic cells obligatorily pass through different stages of the cell cycle in a specific sequence. The cell cycle is divided into 5 phases: G0, G1, S, G2 and mitosis. G0 is the resting phase and not strictly part of the cycle. Cells leave the cycle to enter G0 and may re-

enter the cycle at a specific point in G1, when the cells need to divide. While in the G1 phase, the cells prepare for DNA replication which occurs during the S or synthesis phase. It is during S phase that histone synthesis also occurs. S phase is followed by the G2 phase, during which the cell prepares for cell division. The latter occurs during mitosis, after which the two new daughter cells again enter the G1 phase, beginning a new cycle.

Because DNA is synthesised during S phase, the methylation of hemimethylated DNA can either be S phase specific (occurring either early or late during S phase) or be delayed (occuring during G2, mitosis, or during the G1 phase of the next cycle). However, the methylation pattern would of necessity have to be completed before the following S phase in order to be clonally heritable. Several reports in the past have focussed on the relationship between DNA replication and methylation. However, conflicting results have complicated the issue. Burdon and Adams (61) suggest that DNA methylation and replication do not occur simultaneously but are separated by as much as one hour. Furthermore they show that once synthesized in this manner, the 5-mC is metabolically stable with no evidence of demethylation. They therefore suggest that methylation of cytosine occurs very shortly after the initial process of DNA synthesis rather than randomly throughout the cell cycle.

Kappler et al (62) were able to show similar results in a mouse adrenal cell line, by monitoring methylation as the incorporation of ^{3}H-methionine into DNA. However, using a more direct approach, where the conversion of deoxycytidine into its methylated derivative is monitored, the same investigators were able to show a lag of 1 minute between DNA synthesis and methylation. Recently Gruenbaum et al (63), using a nick translation assay and nearest-neighbour analysis investigated the extent of methylation at CpG sites. Cells were permeabilised and nick-translated *in vitro* with $|^{32}P|$-dGTP, thus avoiding the effects of precursor pool sizes, rates of precursor biosynthesis and the need for synchronisation. In this study they were able to show that methylation at the replication fork proceeds with a lag of 75 seconds, while methylation upstream from the fork shows no apparent lag period. The latter is suggested as a model for the methylation of repaired DNA. Their study was, however, unable to answer the question of whether the methylation of a minor fraction of CpG sequences is delayed or not. Methylation of DNA at other stages of the cell cycle has been reported by Woodcock et al (64,65), who found that in asynchronously growing cells of both human and hamster origin, some cytosine methylation is delayed for several hours after strand synthesis, and that this delayed methylation is completed before the DNA strand itself acts as a template for replication, during the next S-phase (66). That this methylation is not due to DNA repair synthesis, is shown by

the lack of ^{3}H-dThd* incorporation into newly methylated DNA. Inhibitors of DNA synthesis (such as ara C, ara A and hydroxyurea) are unable to eliminate delayed methylation, while S phase specific methylation is dramatically reduced. In a more recent report by the same group (67), use of inhibitors of methylation showed that delayed methylation was specifically inhibited by certain inhibitors, while S phase methylation was unaffected (e.g. Tubericidin plus homocysteine). Other inhibitors affected both delayed and non-delayed methylation to the same extent (e.g. cycloleucine, ethionine and 5'-deoxy-5'-methylthioadenosine (MTA)), while 5-azacytidine affected the S-phase specific methylation more than the delayed methylation. In the light of these and other findings, these authors suggest that delayed and non-delayed methylation is achieved by different methylase enzymes. They therefore suggest the existence of at least two different methylases in their system. Results obtained in this study, using a different assay system, also suggest the existence of more than one methylase that is capable of distinguishing between parental and newly replicated DNA (See Ch. 3, section 3.3).

If more than one class of DNA methylases exists for performing delayed and non-delayed methylation reactions, then this implies that either the DNA sequences differ or that chromatin structure and/or localised secondary structure of the DNA duplex differs, thus enabling the two

* Deoxythymidine

classes of methylases to distinguish between delayed and non-delayed methylatable sites. This implies a differential function for delayed and non-delayed methylation and is consistent with the model proposed by Taylor et al (60), who suggest that a special set of symmetrically methylated DNA sequences constitutes the activated initiation site for DNA replication in mammalian cells. Following replication of these sequences, the methylation is delayed, maintaining the sequence in the hemimethylated form until after the end of S phase. In doing so, these sites are kept in an inactive state as initiation sites for DNA replication, ensuring that each DNA segment of the cell is replicated once and once only during each cell cycle. These sequences are then methylated prior to the next S phase in order to regenerate DNA replication initiation sites.

Furthermore, possibly this is how gene activation occurs, where DNA sequences acquire the property of delayed methylation and are not remethylated until before the next S phase. This is consistent with the partial loss of methylation sites near activated DNA sequences (3,8,9,39).

In this study, cells were synchronised and S phase methylation examined to determine:

(1) the time period between DNA synthesis and bulk methylation

(2) whether DNA methylation levels are constant or fluctuate during the S phase

(3) whether delayed methylation can be detected early in the G2 phase of the cell cycle.

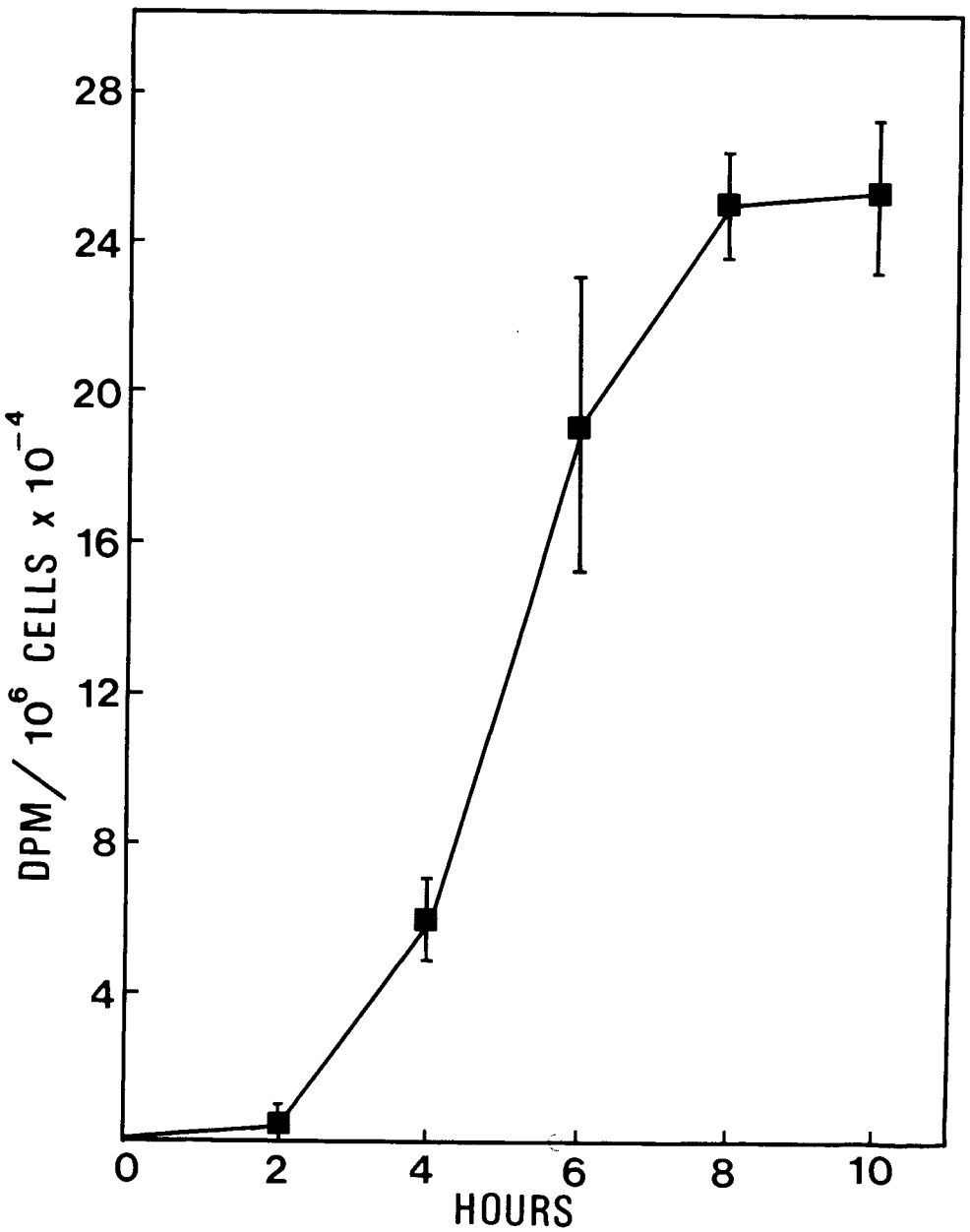
Two approaches were used to probe for methylation during the S phase. The first method monitors DNA and RNA methylation using ^{3}H-methionine as the methyl group donor, while the second method investigates the conversion of labelled deoxycytosine to 5-methyldeoxycytosine in DNA, during the S phase.

2.2. RESULTS

The double thymidine block technique was used to synchronise the human embryonic lung fibroblast cell line (WI-38 cells), at the G1/S boundary as described in Materials and Methods (6.2). The first thymidine block prevents about 70% of the cells from entering the S phase, the cells becoming trapped at the G1/S boundary as they traverse the cell cycle. Those cells already in S phase during the thymidine treatment cannot leave the S phase. Release from the block for about 9 hours, allows those cells that were trapped in the S phase to exist, and furthermore those cells that were situated at the G1/S boundary are able to traverse and exit the S phase, which is usually about 8 hours long. The second thymidine block then synchronises approximately 95% of the cells at the G1/S boundary. Fig. 2.1 shows the degree of synchrony achieved after WI-38 cells were treated with 2 mM dThd, as described above. After release from the block, the incorporation of ^{3}H-dThd into TCA precipitable material was monitored and expressed as dpm/10^{6} cells. From Fig. 2.1, the duration of the S phase was clearly 8 hours, for a plateau was reached 8-10 hours after release from the thymidine block.

FIG. 2.1 Cell synchronisation using a double thymidine block

Cells were plated at $5x10^4$ cells/30 mm² dish, allowed to attach to the dish and grow for 24 hrs, followed by treatment with 2 mM thymidine as described in Materials and Methods (6.2). After release from the second thymidine block, the cell layers were rinsed twice with PBS, and fresh medium added. The cells were then pulsed every 2 hours with 1 μCi/ml dThd, the amount of radiolabel incorporated into TCA insoluble material was determined and expressed as Dpm/10^6 cells.



2.2.1. Monitoring the methylation of DNA and RNA during the cell-cycle, using ^{3}H-Methionine as the methyl-donating group:

After release from the second thymidine block, synchronised WI-38 cells were transferred to methionine-free medium that had been supplemented with 0,1 M glutamine. At two hourly intervals, labelled methionine was added to the synchronised cells. After each 2 hour pulse, the cells were collected, and the DNA and RNA extracted according to the protocol described in Materials and Methods (6.9.1). As a further purification step, the nucleic acids were electrophoresed on 1.5% soft agarose gels and the corresponding bands eluted from the gel for further analysis. The DNA concentration was determined fluorometrically using a Hoescht 33258 dye that binds specifically to AT rich sequences in the DNA, see Materials and Methods (6.3).

Fig. 2.2 shows the standard curve of WI-38 DNA,that was used to determine the concentrations of unknown DNA samples. Absorbance readings at 260 nm were taken to determine the RNA concentrations. The amount of label incorporated during each pulse was determined by β-counting, and the specific activity expressed as dpm/µg DNA or RNA. The results are represented graphically in Fig. 2.3. Cell synchrony was monitored as detailed before, except that it is represented (in Fig. 2.3) as a change in

FIG. 2.2 Standard curve of WI-38 DNA to determine unknown DNA concentrations

Known concentrations of DNA were used in the Hoescht dye assay described in Materials and Methods (6.3) in order to establish the standard curve represented below. The fluorescence was measured using a Perkin Elmer LS-5 luminescence spectrometer, at an excitation λ = 356 nm and emission λ = 458 nm.

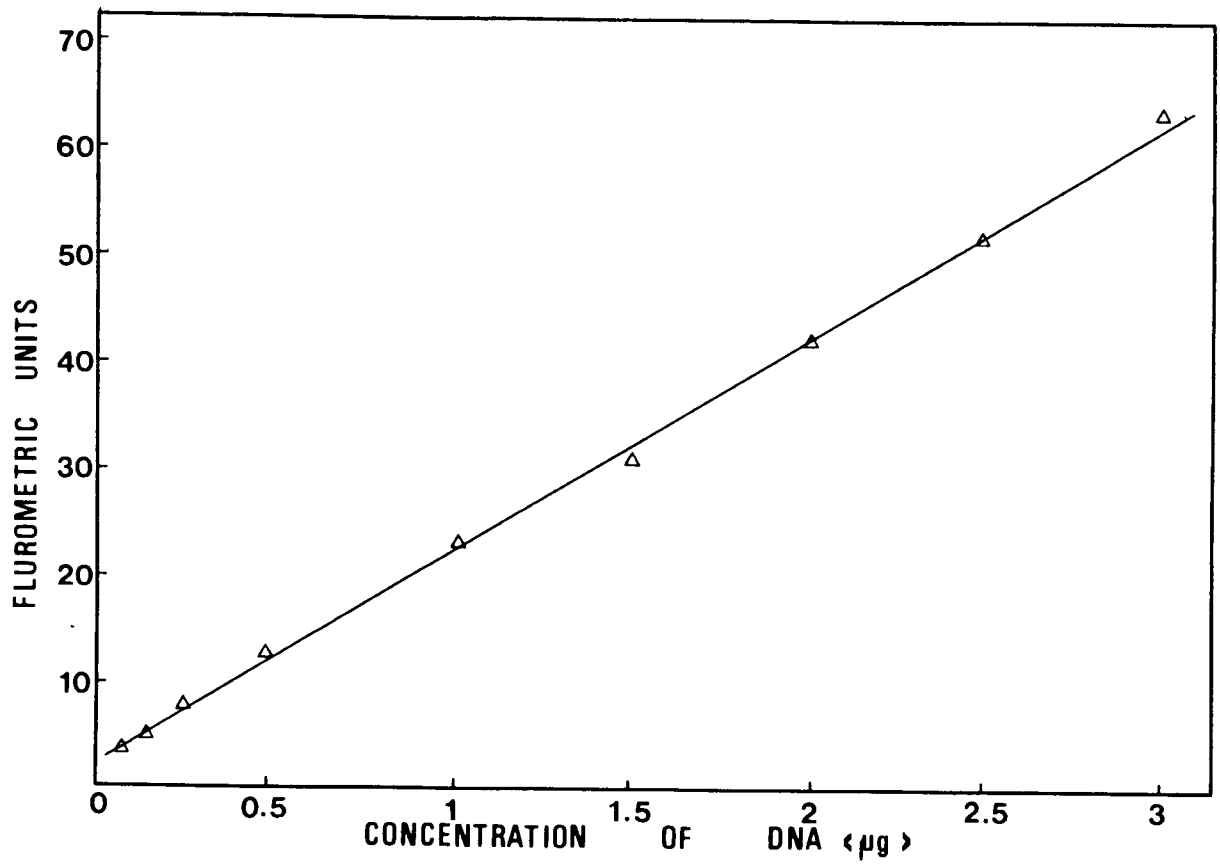
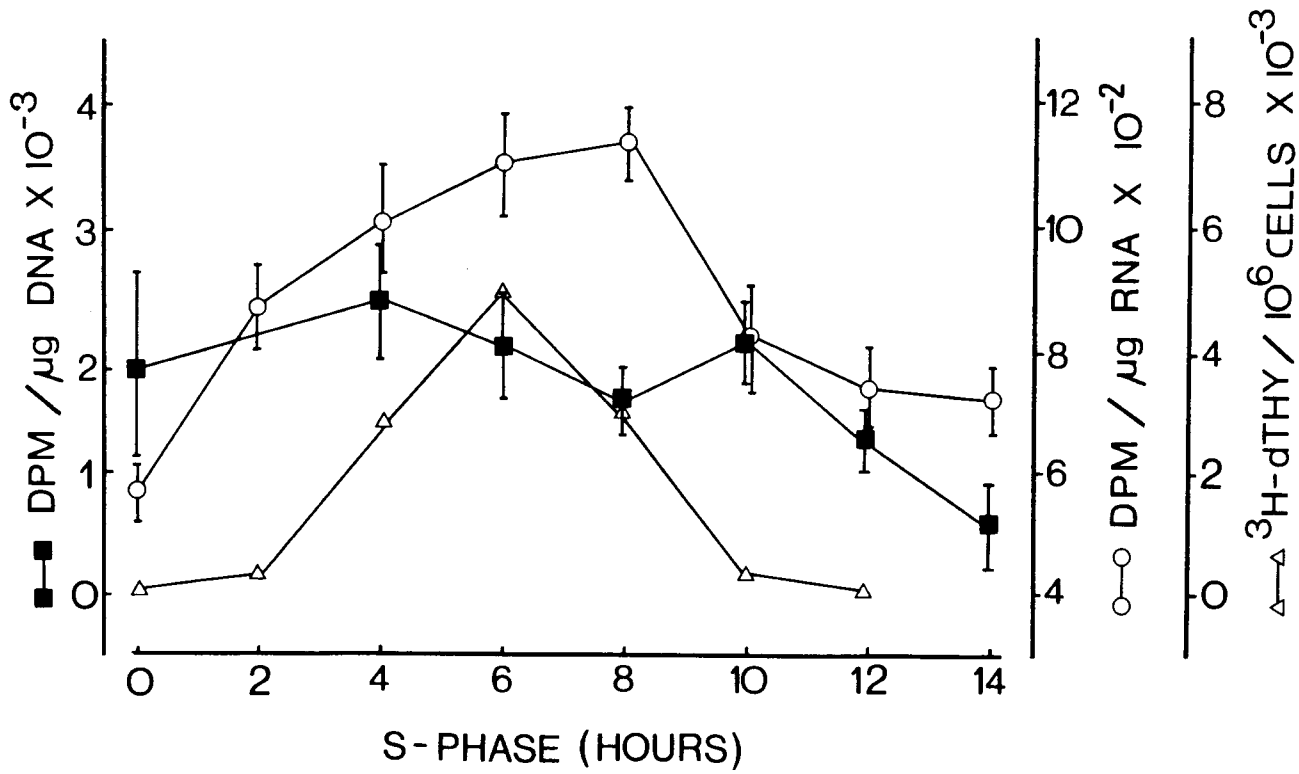


FIG. 2.3 Incorporation of ^{3}H-methyl groups into DNA and RNA during the S phase and early G_2 phase of the cell cycle

WI-38 cells were synchronised using the double thymidine block technique described in Materials and Methods (6.2). After release from the second thymidine block, cells were pulsed with ^{3}H-methionine at 2 hour intervals. DNA was extracted as described in Materials and Methods (6.9.1). Methylation was measured as the incorporation of ^{3}H-methyl groups into DNA (■—■) and RNA (○—○) and expressed as the amount of radiolabel incorporated per µg DNA or RNA. Cell synchrony was monitored as the incorporation of ^{3}H-dThd into TCA insoluble material (△—△).



the rate of ^{3}H-dThd incorporated, over 2 hour intervals. The incorporation of ^{3}H-methyl groups into DNA and RNA showed differential responses during the S phase. Incorporation of label into RNA increased steadily during the first 8 hours of the S phase, after which it returned to pre-S phase levels. It thus appears that RNA methylation fluctuates during the S phase, being maximal 6-8 hours after release from the block. However, the incorporation of radiolabel into DNA did not fluctuate during the S phase. Of interest is the observation that no decrease of labelled incorporation into DNA was noted 10 hours after release from the thymidine block, as was seen with RNA methylation. Because S phase is completed 8 hours after release from the block (see Fig. 2.2), it therefore appears that the incorporation of methyl groups into DNA continues into the "early" G2 phase.

2.2.2. Use of high pressure liquid chromatography (HPLC) to analyse the 5-methylcytosine content during the cell-cycle:

WI-38 cells were synchronised using the double thymidine block technique as discussed in section 2.2. After release from the block, the cells were either given 6-^{3}H-uridine at 2 hourly intervals, or the label was administered for 4 hours, followed by a cold chase at 2 hourly intervals (uridine is converted into dCTP which is incorporated into DNA, via the following pathway:

Uridine → uridylate → UDP → UTP → CTP → CDP → dCDP → dCTP.

Labelled uridine is also detected in thymine after base analysis on HPLC. Conversion to dTTP occurs via the following pathway:

Uridine → uridylate → UDP → dUDP → dUMP → dTMP → dTDP → dTTP.

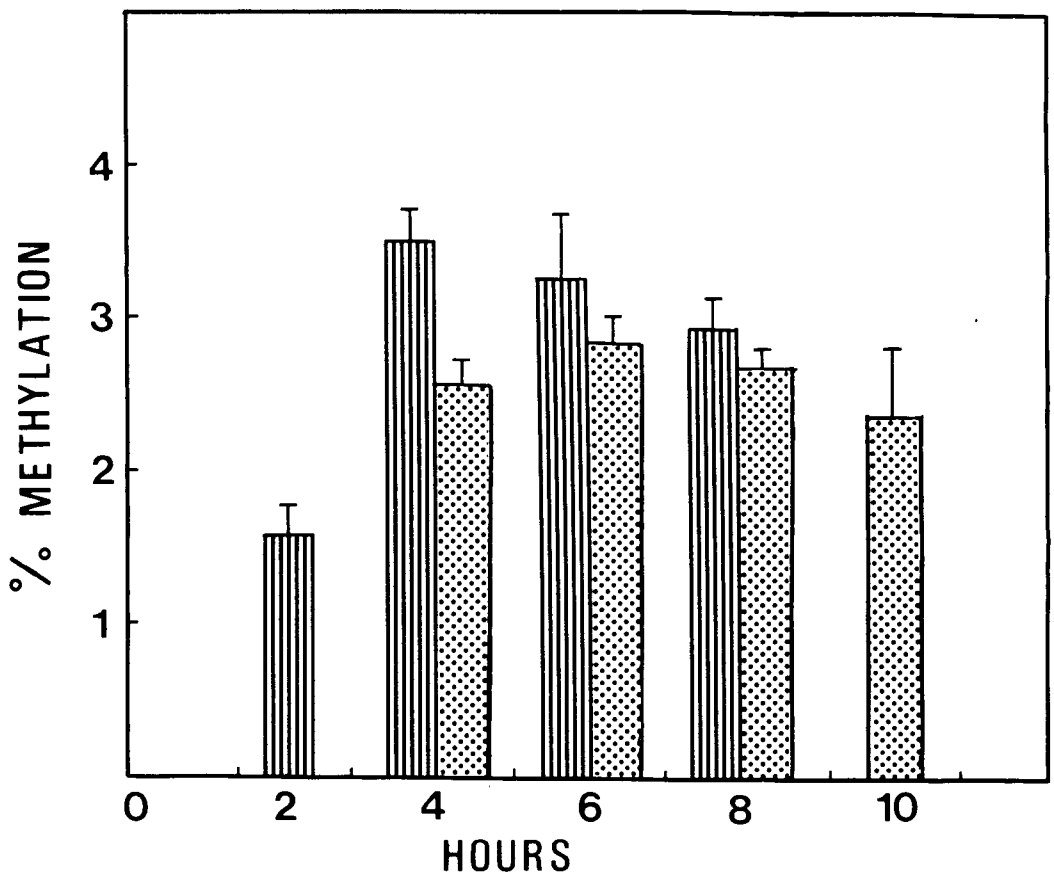
The DNA was isolated according to the protocol of Wilson and Jones (see section 6.9.2), hydrolysed in 88% formic acid and separated on a cation exchange column using sodium acetate pH 3.35 to elute the bases.

2.2.2.1. Analysis of the 5-methylcytosine content of synchronised cells at 2 hourly intervals during the S phase:

Fig. 2.4 shows the results obtained when the 5-mC content of WI-38 cells was monitored at 2 hourly pulses during the S phase. During the first 2 hour pulse, a 5-methylcytosine content of 1,67 ± 0,27% was obtained. This value increased to 3,5 ± 0,32% during the next 2 hour pulse and was maintained at this value for the remaining pulses. Thus an initial lag period of approximately 2 hours was noted, during which no methylation of newly synthesised strands occured. After two hours, the value returned to that of the fully methylated species (WI-38 cells are

FIG. 2.4 5-Methylcytosine levels during the S phase of the cell cycle

WI-38 cells were synchronised as described in Materials and Methods (6.2). Synchronised cells were either pulse-labelled every 2 hours with 6-^{3}H-uridine (▥) or label was given for 4 hours after which it was chased at 2 hourly intervals (▩). DNA was prepared as described in Materials and Methods (6.9.2) and the 5-methylcytosine content determined by HPLC analysis (6.10).



reported to have a 2,94 ± 0,28% 5-methylcytosine content by Diala et al (68)). This percentage was then maintained throughout the 8 hour S phase. It is of interest to note that the 5-mC content for the first 2 hour pulse is approximately half of that obtained during the subsequent pulses. This is what one would expect of hemi-methylated DNA, where one strand is methylated while the newly replicated strand is not. Thus methylation of newly synthesised DNA appears to lag behind DNA replication by about 2 hours at the beginning of the S phase.

2.2.2.2. Analysis of the 5-mC content during a pulse chase experiment:

This experiment investigated whether DNA that was synthesised in early S-phase was methylated immediately or whether possibly, as reported by others, delayed methylation occurred during late S-phase or early G2 phase. Cells were released from the thymidine block and 6-^{3}H-uridine added for 4 hours, after which it was removed, and fresh medium added. The incorporated label was then chased at 2 hourly intervals and the DNA analysed for its 5-mC content.

Fig. 2.4 shows that during the first 4 hours, when label was present, a 5-mC content of 2,58 ± 0,17% was obtained, which corresponded to that of fully methylated DNA.

Chasing the labelled DNA for a further 6 hours did not alter the 5-mC content.

2.3. DISCUSSION

Both methods discussed above to monitor changes in methylation levels during the S phase of the cell cycle, rely on the equilibration of either the ^{3}H-methionine or 6-^{3}H-uridine with de novo pools of these precursors in the cell. It must therefore be remembered that other factors may have contributed to the results obtained. For this reason, RNA methylation was also monitored in section 2.2.2.1, to show that the lack of fluctuation of labelled methyl-group incorporation into DNA was not due to an interference of SAM pools (S-adenosyl methionine (SAM) is the cofactor required by the methyl transferase enzyme as the methyl donating group). A 65% increase in labelled incorporation into RNA was noted 6-8 hours after release from the thymidine block.

That no fluctuation in incorporation of label into DNA occurred during the S phase, implies that bulk methylation of DNA probably occurs throughout S phase, that is, immediately after the synthesis of new DNA strands, they are converted to the fully methylated form and not stored as hemi-methylated DNA to be methylated at a later stage during the S phase. Although this experiment does not

indicate how soon after synthesis DNA strands are methylated, it does indicate that no gross stage specific methylation occurs in mammalian cells. The level of methyl-group incorporation decreased 12-14 hours after release from the thymidine block, a time period corresponding to the early G2 phase of the cycle. However, the decrease was gradual, possibly implying that delayed methylation occurred at some sites.

Thus methylation of DNA appears to be maintained at a constant level during the S phase. From the HPLC data, methylation again appeared to occur throughout the S phase. In agreement with other workers (61), we also observe that methylation and DNA synthesis do not occur simultaneously, but that a ± 2 hour lag period occurred before fully methylated DNA species were detected. Burdon et al (61) show a 1 hour lag period. Unfortunately, this discrepancy with the results of Burdon et al. cannot be solved by looking at the ^{3}H-methionine incorporation rates, for, as detailed in Chapters 3 and 4, inhibition of DNA synthesis resulted in hypermethylation of the DNA. This could explain why the incorporation of labelled methyl-groups into DNA was already high at the zero time point in Fig. 2.3.

The pulse-chase experiment showed that DNA which had been synthesized during the first half of the S phase, became

fully methylated during the first 4 hours of S. The 5-mC percent did not alter during the chase period, implying that those methyl groups added during early S phase are stably maintained during the latter part of the S phase. This is in agreement with results reported by other workers (Burdon et al (61)).

Thus in conclusion, it appears that methylation occurs throughout the S phase and possibly into the early G2 phase. DNA synthesis and methylation do not occur simultaneously, but methylation follows closely behind DNA synthesis, with an approximate 2 hour lag period at the beginning of S phase. Furthermore, once added during S phase, the methyl groups appear to be stably maintained in the DNA.

CHAPTER 3

THE EFFECTS OF DNA SYNTHESIS INHIBITORS ON DNA METHYLATION

3.1 INTRODUCTION

The clinical use of agents that inhibit cell proliferation in culture has been investigated in several studies (70,71,72). These agents have shown tremendous antitumor and antiviral potential. A number of cancers have responded favourably to treatment with these drugs in clinical trials (69,70). At the biochemical level, many fundamental questions governing the mode of action of these drugs remain unanswered. It is obvious that, in order to achieve better chemotherapeutic results, a deeper understanding of their biochemical mode of action is essential.

Already some drugs that show promise as cytotoxic agents in certain cancer trials have been investigated in fair detail at the biochemical level (71.72.73). In particular, the mode of action of 1-β-D-arabinofuranosylcytosine (ara-C), which has been used in patients with acute myelogenous leukemia, has been examined in a number of studies (52,74,75,76). Other drugs that have been examined include hydroxyurea (used primarily to treat chronic granulocytic leukemias(77,78)), daunorubicin (74,79),

aphidicolin (80,81), methotrexate and 5-flurodeoxyuridine (82).

Various combinations or modifications of these agents have been investigated to obtain effective cytotoxicity of the transformed cells at doses that are non-toxic to normal cells. This approach provides a way of determining the effectiveness of a drug, but in not knowing the precise mode of action, it is impossible to determine whether these drug affect other vital processes. Thus further biochemical analysis into their mode of action is required.

In this study, the effects of three DNA synthesis inhibitors (namely, 1-β-D-arabinofuranosylcytosine (ara-C), hydroxyurea (HU) and aphidicolin) were investigated in normal and transformed cells in culture. The three inhibitors were chosen because of their ability to inhibit DNA synthesis by different mechanisms.

Of the three, the mode of action of ara-C has been studied most extensively. Ara-C is a pyrimidine nucleoside analogue that resembles deoxycytidine, except that the 2' carbon of the sugar has a hydroxyl group in an anti position to that of a ribose sugar. This causes a slight conformational change in its structure compared with deoxycytidine. After entry into the cell, ara-C is phosphorylated to 1-β-D-arabinofuranosylcytosine 5'-monophosphate by deoxycytidine kinase (83), this being the

rate-limiting step in the conversion of ara-C to its nucleotide derivatives. Subsequent kinases convert the ara-C monophosphate to ara-CTP, the active inhibitor of DNA synthesis. Two modes of inhibition have been postulated for ara-CTP. The first is a direct effect on the DNA polymerase itself, while the second involves incorporation into the DNA and subsequent chain termination of DNA synthesis (75). In vitro studies have shown that ara-CTP inhibits the DNA polymerase-α isolated from calf thymus (84). Moreover, DNA polymerase-α is more sensitive to this inhibition than DNA polymerase-β. The kinetics of the DNA polymerase-α inhibition show it to be competitive with respect to dCTP, having an inhibitory constant (Ki) that is 8 times higher than that of DNA polymerase-β. However, the Ki of ara-CTP is approximately the same as the Michaelis constant (Km) for dCTP, implying that their binding affinities for the catalytic site of the DNA polymerase are similar. Thus ara-CTP is a weak competitive inhibitor in vivo, and cannot be the major mechanism by which DNA synthesis inhibition is achieved (76).

The second mechanism involves the incorporation of ara-CMP into DNA, producing termination of DNA chain elongation. When ara-CTP is added at the 3'-terminal end of the growing DNA chain, the presence of the 2'-hydroxyl of the arabinose moiety produces a conformational change in the template, thus preventing further enzymatic addition of other

nucleotides. Studies with radioactive ara-CTP and purified mammalian DNA polymerase have shown that chain termination does occur in vitro (85,86). However, the situation is more complex when studying intact cells. Very small amounts of radioactive ara-C is incorporated into DNA in vivo (which is consistent with the chain termination action), but enzymatic digestion studies show that much of the radioactivity is situated at internucleotide linkages and not at the terminal ends (76). Thus ara-C is not an absolute chain terminator, with termination possibly depending on the DNA sequence at the site where ara-CTP is incorporated. In vivo studies on Chinese hamster ovary cells (87) have shown that ara-C slows down the rate of chain elongation without altering the site at which DNA replication is initiated within individual replicons.

The effectiveness of ara-CTP as an antitumor drug has been correlated with its intracellular retention. Because the conversion of ara-C to ara-CMP, via deoxycytidine kinase is rate-limiting, any process that either enhances or suppresses this enzyme activity will affect the effectiveness of the drug. dCTP is a negative feedback inhibitor of deoxycytidine kinase, hence any fluctuations in dCTP pools could affect ara-CTP metabolism and activity. Other factors that affect dCTP pools (e.g. increasing the concentration of dThd will inhibit the ribonucleotide reductase and deplete dCTP pools) would also affect ara-CTP

activity. Thus pool size considerations are important when administering ara-C. By administering dThd and ara-C simultaneously (as has been already done in clinical trials (88), it is possible to increase the incorporation of ara-C into nucleoside triphosphate pools and thus increase its effectiveness at lower doses. Other drugs have been tested together with ara-C, in in vitro studies to improve its activity, e.g. HU and ara-C administered simultaneously cause enhanced ara-CTP activity (89); and daunorubicin shows synergistic activity with ara-CTP (74). Because of the rapid inactivation and excretion of ara-C (it is deaminated and rendered ineffective in the liver), macromolecular conjugates of ara-C have been investigated to enhance its efficacy. Poly-L-glutamic acid was shown to be effective as a carrier in one study (90).

Although it is now known that ara-C inhibits DNA synthesis, other biochemical mechanisms may be affected by this drug. Recently, Boehm and Drahovsky (54) showed elevated levels of 5-methylcytosine in a mouse mastocytoma cell line after treatment with ara-C. Furthermore, they show that the hypermethylation is permanent after removal of the drug, and suggest that ara-C may mediate the variety of cellular effects noted during treatment by altering gene expression.

HU was first synthesized by Dresler & Stein in 1869 and has been in clinical use since 1960. HU is an artificial monohydroxamic acid that inhibits DNA synthesis in a number

of systems (Hela cells (91), ascites tumor cells (92), regenerating rat liver cells (93) and bacteria 94)). It has also been shown to inhibit histone synthesis (95), but the major effect is on DNA synthesis inhibition. Clinical use of HU has led to improvements in a variety of solid tumors as well as in acute and chronic leukemia.

The major cytotoxic effect of hydroxyurea is thought to reside in its ability to inhibit ribonucleotide reductase activity (96,97,98), resulting in depleted deoxyribonucleotide pools required for DNA synthesis (97,99,100) (Ribonucleotide diphosphate reductase is responsible for the conversion of ribonucleotides to deoxyribonucleotides). Because of depleted deoxyribonucleotide pools, synthesis of new DNA strands is inhibited, resulting in the accumulation of short DNA fragments that cannot be elongated. However, controversy still surrounds the mechanism of the HU-induced DNA synthesis inhibition. Other mechanisms have been proposed that possibly occur simultaneously with ribonucleotide reductase inhibition, e.g. HU is thought to block pyrimidine synthesis at a step prior to orotic acid formation (82). Because HU affects repair synthesis to a much lesser extent than replicative DNA synthesis, and the fact that cells are not blocked at the G1/S boundary, but start replication and accumulate early in the S phase of the cell cycle, has indicated that the inhibition of ribonucleotide reductase is probably not the only mechanism by which HU affects DNA synthesis. Wawra and Wintersberger

(78) propose that HU interferes with the processing of early replication products, preventing the formation of longer intermediates by a mechanism that is either independent of the ribonucleotide diphosphate reductase or caused by the inhibition of a function of this enzyme at a later step of replication, which could explain why replicative synthesis is inhibited early in S phase and why DNA repair synthesis is relatively insensitive to the drug.

HU in conjunction with ara-C enhances the uptake of ara-CTP into DNA by reducing the intracellular dCTP pools through a reduction in the conversion rate of CDP to dCDP (89). Thus the use of HU simultaneously with other cytotoxic drugs may prove successful in the treatment of some leukemias. Ultimate success however depends on a better understanding of its mode of action within the cell.

Aphidicolin, the third inhibitor examined in this study, is a tetracyclic diterpene tetraol obtained from Cephalosporium aphidicola. It has a steroid-like structure but contains an additional ring system (73). It inhibits eukaryotic cell growth by inhibiting replicative DNA synthesis, due to specific inhibition of the DNA polymerase-α (81,101,102). DNA polymerase-β and γ are resistant to the drug (102). Protein and RNA synthesis are also unaffected by aphidicolin (103,104). Kinetic analysis of the mechanism of action on DNA polymerase-α suggests that the inhibition is competitive with respect to

dCTP and non-competitive with respect to DNA and other dNTPs (105,106). Other data, where DNA synthesis is monitored in intact nuclei, have shown that the inhibition is competitive with all four dNTPs. This contradiction possibly reflects a variation in pool sizes in the different systems, for it has been shown that in the presence of high concentrations of aphidicolin, all intracellular nucleotide pools increase (107). It has been proposed that the inhibition of DNA polymerase-α by aphidicolin arises by the binding of the drug to a site involved in the regulation of enzyme function rather than by binding to the active site (81). Furthermore, Yoshida et al (84) were able to show that DNA polymerase from calf thymus can be separated into subunits, some of which are resistant to aphidicolin.

At low aphidicolin concentrations, cells are able to overcome the DNA synthesis block after a few hours (108), probably due to an increase in nucleotide or DNA polymerase pools, or degradation of the drug by the cells. Aphidicolin does not cause additional DNA strand damage when added to cells after UV irradiation to assess single or double stranded breaks (108). Thus aphidicolin does not appear to affect DNA directly. Of the three drugs used, the least is known about the mechanism of action of aphidicolin. Whether these drugs affect other cellular functions is unknown at this stage. In order for these compounds to be effective as chemotherapeutic agents,

further knowledge of their biochemical mode of action is required.

To summarise, the major target areas are:

ARA-C:

1. Inhibition of the α-polymerase: a weak competitive inhibition
2. Incorporation into DNA and subsequent chain termination

HU:

1. Inhibition of the ribonucleotide reductase
2. Blocks pyrimidine biosynthesis at a site prior to orotic acid formation

Aphidicolin:

Inhibition of the α-polymerase: possibly a competitive inhibition with respect to dCTP.

Because ara-C induces a marked increase in the 5-methyl-cytosine content of mouse mastocytoma cells in culture (54), and because methylation patterns of DNA have been correlated with gene control, DNA methylation of normal and transformed cells that were treated with these drugs, was examined in this study. It was necessary to first establish the extent of inhibition caused by these drugs. This was achieved by monitoring cellular growth and DNA synthesis in the presence of the inhibitors.

Furthermore, the methylation status of the parental as well as the daughter DNA strands was investigated in both the normal and the two transformed cell lines.

3.2. RESULTS

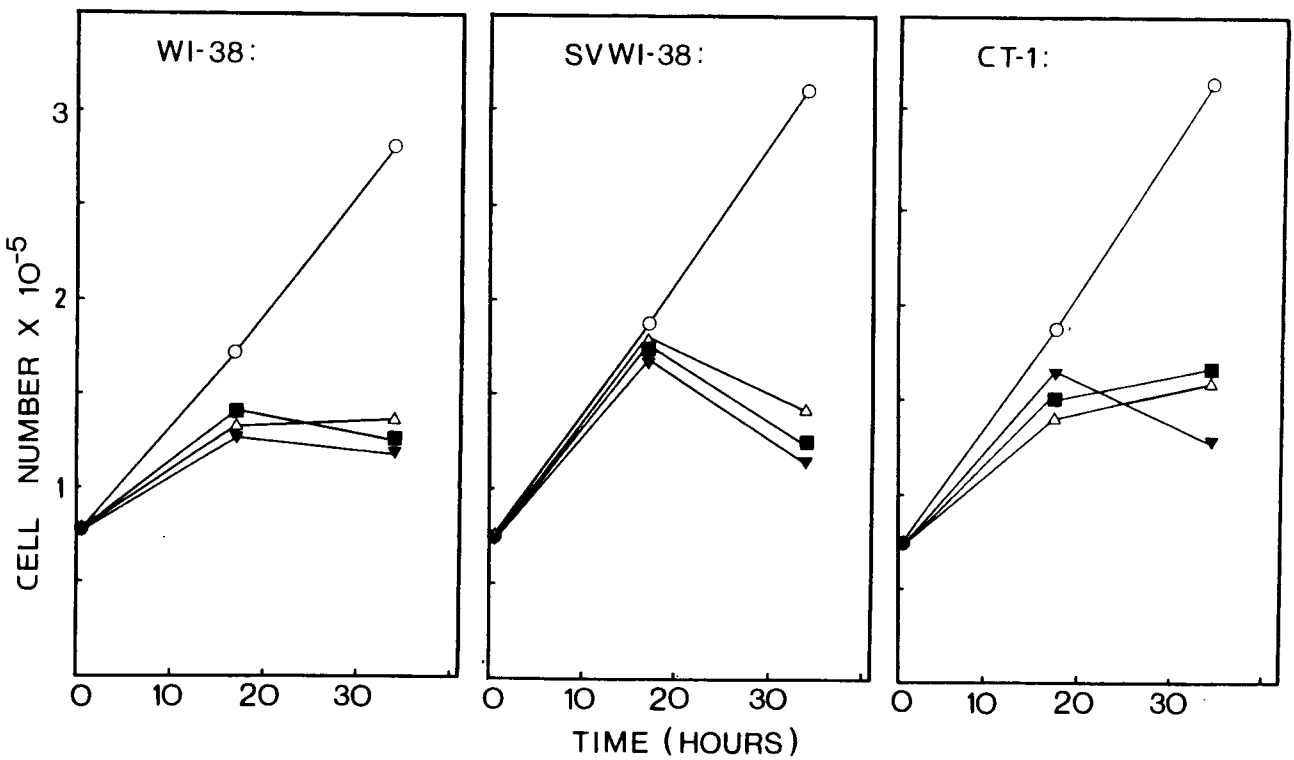
3.2.1. Cell growth in the presence of DNA synthesis inhibitors:

The effects of ara-C, HU and aphidicolin on WI-38 cells (normal embryonic lung fibroblasts) and two transformed cell lines, SVWI-38 (SV-40 transformed WI-38) and CT-1 cells (γ-irradiation transformed WI-38) were investigated. The concentrations used were those that inhibited DNA synthesis by 95-97% in other systems (109). Inhibitors were prepared and sterilised as detailed in Materials and Methods (6.4) and added to quadruplicate dishes of the three cell types. At time points of 18 hours and 36 hours, the cells were trypsinised and counted (see section 6.5 for details).

Fig. 3.1 compares the results obtained for the three cell lines after exposure to the drugs. The WI-38 cells and the virally transformed counterpart (SVWI-38) showed a marked difference in response to the added inhibitors. Over the first 18 hours, virtually no inhibition of growth was detected in the transformed cells in the presence of the inhibitors, whilst noticeable inhibition of growth occurred

FIG. 3.1 Cellular growth in the presence of inhibitors of DNA synthesis

Cells were plated at $5x10^4$ cells per 30 mm^2 dish and allowed to attach and grow before commencing treatment. Treatment with ARA C (■), HU (▼), and aphidicolin (△) was as described in Materials and Methods (6.4). Dishes were harvested at 18 and 36 hours after treatment. The cells were trypsinised and counted as described in section 6.1. (○) represents control dishes (i.e. no inhibitor treatment).



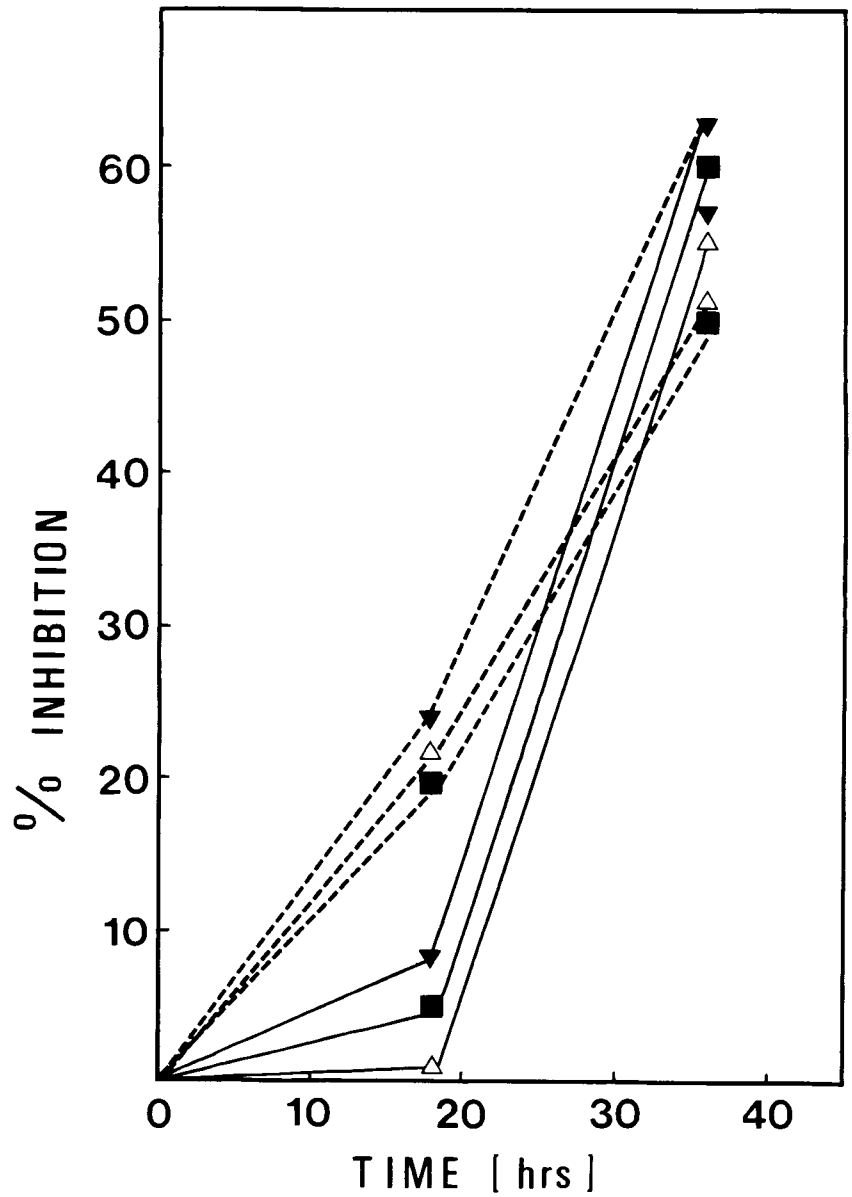
in the normal cells. These results became clearer when expressing the inhibition as a percentage of the control (see Fig. 3.2). Clearly the growth of the normal cells is more retarded than the transformed cells. It is interesting to note that in both WI-38 and SVWI-38, HU caused the greatest inhibition of growth over the first 18 hours. In the presence of ara C and aphidicolin, the γ-radiation transformed cells showed more growth inhibition than the SV-40 transformed cells, whilst the HU induced inhibition was virtually the same for both cell lines (see Fig. 3.1).

Continued growth in the presence of inhibitors for a further 18 hours did not allow further cell growth of any of the cell lines. The inhibitors had a cytostatic effect on the normal cells, while all three inhibitors had cytotoxic effects on the SVWI-38 cells, resulting in a negative growth slope. HU caused the most cell death in the SVWI-38 and CT-1 cells. Interestingly, aphidicolin allowed a slight increase in cell growth, in both the WI-38 and CT-1 cells. This effect has been observed by Iliakis et al (108) where the effect of aphidicolin on DNA synthesis inhibition was partially reversed, after longer treatment times (24 hours).

These different responses by normal and transformed cells probably reflect differences in nucleotide precursor pool sizes required for DNA synthesis.

FIG. 3.2 Inhibition of growth of WI-38 and SVWI-38 cells

The results obtained after treatment of WI-38 and SVWI-38 cells with the various inhibitors (Fig. 3.1) were expressed as a percentage of the untreated cells. (▼) represents treatments with HU, (△) represents treatment with aphidicolin and (■) represents Ara-C treatment, at concentrations giving 95-97% DNA synthesis inhibition. (See Materials and Methods (6.4)). (----), WI-38 cells and (——), SVWI-38 cells.



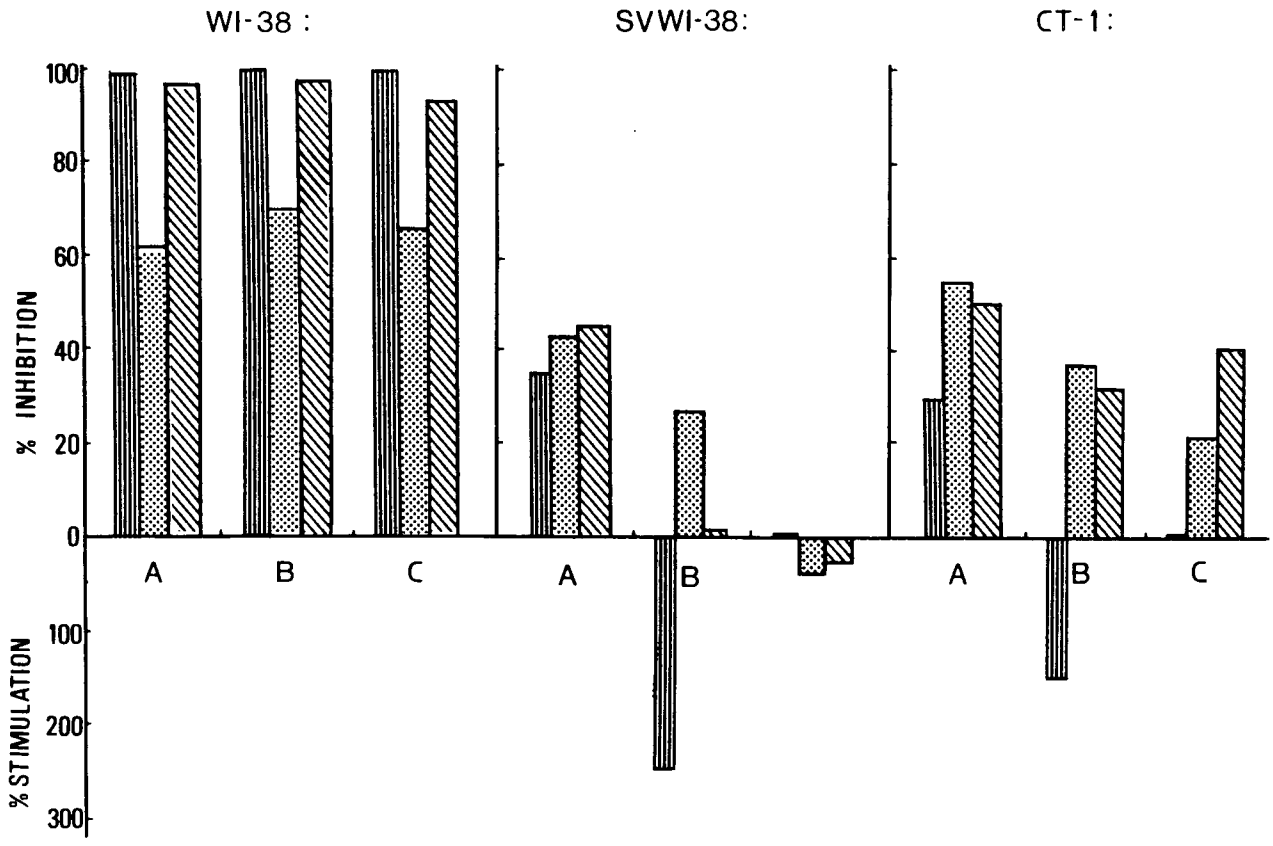
3.2.2. DNA synthesis inhibition:

To determine the extent of DNA synthesis inhibition induced by the three drugs, cells were incubated with ^{3}H-thymidine and the indicated inhibitor (at the specified concentrations) simultaneously for 16 hours. Cells were harvested, an aliquot counted for cell number determination and the radioactivity determined in TCA insoluble material, as described in Materials and Methods (6.7).

The results are shown in Fig. 3.3, where A, B and C represent treatments with HU, ara-C and aphidicolin respectively. The degree of DNA synthesis inhibition obtained in the WI-38 cells agrees favourably with that of the literature (108) where an inhibition of ±95-97% was obtained at these concentrations. The transformed cells showed somewhat surprising results. HU produced a ±30-35% inhibition of DNA synthesis in both the SVWI-38 and CT-1 cells, while no inhibition of DNA synthesis occurred in either the SVWI-38 or CT-1 cells treated with HU. Ara-C appeared to induce a 150 and 200 fold increase in ^{3}H-dThd uptake in the CT-1 and SVWI-38 cells respectively! From these results it therefore appears that the inhibitors are either able to stimulate DNA synthesis in transformed cells, or are unable to produce the same extent of DNA synthesis inhibition, possibly due to variations in intracellular thymidine pools, compared with WI-38 cells.

FIG. 3.3 Use of different radiolabelled precursors to monitor DNA synthesis in WI-38, SVWI-38 and CT-1 cells

Cells were plated at $5x10^4$ cells/30 mm^2 dish, and allowed to attach and grow before commencing treatment. A, B and C represent treatments with 10 mM HU, 1.5 mM Ara-C and 100 µg/ml aphidicolin respectively; the labelled markers were ^{3}H-Thd ▥, 5,6-^{3}Hdeoxyuridine ▨ and ^{3}H-Ade ▩. Treatment with inhibitor and radiolabelled precursor was for 16 hours, after which the cells were trypsinised, an aliquot counted and another aliquot precipitated with 10% TCA. DNA synthesis was expressed as Dpm/10^6 cells and the percent inhibition or "apparent stimulation" expressed as percentages of untreated cells (i.e. no inhibitor treatment).



To investigate this problem further, use was made of two other labelled nucleotides to monitor DNA synthesis. ^{3}H-deoxyuridine (dU) (which is incorporated into DNA after conversion into dThd and deoxycytidine) and deoxyadenosine (dAde) were added to the cells for 16 hours, as detailed in section 6.7. When using dU as a marker for DNA synthesis, all three inhibitors caused a 95-97% inhibition of DNA synthesis in WI-38 cells. The percent inhibition was reduced to ±60% when monitoring DNA synthesis with dAdenosine (see Fig. 3.3).

The results obtained with the transformed cells were interesting. In SVWI-38 and CT-1 cells, inhibition of DNA synthesis with HU as the inhibitor, gave results similar to those seen with dThd, namely a ± 40-50% inhibition of dU or dAde uptake. However, in ara-C treated cells, the apparent "stimulation" of dThd incorporation was reduced when using dU or dAde as markers (in fact, inhibition of incorporation occurred in both cell lines). In the SVWI-38 cells, a ± 40% inhibition of dAde uptake was noted, where previously a 200 fold stimulation of dThd uptake had occurred. Similarly the CT-1 cells showed a ± 40% inhibition of DNA synthesis, where a 150 fold stimulation had occurred previously. A slight inhibition (± 5%) of dUridine incorporation was noted in ara-C treated SVWI-38 cells, while CT-1 cells showed a ± 35% inhibition of incorporation (Fig. 3.3). Both transformed cells

responded differently in their uptake of ^{3}H-dAde and dU into TCA insoluble material when treated with aphidicolin. The SVWI-38 cells showed a slight stimulation of incorporation (± 50%) for both dAde and dU, while the CT-1 cells showed a ± 30 and ± 40% inhibition of dAde and dU uptake respectively.

Of importance is the fact that use of alternate labels as markers did not result in the same apparent "stimulation" of label incorporated in the transformed cells. This rules out the possibility that these inhibitors (especially ara-C) are capable of stimulating DNA synthesis in transformed cells. Two explanations as to why the transformed cells responded differently to their normal counterpart (i.e. a 98-99% inhibition was never achieved with any of the inhibitors in the transformed cells) can be proposed:

(a) The normal cells may be more sensitive to the effects of the three inhibitors. If the target area of inhibition is an enzyme, as is the case with HU and aphidicolin, then normal enzyme kinetics must be considered. For example, the number of enzyme molecules present in the cell: the more enzyme molecules, the more inhibitor required to produce the same extent of inhibition. Competition kinetics with normal substrates will also affect the degree of inhibition produced. Thus transformed cells may have more target enzymes or the affinity of the inhibitor for

the enzyme may be less in transformed cells, resulting in reduced inhibition at equivalent inhibitor concentrations in transformed cells compared with normal cells.

(b) Nucleotide precursor pool size variations may account for these results. The apparent reduction in the amount of inhibition caused by the three inhibitors in the transformed cells could be explained if transformed cells have smaller nucleotide pool sizes, e.g. although HU appeared to cause only a 35% inhibition of dThd uptake in SVWI-38 cells, DNA synthesis inhibition may in fact be the same as that of the WI-38 cells. The inhibition is then reduced from 90-95% to 35% due to the limited DNA synthesis that continues in the presence of the inhibitor (5-10%), incorporating ^{3}H-dThd of higher specific activity into DNA, resulting in the synthesis of DNA that is of a higher specific activity.

3.2.3. DNA methylation in the presence of inhibitors of DNA synthesis:

The work of Drahovsky et al (54) on the treatment of mouse mastocytoma cells with 0,1 µg/ml ara-C (a dose that gave a 50% inhibition of DNA synthesis) was the first demonstration of the hypermethylation of DNA during treatment with ara-C. In this study, two approaches were undertaken to investigate whether hypermethylation could be induced by other DNA synthesis inhibitors as well as ara-C.

Cells were either pre-labelled with 6-^{3}H-uridine for 72 hours and then treated with the drug, or the drug and label were added simultaneously to the cells, at a concentration of inhibitor which gave 50% inhibition of DNA synthesis. This enabled the investigation of parental as well as daughter DNA strand methylation patterns.

The following problems were addressed:

(1) Does ara-C induce hypermethylation of DNA in other cell lines and if so, do normal and transformed cells respond differentially to treatment (Drahovsky and coworkers examined the effects of ara-C on transformed cells only)?

(2) Are the methylation patterns of DNA in normal and transformed cells affected by either HU or aphidicolin?

(3) Is the hypermethylation of parental and daughter DNA strands induced to the same extent within the same cell type?

3.2.3.1. Methylation of the parental DNA strand:

Prelabelled DNA was exposed to the indicated doses of HU, ara-C and aphidicolin for 16 hours, and the 5-methylcytosine content determined as detailed in Materials and Methods (6.9 and 6.10).

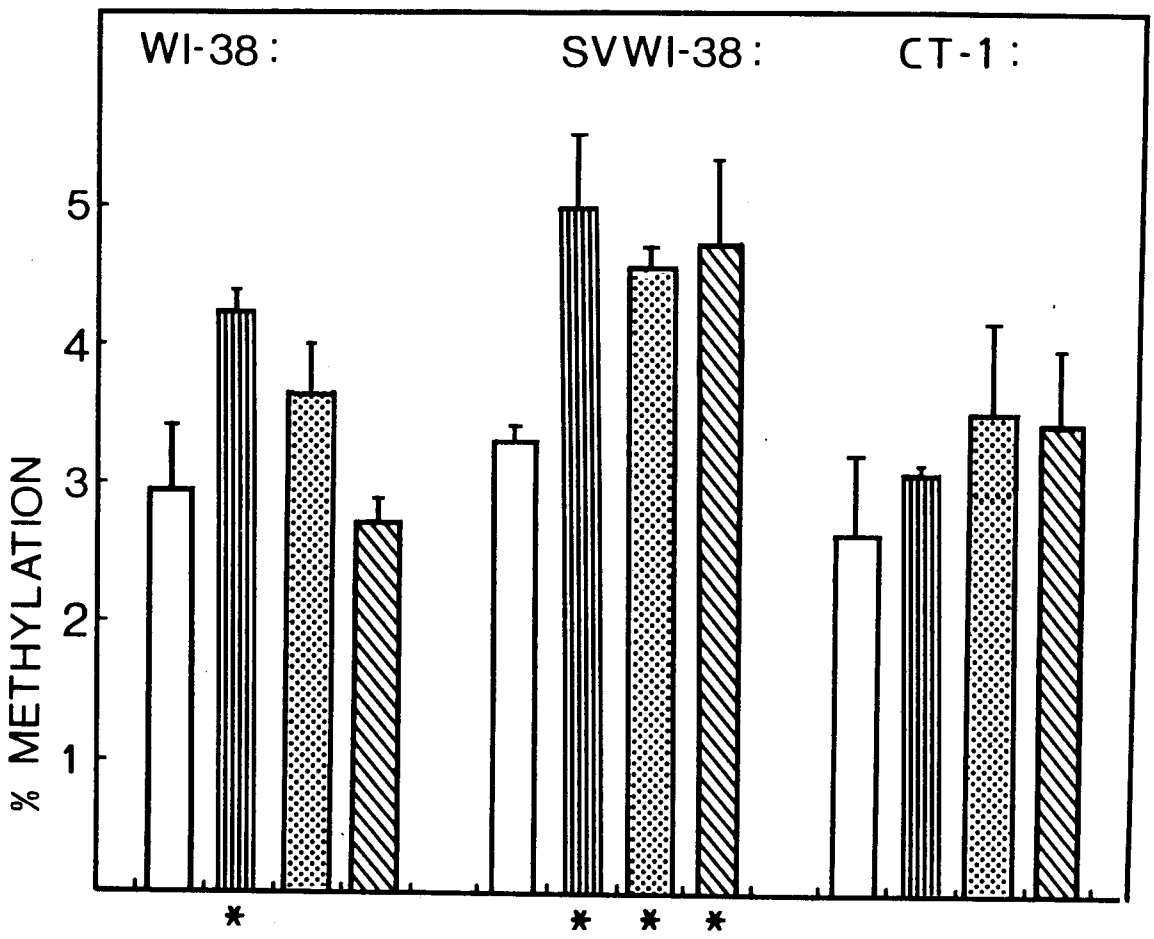
Fig. 3.4 shows the results obtained when WI-38, SVWI-38 and CT-1 cells were treated with the three DNA synthesis inhibitors. HU induced a slight increase in DNA methylation in both WI-38 and SVWI-38 cells, whereas ara-C and aphidicolin did not cause any noticeable increase in the 5-methylcytosine content of WI-38 cells. None of the three inhibitors affected the methylation status of the CT-1 cells. Ara-C and aphidicolin did however induce an increase in the parental DNA strand methylation of the SVWI-38 cells. Thus, of the three cell types, the SV-40 transformed cells showed the greatest extent of hypermethylation of the parental DNA strand when treated with either HU, ara-C or aphidicolin, while only HU was able to induce hypermethylation in WI-38 cells. The CT-1 cells remained unaffected by the three drug treatments.

3.2.3.2. Methylation of the daughter DNA strand :

Although Drahovsky et al (54) did not distinguish between parental and daughter strand DNA, the protocol (where the drug and label were added simultaneously) was such that newly replicating DNA or daughter DNA strands were investigated. In this study, daughter DNA strand methylation levels were investigated at concentrations that caused 50% and 95-97% DNA synthesis inhibition respectively. Although 95-97% DNA synthesis inhibition was achieved, sufficient label was incorporated into the DNA, due to the

FIG. 3.4 Hypermethylation of parental DNA strands in the presence of various DNA synthesis inhibitors

Cells were labelled with 1 μCi/ml of 6-^{3}H-uridine for 3 days after which the cell layers were rinsed and replaced with fresh medium containing either 10 mM HU (▥), 1.5 mM Ara-C (▩) or 100 μg/ml aphidicolin (▨) for 16 hours. The medium was removed, the cells lysed in SDS/NaOH, the DNA extracted and the 5-mC content determined as described in Materials and Methods (6.92 and 6.10). * Indicates those treatments where methylation values were significantly increased above that of control cells (□). Bar (⊤) indicates standard deviation (n=5).



3-5% DNA synthesis that continued in the presence of the inhibitors.

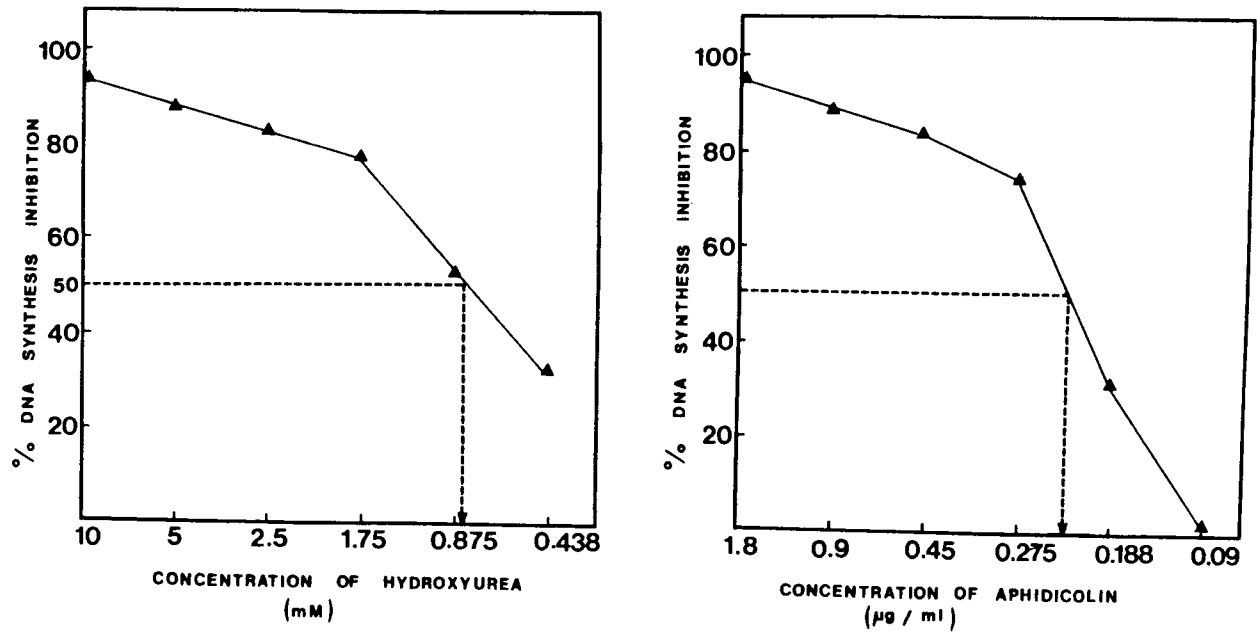
To establish the concentration required to give a 50% inhibition of DNA synthesis, cells were treated with increasing doses of the inhibitor, and the incorporation of ^{3}H-thymidine into TCA insoluble material was determined as detailed in Materials and Methods (6.7).

Fig. 3.5 shows the inhibition curves of WI-38 cells treated with increasing inhibitor concentrations. From these results, the concentration required to give ±50% inhibition of DNA synthesis was 0,88 mM for HU and 0,232 µg/ml for aphidicolin. The 50% inhibition concentration obtained for the latter agrees well with Iliakis et al (108), where a concentration of 0,2 µg/ml produced 50% inhibition after a 24 hour treatment period. It was noted that a concentration of only 0,075 µg/ml aphidicolin was required if the cells were treated for 6 hours instead of 24 hours. Cells appear to overcome the aphidicolin block when treated for longer periods and thus require higher doses to produce the same extent of inhibition. The value of Drahovsky et al (54) of 0,1 µg/ml was used to obtain the 50% inhibition with ara-C.

To investigate daughter DNA strand methylation at 95-97% DNA synthesis inhibition, cells were treated with 5 µCi of ^{3}H-uridine and HU, ara-C or aphidicolin for 16 hours. The

FIG. 3.5 Determination of the concentration of inhibitor required to give 50% inhibition of DNA synthesis

WI-38 cells were treated with the indicated concentrations of either HU or aphidicolin plus 1 μCi/ml ^{3}H-dThd for 16 hours to determine the extent of DNA synthesis inhibition. After removal of the medium, the cells were trypsinised, one aliquot was counted and another TCA precipitated; DNA synthesis was thus expressed as Dpm/10^{6} cells, and DNA synthesis inhibition was expressed as a percentage of control cells (i.e. no inhibitor treatment).



DNA was isolated and the 5-methylcytosine content analysed by HPLC, as detailed in section 6.10. Fig. 3.6 shows the results of this experiment.

No significant increase in methylation was observed for WI-38 cells treated with HU, ara-C or aphidicolin. Treatment with HU gave an apparent hypomethylation of daughter strand DNA. Both ara-C and aphidicolin caused significant hypermethylation of daughter DNA strands in the SVWI-38 cells, while only aphidicolin caused significant hypermethylation in CT-1 cells.

To investigate daughter strand methylation at 50% DNA synthesis inhibition, cells were treated with the required drug doses. Both ara-C and aphidicolin produced significant hypermethylation of daughter DNA strands in SVWI-38 cells (Fig. 3.7). Similarly, these two inhibitors also caused hypermethylation of daughter DNA strands in the CT-1 cells. Only HU caused a marked increase in methylation in the WI-38 cells, while the other two inhibitors did not affect the methylation status at all.

A comparison of the above two experiments allows the following deductions to be made:

(1) High concentration of HU reduced the methylation in WI-38 cells and hypomethylation resulted.

FIG. 3.6 Methylation of daughter DNA strands in the presence of inhibitors of DNA synthesis (95-97% inhibition)

Cells were treated with 1 μCi/ml 6-^{3}H-uridine and 10 mM HU (▥), 1.5 mM ara-C (▩) and 100 μg/ml aphidicolin (▨) simultaneously for 16 hours. The medium was removed, the cells were lysed, the DNA prepared and the 5-mC content determined, as described in Materials and Methods (6.9.2 and 6.10). * Indicates significantly hypermethylated DNA (n=5).

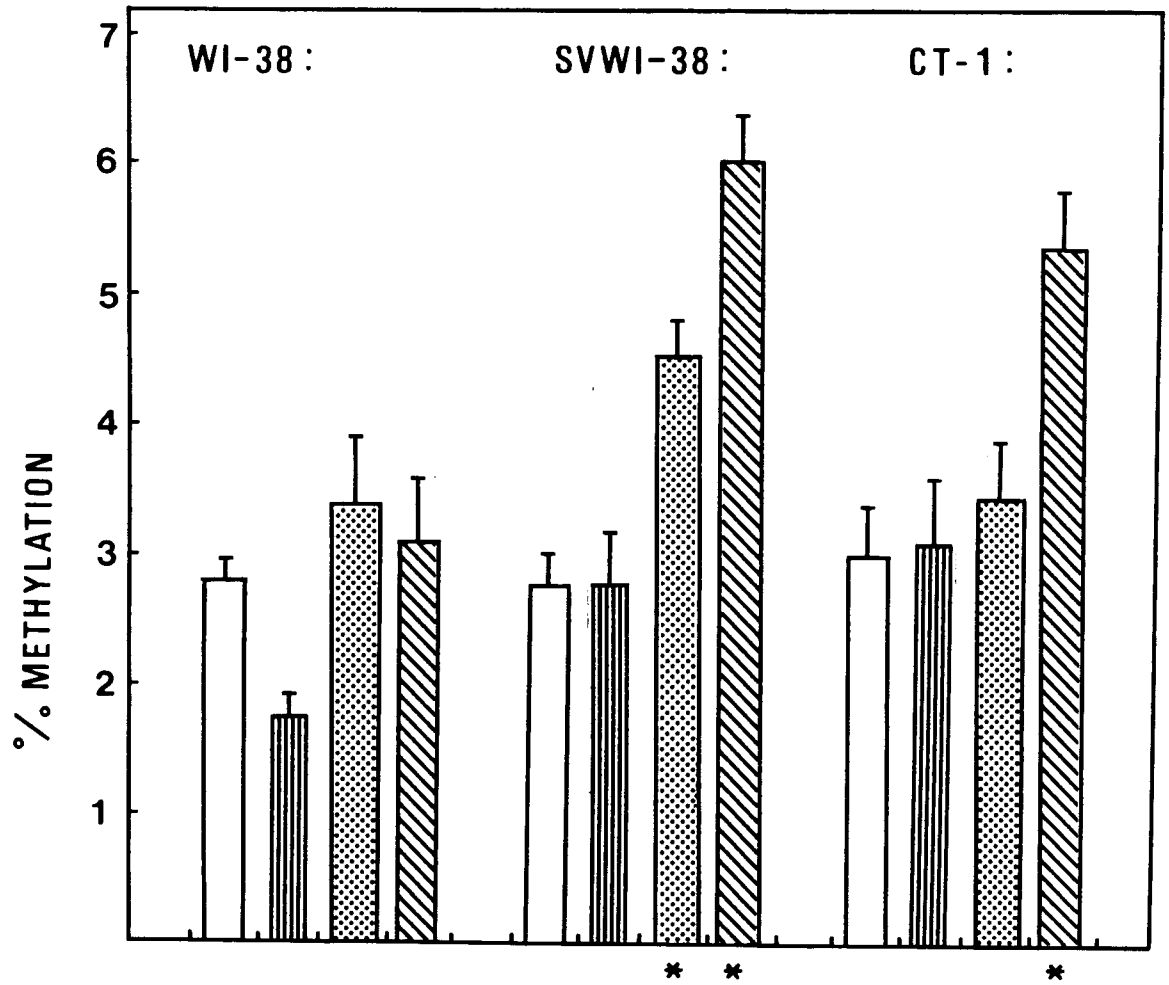
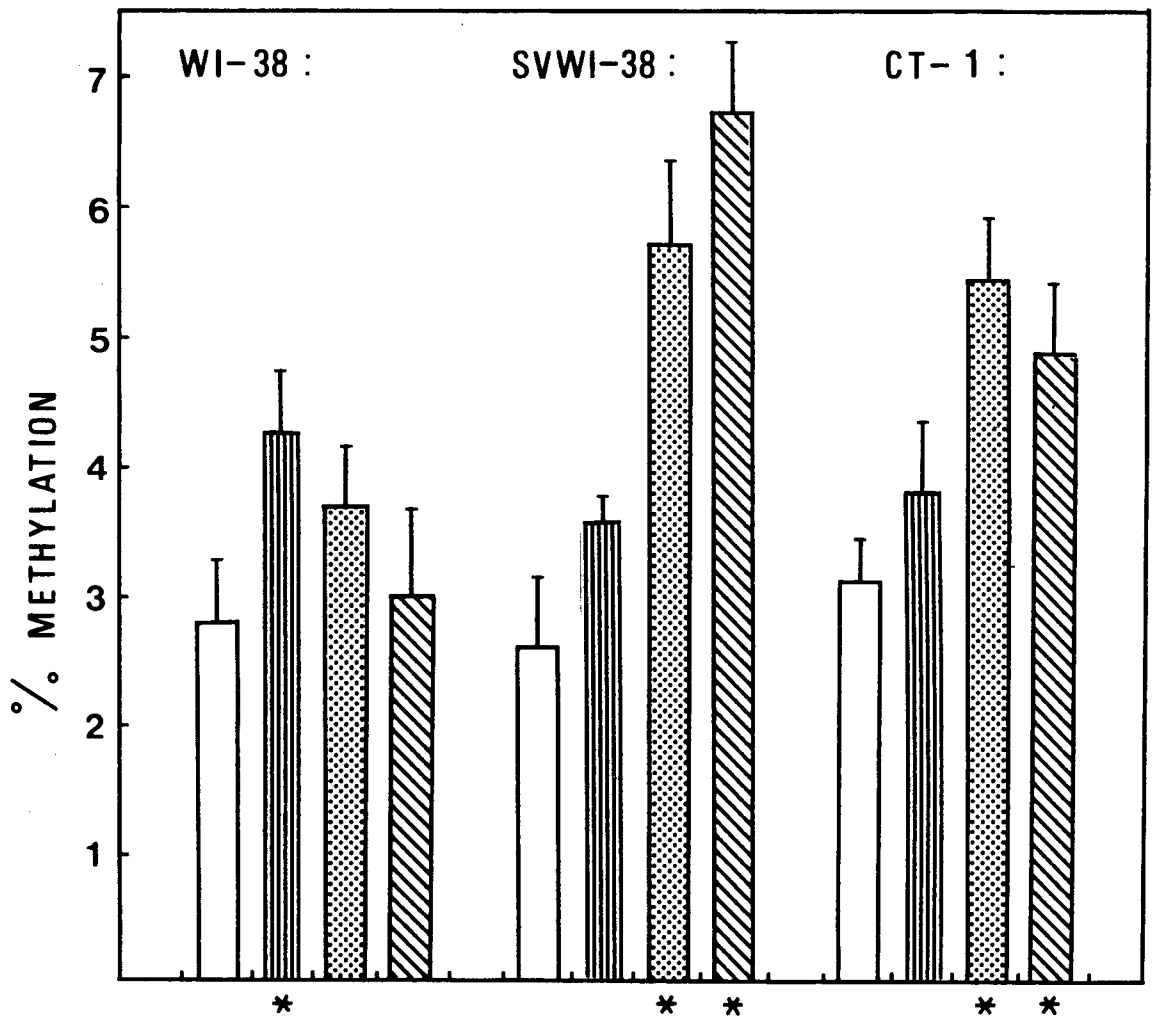


FIG. 3.7 Methylation of daughter DNA strands in the presence of inhibitors of DNA synthesis (50% inhibition)

Cells were treated as described in the legend to Fig. 3.6 except that concentrations required to give 50% DNA synthesis inhibition (as determined from Fig. 3.5) were used; i.e. 0,875 mM HU, 0,232 µg/ml aphidicolin and 0.1 µg/ml Ara-C (54). ▥ represents HU; ⊡ represents ara-C and ▨ represents aphidicolin.



(2) An increase in the concentration of either ara-C or aphidicolin did not abolish the hypermethylation of SVWI-38 cells, although a slight decrease in the extent of hypermethylation is evident in both cases.

(3) Hypermethylation was not induced in CT-1 cells at higher ara-C doses.

Thus the overall effect of increasing the inhibitor concentration is a reduction in the extent of hypermethylation, this being partially reduced in the transformed cells and resulting in hypomethylation in the WI-38 cells.

3.3. DISCUSSION

This study has made use of a normal cell line and its two transformed counterparts to investigate the effects that various DNA synthesis inhibitors have on DNA methylation. Ara-C has been used extensively in clinical trials for the treatment of certain leukemias (in particular, acute myelogenous leukemia (69,71) and acute non-leucocytic leukemia (88)), while hydroxyurea has been used to treat patients with chronic granulocytic leukemia (110). It is important to realise that these drugs will encounter normal as well as malignant cells during treatment. The need therefore arises to establish precisely what effects these drugs have on normal and transformed cells.

Drahovsky et al (54) were able to show that treatment of mouse mastocytoma cells with ara-C led to DNA hypermethylation. This was the first report detailing such an effect by inhibitors of DNA synthesis, although Taylor and Jones (111) had previously suggested the possibility. However, Drahovsky and coworkers investigated these effects on transformed cells only. It was decided in this study to investigate the effects of ara-C on both normal and transformed cells. Ara-C is known to cause DNA synthesis inhibition by affecting the DNA polymerase, but more specifically through its incorporation into DNA and subsequent chain termination. Two other known DNA synthesis inhibitors, whose modes of inhibition differ from ara-C, were also included in this study, to determine whether the incorporation of the cytidine analogue into the DNA could be a reason for the observed hypermethylation (the other two inhibitors are not incorporated into the DNA). It was first necessary to establish what effects these inhibitors had on cell growth and DNA synthesis inhibition.

Growth curve analysis of the normal and transformed cells showed different responses to the three inhibitors, over a 36 hour treatment period. The transformed cells (especially the SVWI-38 cells) were virtually unaffected by drug treatment during the first 18 hours, while the normal cells showed a $\pm$ 20% reduction in cell growth. Prolonged growth in the presence of the inhibitors resulted in

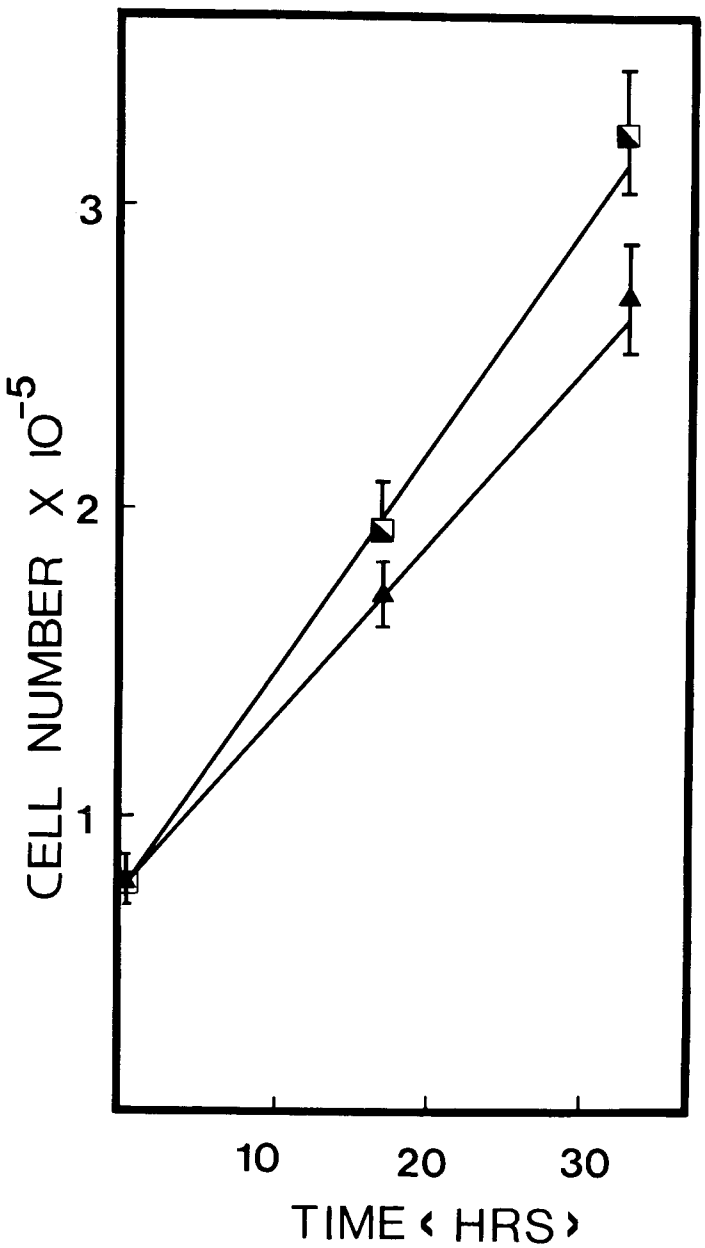
cytotoxicity of the SVWI-38 cells (cells must either have lysed or peeled off the dish, thus giving the negative growth curve), while the normal cells were prevented from dividing but remained viable. Comparison of the growth of the transformed cells showed that CT-1 cells were more affected than the SVWI-38 cells. From these results it could be deduced that such treatments should be given for longer time periods, in order to cause maximum cytotoxicity of transformed cells. The inhibitors affected the normal cells to a lesser extent, that is, the drugs had a cytostatic effect on the WI-38 cells. The initial drug resistance of the transformed cells could be related to the availability of just sufficient precursors to sustain one round of replication, whereas the normal cells have larger precursor pools.

Most interesting was the finding that transformed cells showed an "apparent" reduction of DNA synthesis inhibition (monitored by deoxythymidine incorporation into DNA). This led to an investigation of the use of other labelled precursors to monitor DNA synthesis in transformed cells. From these results it was evident that DNA synthesis inhibition was in fact not reduced or stimulated (as was seen with ara-C, where an apparent stimulation of deoxythymidine incorporation into DNA occurred), for the same inhibitor treatment gave different amounts of DNA synthesis inhibition with the various labelled precursors. Two mechanisms that could account for this are proposed:

(1) A reduction in the size of nucleotide precursor pools in transformed cells. The reduction of pool sizes could be due to either a general or inhibitor mediated reduction of de novo nucleotide synthesis in transformed cells. Irrespective of the mechanism of nucleotide pool reduction, addition of labelled precursor to the cells results in precursor pools of higher specific activity. Incorporation of these nucleotides into DNA will give the "apparent stimulation" or less inhibition that was noted for the transformed cells. If reduced nucleotide pools are a feature of transformed cells, then this could be related to the length of time spent in the G1 phase of the cell cycle. It is within this phase that the cell prepares for the events that are to follow, that is, the synthesis of nucleotides required for DNA synthesis during the S phase (112). Transformed cells generally have reduced cell cycle times. From results presented in this study, the SVWI-38 cells have a doubling time of 14 hours compared with an 18 hour doubling time of WI-38 cells (see Fig. 3.8). Reduced cycling time is a consequence of a reduced G1 phase. Many transformed cells do not exhibit any G1 phase at all and traverse the S phase immediately after mitosis. The shortened G1 phase may explain the reduction in nucleotide precursor pool size, for transformed cells consequently may have less time to synthesize the same amount of nucleotide precursor as normal cells.

FIG. 3.8 Population doubling of WI-38 (▲); SVWI-38 (■) and CT-1 (□) cells

Cells were plated at $5x10^4$ cells per 30 mm² dishes, allowed to attach and grow. At t=0, the medium was removed and replaced with fresh medium. Cell number determinations were made at T=0, 18 and 36 hours. (Cells were trypsinised and counted as described in Materials and Methods (6.1)). Each time point represents an average of 5 determinations. WI-38 cells have a slope = 1,0 while SVWI-38 and CT-1 cells both have a slope = 1,28 which corresponds to doubling times of 18 and 14 hours respectively.



If transformed cells have reduced nucleotide precursor pools, then an interesting paradox exists. Transformed cells, as already discussed, have shorter cell-cycling times and therefore have faster cellular growth than normal cells. One would therefore expect that the reduced nucleotide pools would be disadvantageous to the rapidly dividing cell, for these precursors are vital for DNA synthesis. Possibly a delicate balance has been established where transformed cells have just sufficient material to ensure rapid DNA synthesis. Possibly it is the reduced precursor pool sizes that are rate limiting in transformed cells with respect to their cellular growth, that is, precursors are synthesized at the same rate as normal cells, but they are utilised faster such that supply just manages to meet demand.

(2) Because the target end-points of the inhibitors are often enzymes, enzyme kinetics must be considered. Both ara-C and aphidicolin affect the polymerase, while HU inhibits ribonucleotide-reductase. If the affinity of the enzyme for its natural substrate is greater than for the inhibitor (i.e. the Ki is low), then the effective inhibition will be reduced. To produce the equivalent amount of inhibition would require a higher inhibitor concentration. The Ki for the polymerase and the ribonucleotide reductase could therefore be lower in transformed cells, which would then explain the reduced DNA

synthesis inhibition observed. Alternatively, transformed cells may have increased numbers of target enzymes, so that at the same inhibitor concentration, the effective inhibition in transformed cells will be less.

It is most likely that variable pool sizes are responsible for the different extents of inhibition observed. Even when using deoxyadenosine as the marker for DNA synthesis, the inhibition never reached 95-97% with either of the three inhibitors in the normal cells. Kinetic considerations probably apply mainly to HU and to aphidicolin, because ara-C has been shown to be a weak competitive inhibitor in vivo (76). Ara-C treatment was responsible for a massive stimulation of thymidine incorporation into DNA in both transformed cells. This was probably due to reduced endogenous thymidine pools. Whether ara-C was responsible for reducing the de novo pool, or whether transformed cells generally have smaller pools of precursor nucleotides, is difficult to say. The opposite effect, where thymidine enhanced the incorporation of ara-C into DNA, due to a negative thymidine feed-back on the ribonucleotide reductase (thus lowering dCTP pools (88)), has been shown.

The main conclusion that can be derived from these studies, is that normal and transformed cells respond differently to different DNA synthesis inhibitors, and that these differences probably arise due to variations in nucleotide

pool size between normal and transformed cells. Care should therefore be taken in interpreting such data when monitoring DNA synthesis in normal and transformed cells. DNA synthesis inhibition is further complicated by the fact that the inhibitors themselves affect nucleotide pools. It has been observed that in the presence of high concentrations of aphidicolin (5 µg/ml) all intracellular nucleotide pools are increased (107).

Analysis of the 5-methylcytosine content of parental and daughter DNA strands has revealed a number of interesting findings. Drahovsky et al investigated the methylation status of daughter DNA strand in a transformed cell line only. This is equivalent to the methylation observed in the SVWI-38 and CT-1 cells treated with 0,1 µg/ml ara-C. Furthermore, these authors reported a hypermethylation of 6,4%. In this study, methylation percentages of 5,7 ± 0,7% and 5,45 ± 0,45% were obtained for the SVWI-38 and CT-1 cells respectively. Our percentages therefore agree with those of Drahovsky et al (54). Of importance was the finding that ara-C did not affect the methylation of daughter DNA strands in the normal cell line. Ara-C therefore specifically enhanced the daughter DNA strand methylation levels of the transformed cells. Even at higher doses of ara-C, the SVWI-38 cells still showed DNA hypermethylation, although the extent of this hypermethylation was slightly reduced. CT-1 cells no longer showed the induced hypermethylation at higher doses

of ara-C, indicating that very high concentrations of inhibitor probably affect the activity of the methylases. This is substantiated by the fact that HU caused significant hypermethylation of WI-38 daughter DNA strands at 50% inhibition of DNA synthesis, while at 95-97% DNA synthesis inhibition, the percent methylation dropped considerably below that of the control (i.e. hypomethylation was induced).

Of the other two inhibitors, only aphidicolin was able to mimic the effects of ara-C in the SVWI-38 and CT-1 cells. The induced hypermethylation in the SVWI-38 cells at both 50% and 95% inhibition, was greater than with ara-C. Aphidicolin was able to induce hypermethylation in the CT-1 cells at 50% and 95% inhibition, whereas at the latter concentration, ara-C was in fact unable to induce hypermethylation of the DNA. Hydroxyurea did not induce hypermethylation of daughter DNA strands in either of the transformed cell lines. This implies that the induced hypermethylation is not due to a general DNA synthesis inhibition, as was speculated by Drahovsky et al (54), but is more specific, for only ara-C and aphidicolin were able to elicit these effects.

Examining the mode of action of the inhibitors suggests that possibly it is the inhibitory effect on the α-polymerase that triggers the induced hypermethylation. Assuming that the inhibitor e.g. ara-C or aphidicolin,

binds to the polymerase once the polymerase has attached to the DNA, maintenance methylases which follow closely behind the polymerases, may recognise the "inhibitor-polymerase" complex and this may then trigger hypermethylation of newly synthesized DNA. Hydroxyurea does not affect the α-polymerase. This could therefore explain why hypermethylation was not observed with hydroxyurea. A further observation can be made; at both 50% and 95% DNA synthesis inhibition, aphidicolin induced a greater extent of hypermethylation than ara-C, in the SVWI-38 cells. If "α-polymerase-inhibitor" binding is the trigger, then this could explain why aphidicolin induced the greater hypermethylation, for aphidicolin is a stronger inhibitor of the α-polymerase than ara-C, which has been shown to be a weak inhibitor in vivo. Thus ara-C would require a higher concentration of inhibitor to cause the same extent of hypermethylation. Other mechanisms could however have led to the observed hypermethylation.

Possibly these inhibitors

(i) increase the number of maintenance methylase molecules (i.e. at the transcriptional level, genes coding for these enzymes are activated)

(ii) maintenance methylases are activated in a similar way to a repressor/activator system, where repressor molecules are bound to the enzyme until a stimulus or activator releases the repressor and the enzyme is then free to perform its function.

(iii) the inhibitor could cause the exposure of more methylatable sites in the DNA to the enzymes, thus methylating sites that are not normally methylated.
(iv) the inhibitors inhibit "demethylating enzymes". It is generally accepted that demethylases do not exist in vivo, for they have not yet been isolated. Two reports have however provided evidence for their existence (37,38). If a demethylase does exist, then methyl-group turnover must occur and methylation values are therefore average values of this turnover. Thus hypermethylation need not necessarily be due to increased methylase activity, but rather due to decreased demethylase activity.

Only the parental DNA strand of SVWI-38 cells was significantly hypermethylated after treatment, all three inhibitors inducing such hypermethylation. HU alone caused hypermethylation of the WI-38 cells, while the CT-1 cells were unaffected by the drugs. Hypermethylation of parental DNA strand is only possible if

(1) Maintenance methylases "back-track" along parental DNA strands and methylate sites that are not normally methylated.
(2) De novo methylases are stimulated, which then randomly methylate non-replicating DNA.

As was seen with daughter DNA strand methylation, parental DNA strand hypermethylation occurs preferentially in the

transformed cells, (daughter DNA strand hypermethylation occurs in both SVWI-38 and CT-1 cells and parental DNA strand hypermethylation in the SVWI-38 cells), while the normal cells are relatively unaffected by drug treatment. Only HU produced significant hypermethylation of both parental and daughter DNA strands (the latter at a 50% inhibition) in the normal cells. HU inhibits DNA synthesis in a totally different manner to ara-C and aphidicolin. This could explain why it alone affected the normal cells. Possibly the ribonucleotide reductase of the WI-38 cells is more sensitive to the inhibitory effects of HU, thus resulting in the increased methylation. It is difficult to speculate how this hypermethylation is induced. Ara-C and aphidicolin caused hypermethylation of parental and daughter DNA strands in the transformed cells only. These inhibitors appear to selectively hypermethylate transformed cell DNA. This could be the manner in which ara-C is able to selectively affect leukemic cells in the treatment of acute myelogenous leukemia (69,71). Alterations of the methylation patterns of DNA could lead to changes in the expression of certain genes and ultimately to faulty gene products. Hypomethylation has been associated with gene activation, while conversely, hypermethylation with inactive genes. These inhibitors could therefore elicit their inhibitory effects in transformed cells, by "switching-off" the synthesis of vital gene products required for the maintenance of life.

From this study the following can be deduced:

(1) Transformed cells appear to have smaller nucleotide precursor pools than normal cells.

(2) After longer treatment periods, the inhibitors are cytotoxic with respect to cell growth of the transformed cells but are cytostatic with respect to cell growth of the normal cells.

(3) Treatment with inhibitors that specifically affect the α-polymerase leads to hypermethylation of DNA, this hypermethylation only being induced in transformed cells.

(4) HU induces hypermethylation of both parental and daughter DNA strands in normal cells, possibly as a consequence of ribonucleotide reductase inhibition.

CHAPTER 4

THE EFFECTS OF SODIUM BUTYRATE ON DNA METHYLATION IN NORMAL AND TRANSFORMED CELLS

4.1 INTRODUCTION

Butyrate is a 4-carbon fatty acid, usually present in lipids and constitutes about 10% of milk fatty acids. It occurs naturally in the body and is formed by the hydrolysis of ethyl butyrate. Detailed studies of butyrate first began when it became established that administration of N_6O_2'-dibutyryl adenosine 3'5'-cyclic monophosphate (dibutyryl cAMP) to cells results in hydrolysis to cAMP and butyrate. It therefore was necessary to include sodium butyrate as a control in those experiments. Results showed that butyrate was in fact responsible for many of the observed effects. Butyrate has proven interesting as a tool in the study of the cells' molecular biology, because it elicits a wide variety of morphological and biochemical effects on cells in culture. That butyrate itself is responsible and not a metabolic product is evident from studies on other fatty acids which are shown to be less effective in eliciting these effects. Table I compares many known effects enhanced by butyrate and other monocarboxylic fatty acids. In all cases studied, the four-carbon fatty acid elicits the greatest response. Furthermore, branched derivatives of butyrate

TABLE 1

A COMPARISON OF A NUMBER OF EFFECTS OF SODIUM BUTYRATE AND OTHER MONOCARBOXYLIC FATTY ACIDS

In all cases, the 4-carbon fatty acid elicits the greatest response to the indicated treatment (114).

Table 1. Comparative effects of various monocarboxylated compounds.

Inhibition of calf thymus histone deacetylase (Cousens et al. 15)

C_2	10%
C_3	60%
C_4	80%
C_5	65%
C_6	30%

Effect on HTC cell histone acetylation (Sealy and Chalkley, 21)

	Acetylated histones
Control	40%
C_2	50%
C_3	72%
C_4	80%
iso C_4	60%

Inhibition of estradiol induced synthesis of transferrin mRNA (McKnight et al. 71)

C_2	18%
C_3	77%
C_4	95%
iso C_4	21%

Correlation between histone acetylation and thyroid hormone receptor level in GH_1 cell line (Samuels et al. 74)

	Relative histone acetylation	T_3 receptor level (% of control)
C_2	20	85
C_3	65	40
C_4	100	30
C_5	65	40
C_6	25	90

Effect on fibroblast growth, as % of control (Hagopian et al. 78)

C_2	82
C_3	45
C_4	0
iso C_4	79
C_5	71

Effect on CGH synthesis in Hela cells (Ghosh et al. 106)

	proportional increase with 5 mM	
	intracellular	extracellular
C_3	2.4	2.2
C_4	197	167
C_5	47.6	94.1
C_6	2.0	25.5

Induction of alkaline phosphatase in HeLa cells (Griffin et al. 109)

	% of control
C_2	170
C_3	160
2-methyl C_3	110
C_4	630
2-methyl C_4	470
C_5	420
C_6	120
C_7	150
C_8	33

Effect on l-isoproterenol stimulated cAMP production in HeLa cells (Tallman et al. 110)

	pmol cAMP/mg protein/min
0	3
C_2	3
C_3	41
C_4	46
C_5	26
C_6	5

e.g. iso-butyrate are not as effective as the straight chain n-butyrate (113,114).

Before proceeding further, it is necessary to highlight the various known effects that butyrate exhibits on cells in culture:

4.1.1 Sodium butyrate and histone hyperacetylation:

Histones H3 and H4 from erythroleukemic and Hela cells, following treatment with 5 mM sodium butyrate, have been shown to be hyperacetylated (115,116) . Both mono- and multiacetylated forms of H3 and H4 are found. In in vitro experiments using partially purified acetylase preparations, histone H4 is acetylated to a greater extent than histone H3, although in vivo results show the reverse situation (117). There are 4 lysine residues on histone H3 and H4 that can become acetylated, these being located close to the N-terminal basic end of the molecule. Acetylation is a way of neutralising the very basic charged end of the histone molecule. In doing so, electrostatic attractions between the basic N-terminal end and the negatively charged phosphate backbone of the DNA are weakened, and this could be a trigger for gene activation. DNase 1 digestion studies have shown that butyrate treatment causes diminished stability of nucleosomal DNA (118,119,120). Furthermore, Simpson et al (121) found that chromatin isolated from Hela cells treated with 5 mM sodium butyrate, was 5-10 fold more rapidly digested by DNase 1,

and released fragments that were highly enriched in acetylated forms of histones H3 and H4.

Histones H2A and H2B are affected to a lesser extent by butyrate treatment. Histone hyperacetylation is reversible in all systems investigated. 24 Hours after removal of butyrate from the culture medium, acetylation levels returned to that of control levels (115), while 15 mins after removal of butyrate, acetylated histones returned to the unmodified form in Hela cells (122). The in vivo acetylation is catalysed by an acetyl transferase which uses acetyl-Coenzyme A as the acetyl group donor (123,124). The butyrate-induced hyperacetylation could result from increased acetylase activity; however, butyrate failed to enhance the activity of acetylase enzymes in in vitro studies (114). Acetyl groups are subject to rapid turnover; 70% are removed with a half-life of 3 mins in HTC cells, the rest with a half life of 30-40 mins (125). A deacetylase enzyme, which has been partially purified, is responsible for the removal of the acetyl groups. Butyrate has been shown to be a non-competitive inhibitor of the deacetylases, having a Ki of 10 μM (i.e. no effect on the Km, but decreased Vmax) (126). Butyrate therefore acts at a site on the enzyme other than the substrate binding site. The deacetylase is associated with a nuclease resistant high molecular weight fraction of chromatin, forming a high molecular weight complex (127,128). Butyrate probably acts as a tight binding

detergent to the deacetylase. This was deduced from the results of Cousins et al (126) where the inhibition was maximal with n-butyrate, while a 40-60% inhibition occurred with n-propionate and n-pentanoate, suggesting a relative lack of specificity for fatty acid chain length. Interestingly, the insertion of polar groups into the fatty acid molecule prevented hyperacetylation, thus no effect on the deacetylase is observed with 3-hydroxybutyrate or acetoacetate (129).

4.1.2 Sodium butyrate and protein phosphorylation:

Sodium butyrate selectively inhibits the phosphorylation of the histone proteins H1 (130,131) and H2A (131). This inhibition is concentration and time dependent, and is reversible after removal of butyrate. These effects, unlike acetylation, are not associated with a change in the rate of removal of the modifying group, i.e. no change in the phosphatase activity. Instead it is probably due to an inhibition of the histone kinase and/or an increased accessibility of histone substrates.

Butyrate also affects the phosphorylation of non-histone nuclear proteins, but in a complex way. Phosphorylation may be stimulated, unaffected or inhibited depending on the protein analysed (131). The HMG proteins 14 and 17 also show hyperphosphorylation during butyrate treatment. These two proteins are associated with actively

transcribing chromatin and thus gene expression promoted by sodium butyrate may be a consequence of the phosphorylation status (132). It is known that sodium butyrate increases the levels of cAMP in some human cells (113). Thus a number of the effects exhibited by butyrate may be mediated through cAMP. Low concentrations of cAMP are known to stimulate the phosphorylation of histone proteins, and possibly the hyperphosphorylation of the HMG proteins is induced in this manner.

4.1.3 Methylation and ADP ribosylation of proteins:

As butyrate concentrations increase, the methylation of histones and non-histone nuclear proteins is inhibited. This inhibition is not due to an inhibition of S-adenosyl (methyl)-methionine (SAM) formation, as analysis of SAM pools showed no difference in control and butyrate treated cells (131). ADP-ribosylation of proteins is stimulated by butyrate, as shown by Boffa et al (122) on Hela histones.

4.1.4 The effect of butyrate on cell growth and morphology:

Butyrate inhibits cell growth in a time and concentration dependent manner (130). Chinese hamster ovary (CHO) cells treated with low concentrations of butyrate (up to 2.5 mM) are still capable of cell growth after 24 hours of

treatment (130). However, at 15 mM butyrate, no growth occurs after 20 hours. Analysis of the DNA content of cells using a fluorescent dye, showed that butyrate traps the cells early in the G1 phase of the cell cycle (130,133,134). The block has been estimated to occur 5 hours prior to the beginning of S phase in 3T6 fibroblast cells (133). Thus butyrate differs from other inhibitors of DNA synthesis e.g. hydroxyurea or flurodeoxyuridine where cells accumulate in early S phase or at the G1/S border. Cells already in S phase during butyrate treatment are not trapped in the S phase of the cell cycle (133), becoming blocked only after a further round of mitosis and entry into the G1 phase. The butyrate block is reversible; 12 hours after release from butyrate, 50% of 3T6 fibroblast cells could synthesize DNA (133). Serum factors are required before and after the butyrate sensitive step if cells are to progress into S-phase. Release from the butyrate block results in asynchronous populations of cells entering S phase, thus butyrate is not useful as a cell synchronising agent. These results led Wintersberger et al (133) to propose that butyrate acts by inhibiting cells at a critical point in G1, possibly at the "restriction point" postulated by Pardee et al (135). Because both low concentrations of butyrate and cycloheximide block cells in G1, it is suggested that butyrate acts by interfering with the synthesis of one or more rapidly turning over cell-cycle specific proteins.

A second effect observed after butyrate treatment is the slowing down of the cell cycle progression through G2 and/or mitosis and possibly through S phase in CHO cells (130). It has been reported that butyrate is capable of inducing cell cycle arrest in G2 in Hela cells as well (136).

Normal and transformed cells differ morphologically in culture. Transformed cells are usually rounder in shape, have smoother edges, lack growth contact inhibition and have decreased cell-cell and cell-substrate adhesion (114). Treatment of transformed cells with millimolar concentrations of butyrate usually induces a morphology that is similar to that of the normal cell. More specifically, butyrate induces neurite-like processes in mouse neuroblastoma cells within a concentration range of 1 µM to 0,3 mM butyrate (57), and extensive processes with flattened cell morphology in Hela cells treated with 2mM butyrate for 24 hours (137). In both cases butyrate does not affect cell viability (137), even at high concentrations, although this has been shown to vary according to the cell type under investigation (113).

Alteration of the cellular cytoskeleton is one of the main effects of butyrate (114). Butyrate acts on the cytoskeleton in a manner similar to that of the antitumor and antiviral agent, interferon, by enhancing the organisation of microfilaments, intermediate filaments and

fibronectin in MSV-transformed cells (138). Addition of butyrate to transformed rat kidney cells results in the assembly of cytoplasmic microfilaments and microtubules (139), resembling a more differentiated state. These effects are particularly noticeable in virally and chemically-induced transformed cells, which could partly explain the effect of butyrate on the morphology and growth in these cells. Butyrate also enhances the production of interferon in MSV transformed cells (139) and in human Namalva lymphoma cells (140), thus enhancing the effect on the cytoskeleton. Butyrate acts specifically on the external cytoskeletal matrix, namely fibronectin. Treatment of NRK cells that are transformed by Kirsten sarcoma virus with butyrate, results in the restoration of the fibrillar fibronectin network at the cell surface (141).

4.1.5 Protein synthesis in the presence of Sodium Butyrate:

Of all the effects induced by butyrate, its ability to stimulate the synthesis of specific proteins in a number of systems, has been the most intriguing. The extent of protein synthesis induction depends entirely on the species and cell type investigated (113). Butyrate enhances the activity of a number of enzymes in both mouse and human neuroblastoma cells. In particular, tyrosine hydroxylase activity is increased twentyfold in mouse neuroblastoma cells (142), while the activities of choline

acetyltransferase (143), acetylcholine esterase (144) and catechol-O-methyltransferase (145) are also markedly increased in mouse neuroblastoma cells. Because of its selective action on human neuroblastoma cells (with respect to cellular growth and viability) together with its ability to induce neurite-like process formation (i.e. a more differentiated state), butyrate has been suggested as a useful chemotherapeutic agent for the treatment of human neuroblastomas (113).

The induction of haemoglobin synthesis was demonstrated in murine erythroleukemic cells derived from mouse spleen, that had been infected with Friend virus complex (146). Butyrate is a potent inducer in this system, requiring less than one hundredth the concentration of dimethyl-sulfoxide (DMSO), needed to produce the same effect (147). Terminal induction has also been demonstrated in a human promyelocytic leukemic cell line (148). For this reason butyrate has been suggested as a useful drug in the treatment of certain leukemias.

Hormonal induction occurs in Hela cells when treated with millimolar concentrations of butyrate (114). Both eutopic (induction of proteins normally synthesized by the cells from which the tumor arose) and ectopic induction (induction of proteins not synthesized in the tissue of origin) is observed in these cells. Ectopic synthesis of human chorionic gonadotropin (HCG) is induced by butyrate

in Hela cells (149). In the same system, butyrate also induces increased follicle stimulating hormone (FSH) production (150). Furthermore, the specific activity of the membrane bound alkaline phosphatase is increased ten-fold by sodium butyrate in Hela cells (151). This is also observed in a human pancreatic tumor cell line (152). A possible mechanism underlying induction or the inhibition of hormonal induction has been suggested by Martel et al (153), where butyrate prevents prolactin action on the induction of casein synthesis, due to a direct effect on the membrane. Being a polar fatty acid, the action of butyrate on membrane lipids is not very surprising. This might also occur during the induction of egg white genes, stimulated by oestrogens (154) and protein factors having receptors on the cell membrane which are inhibited or stimulated by butyrate.

Induction of β-adrenergic receptors by butyrate has been demonstrated in Hela cells (155). Although 5-azacytidine (aza-C), a nucleoside analogue of cytosine and a known inducer of differentiation, is also capable of inducing β-receptors, exposure to both butyrate and aza-C resulted in induction levels that were 2 to 3 times greater than the sum of those found with butyrate or aza-C alone (155). This implies that the induction of β-receptors by the two agents involves separate mechanisms.

Concordant expression of "early placental" alkaline phosphatase, pregnancy-specific β1-glycoprotein and the β subunit of human chorionic gonadotropin is induced by 3 mM sodium butyrate in a uterine cervical cancer cell line (SKG-IIIa) (156). Induction of these oncodevelopmental proteins suggests the re-expression of sets of development phase-specific genes in cancer cells. Of importance is the finding that butyrate induces adenylate cyclase activity in Hela cells, erythroleukemic cells and rat foetal hepatocytes (114), resulting in an increase in cellular content of cAMP. This may mediate many of the observed effects of sodium butyrate.

Butyrate also increases the production of the ganglioside BM3 in Hela cells (157) (these are acidic glycolipids that are found in cellular membranes). Other gangliosides and neutral glycosphingolipids are unaffected by butyrate. This is attributable to the ability of butyrate to activate sialyltransferase 1 activity, an enzyme directly involved in BM3 synthesis. The specific activity is increased 10-20 fold, which is prevented by cycloheximide and actinomycin D, suggesting that butyrate induces the synthesis of this enzyme.

Differential responses to butyrate are seen in different tumor cell lines of the same tumor. For example, carcinoembryonic antigen is stimulated in some cell lines of human colonic adenocarcinoma cells in culture (158),

while in other cell lines, antigen production is not stimulated. These results suggest that butyrate may not affect gene expression through a general phenomenon. Although most studies show induction of protein synthesis as a result of butyrate treatment, a number of reports also show inhibition of protein synthesis. For example, 7 mM butyrate inhibited the synthesis of the transformation related protein p53 in 3T6 mouse fibroblasts (159), while creatine phosphokinase activity was inhibited in presumptive myoblasts that are prevented from fusing (160). Furthermore, general properties associated with neoplasia (161) are also inhibited by butyrate.

Of utmost importance is the ability of butyrate to induce differentiation in many transformed lines. Butyrate has tremendous potential as a chemotherapeutic agent for the treatment of human neuroblastomas (113) and acute leukemias (55). Already this agent has been used clinically on a patient with acute myelogenous leukemia, resulting in the elimination of peripheral myeloblasts with a concomitant increase in the number of mature myeloid cells (55). If sodium butyrate is to have significant clinical application, knowledge of its precise mode of action in the cell is vital.

In this study various effects of sodium butyrate on tissue cultured cells were investigated. Most of the research done in this field has investigated the effects of butyrate

on transformed cells,e.g. human pancreatic tumors (152), uterine cervical cancer cells (SKG-IIIa) (156), Hela cells (150,162,163) and many others (113,114,164). The lack of a suitable normal cell line for comparison has been a problem in many studies. Throughout this study, use was made of a normal cell line and its two transformed counterparts, allowing a direct comparison of the effects of butyrate on normal and transformed cells. It is felt that this is important since butyrate will interact with normal as well as transformed cells when used clinically.

The following aspects have been examined:

(1) Cell growth in the presence of increasing butyrate concentrations,

(2) The effects of butyrate on total DNA, RNA and protein synthesis,

(3) ADPRT activity as a measure of DNA repair,

(4) The effect on proteins synthesized in the presence of butyrate, in both normal and transformed cells, and

(5) The effect of butyrate on DNA methylation.

A link has been established between DNA methylation and gene activity, i.e. demethylation is correlated with gene

activation. Because butyrate is able to induce new gene transcripts in some systems, while inhibition of specific gene expression occurs in other systems, we investigated the effect that butyrate has on total DNA methylation in both normal and transformed cells. In this study, butyrate is shown to induce hypermethylation in both parental and daughter DNA strands. Furthermore, maintenance of the hypermethylated state is only possible if methylation occurs during DNA replication.

The results of this study show the differential responses of normal and transformed cells to millimolar concentrations of butyrate.

4.2 RESULTS

4.2.1. Cell growth and toxicity in the presence of butyrate:

The effects of butyrate on cell growth and toxicity were examined on three cell lines. These cell lines were WI-38 fibroblasts, a normal human embryonic lung cell line and two transformed counterparts, SVWI-38 (simian virus 40 (SV40) transformed) and CT-1 cells (a γ-radiation transformed cell line). Cells were treated with increasing concentrations of sodium butyrate until they showed altered morphology when examined under the light microscope, after 14 hours of treatment. This was done in order to determine the threshold concentration of sodium butyrate required for cytotoxicity in all 3 cell lines. Although most studies on the biochemical effects of butyrate have been conducted within the 1 to 20 mM range, the normal cells and their transformed counterparts showed no gross morphological alterations after treatment with butyrate at concentrations as high as 100 mM. Concentrations higher than 100 mM did however affect cell morphology and cell death was evident at 150 mM butyrate and above for the 3 cell lines examined. Trypan blue exclusion tests showed that those cells attached to the surface of the tissue culture dish were still viable after

butyrate treatment, even at concen-trations as high as 100 mM butyrate, for WI-38, SVWI-38 and CT-1 cells. (See section 6.6 of Materials and Methods.)

On microscopic examination, both transformed cell lines showed an increased number of mitotic figures, compared with the WI-38 cells. Fig. 4.1 shows the response of the WI-38 and SVWI-38 cells to increasing sodium butyrate concentrations. Clearly visible was the increase in mitotic figures of the SVWI-38 cells, while the WI-38 cells were not affected by the fatty acid. To investigate the effect of butyrate on cell toxicity, cells were treated with 0, 5, 10 and 20 mM butyrate for 14-16 hours. The cells were trypsinised (as detailed in section 6.1) and counted.

Fig. 4.2 shows that the greatest toxicity occurred in the transformed cells. The effects of sodium chloride on the three cell lines was included as a control to show that hypertonicity of the medium was not responsible for the observed changes. No toxic effects were observed within the 0 to 10 mM range in the normal cell line. A ± 20% decrease in the number of viable cells was however noted at 20 mM, where no effect was observed with sodium chloride. Both transformed cell lines (SVWI-38 and CT-1) showed a ±30% decrease in viable cell number at 5 mM butyrate, while no decrease was observed in the cells treated with sodium

FIG. 4.1 Comparison of WI-38 and SVWI-38 cells treated with 0, 10 and 20 mM butyrate

Cells were treated with the indicated concentrations of sodium butyrate for 16 hours, after which photographs were taken using a Nikon M-35 S camera. Of interest is the increase in the number of mitotic figures (rounded cells) in SVWI-38 cells with increasing butyrate concentrations, while no such increase is noted in the WI-38 cells at equivalent concentrations.

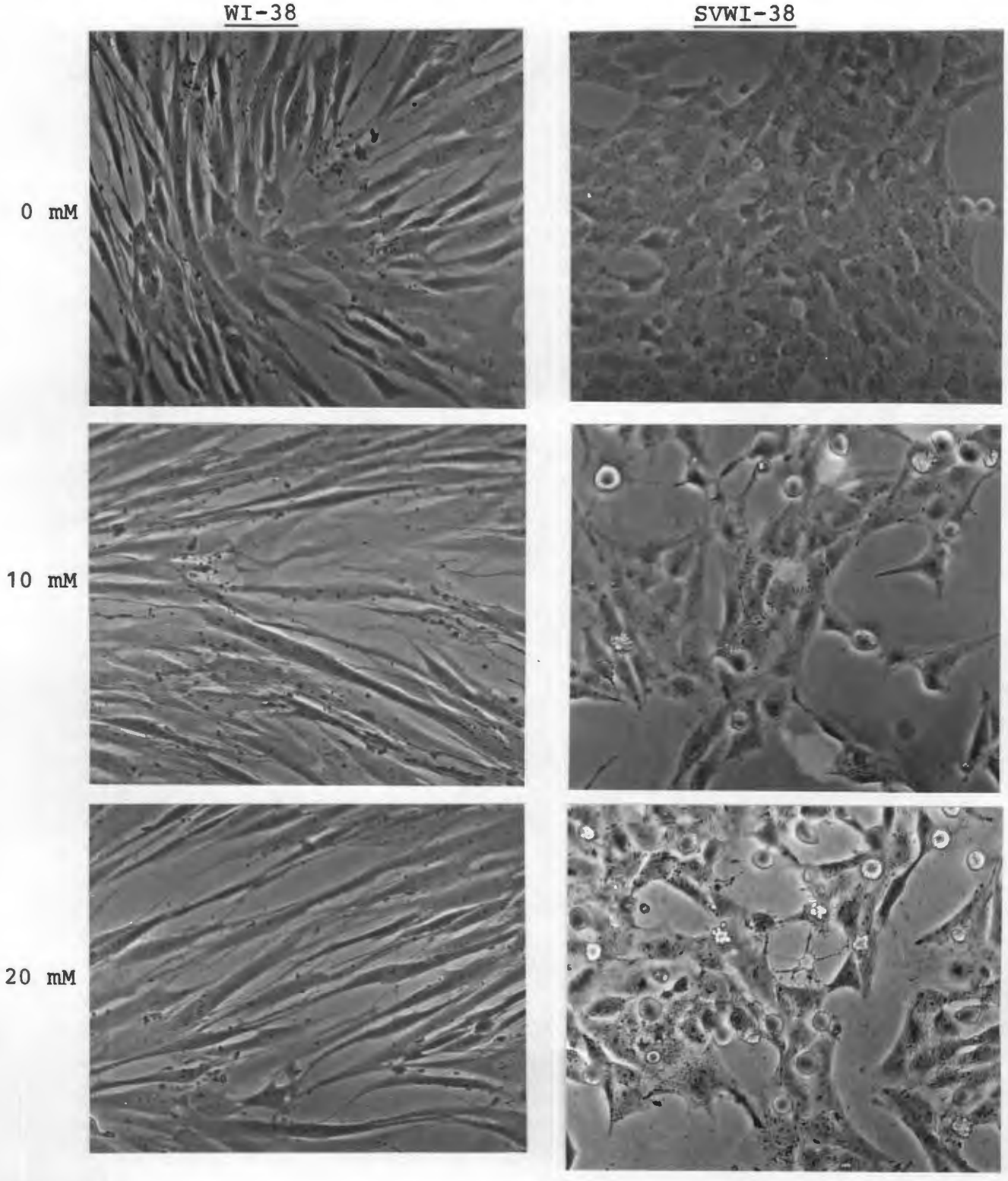
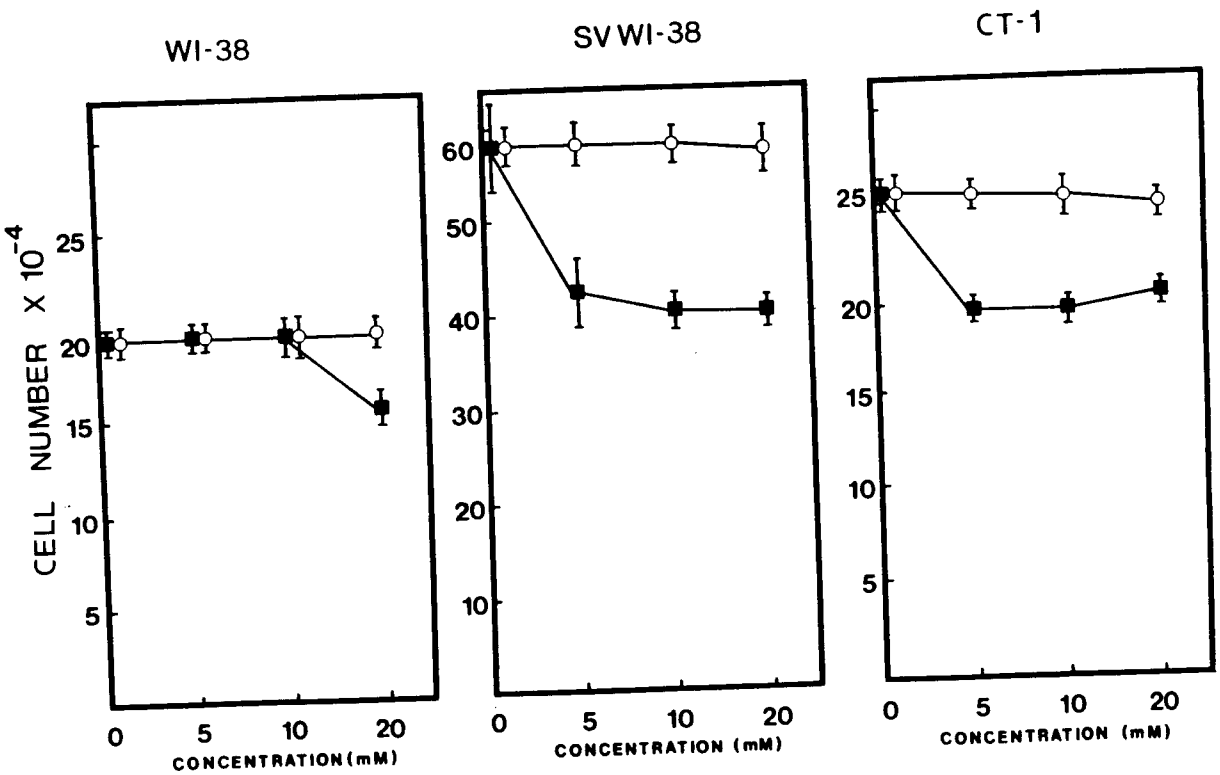


FIG. 4.2 Cell toxicity in the presence of increasing concentrations of sodium butyrate or sodium chloride

Cells were plated at $5x10^4$ cells per 30 mm^2 dish and allowed to attach to the dish and grow before commencing treatment. After sufficient cell growth, the medium was replaced with fresh medium containing the indicated concentrations of either sodium butyrate (■—■) or sodium chloride (○—○), for 16-20 hours. Cells were trypsinised and counted as described in Materials and Methods (6.1).



chloride. The effects were thus butyrate-specific and not due to the hypertonicity of the medium.

At higher butyrate concentrations, the toxic effects were even more evident in the transformed cells. Fig. 4.3 shows the response of the 3 cell lines to higher concentrations of butyrate, where the number of viable cells was monitored as the percent of cells still attached to the tissue culture flask after 14 hours of treatment, compared with untreated cells. Cell toxicity was less than 10% in the normal cells up to 150 mM, while the values for both transformed lines were 40% at 75 mM butyrate. The CT-1 cells displayed greater toxic effects than the SVWI-38 cells in the 25 to 75 mM concentration range. At higher concentrations, the toxic effect on the SVWI-38 cells was more evident than on the CT-1 cells.

Butyrate has been shown to affect cell growth in a time and concentration dependent manner in several other systems investigated (113). To determine whether this occurred in our system, three concentrations of butyrate were used in the different cell lines over an 18 hour time period and cell number determinations were made at two-hourly intervals. Fig. 4.4 shows the results obtained when WI-38, SVWI-38 and CT-1 cells were treated with 75, 120 and 200 mM sodium butyrate for 18 hours.

FIG. 4.3 Cell toxicity at higher sodium butyrate concentrations

Cells were treated as described in the legend to Fig. 4.2, except that the doses of sodium butyrate added to the cells were those shown below. Toxicity is represented as a percentage of control dishes where no butyrate was added. WI-38 cells are represented as (○—○), SVWI-38 cells as (▲—▲) and CT-1 cells as (□—□).

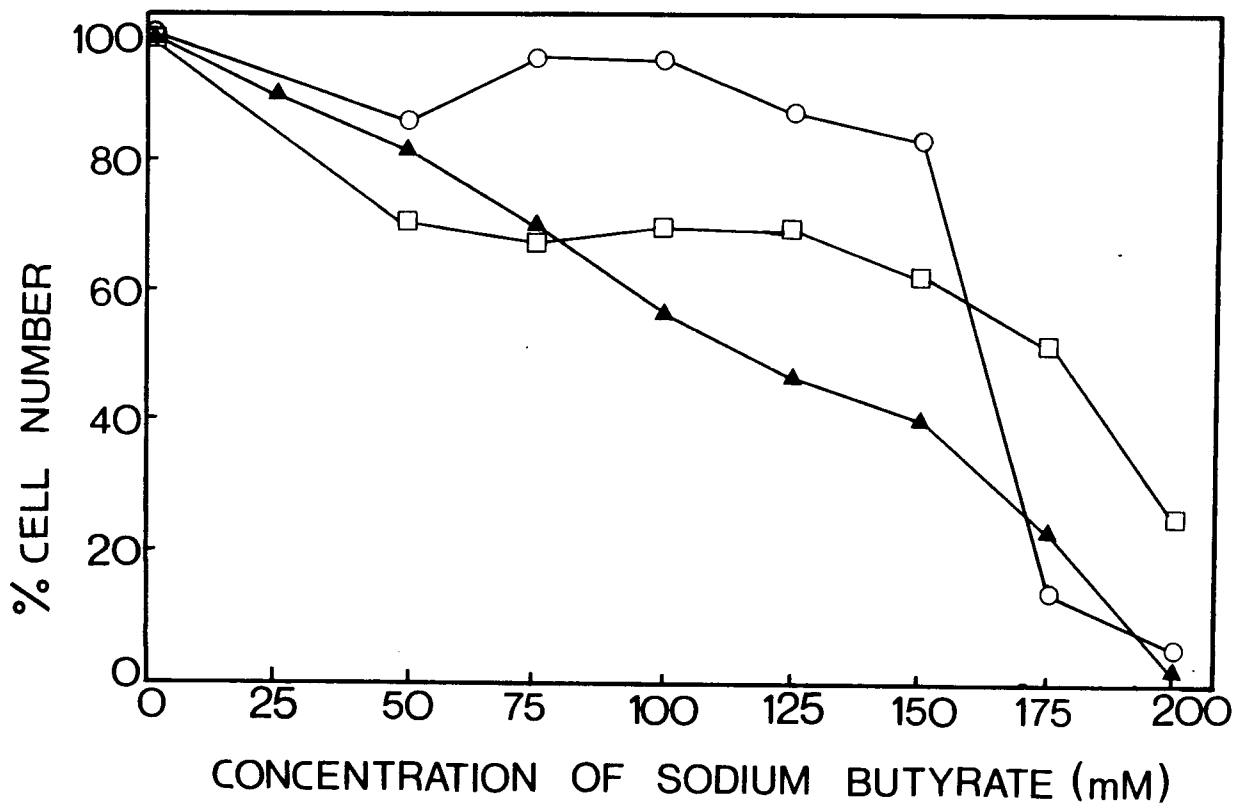
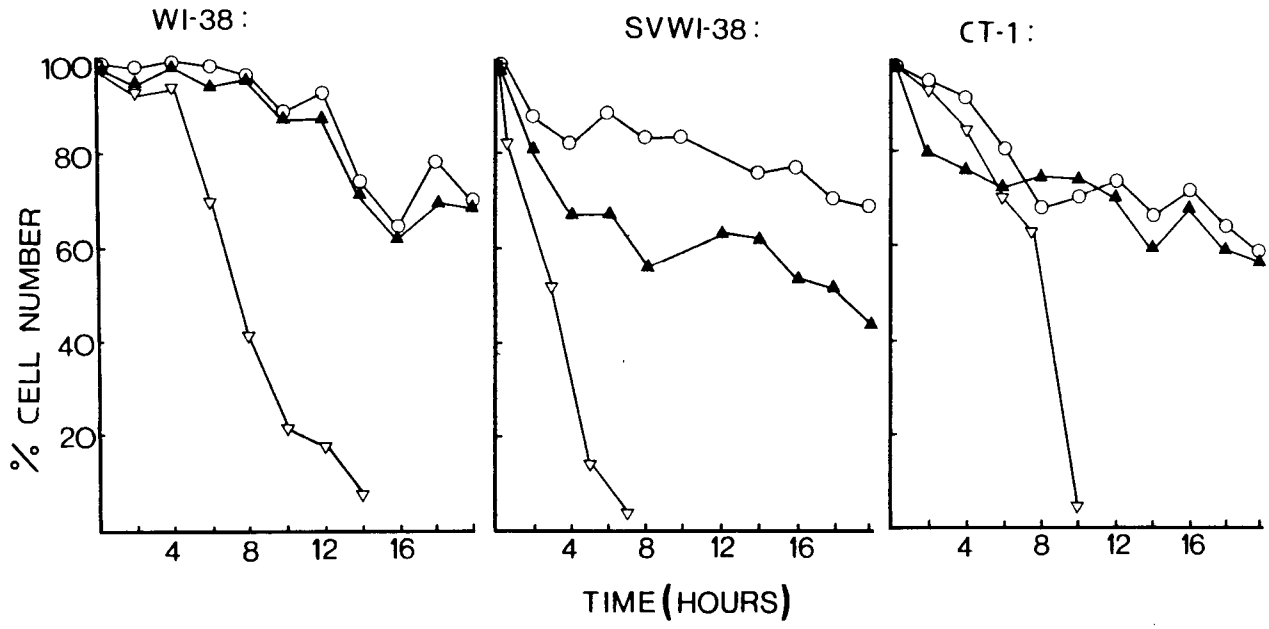


FIG. 4.4 Cellular growth in the presence of various concentrations of sodium butyrate

Cells were plated at $5x10^4$ cells/30 mm² dishes and allowed to attach before adding either 75 mM (○—○), 120 mM (▲—▲) or 200 mM (▽—▽) sodium butyrate to the dishes. Quadruplicate dishes were trypsinised and the cell numbers determined every 2 hours for 18 hours. Cellular growth at the various concentrations was expressed as a percentage of untreated cells.



Each cell line showed a concentration and time dependent response to butyrate in the tissue culture medium. Again the greater susceptibility of the transformed cell lines to increasing butyrate concentrations is apparent. For example, minimal (± 10%) decrease in cell number occured in the WI-38 cells treated with 120 mM butyrate over the first 10 hours, while, the SVWI-38 and CT-1 cells showed a ±40% and ±30% reduction in cell number compared with untreated cells.

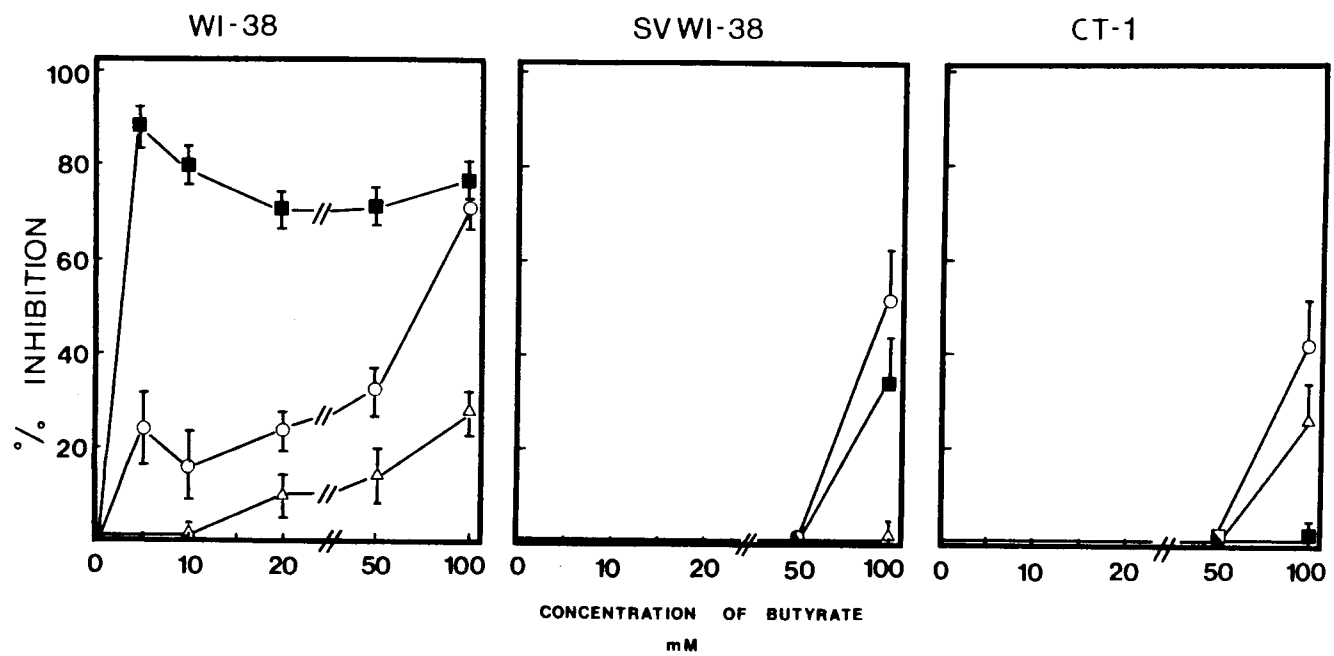
4.2.2. DNA synthesis inhibition:

The inhibitory effect that butyrate has on DNA synthesis was monitored by the incorporation of ^{3}H-thymidine into TCA insoluble material over a 14-16 hour time period, as detailed in Materials and Methods (6.7). The effect of both low and high concentrations of butyrate was investigated on the three cell lines. In order to show that these results were butyrate-specific and not due to increased medium hypertonicity, DNA synthesis was monitored in the presence of equivalent concentrations of sodium chloride.

The amount of DNA synthesis inhibition obtained when WI-38, SVWI-38 and CT-1 cells are treated with 0, 5, 10 and 20 mM butyrate, is shown in Fig. 4.5. DNA synthesis of WI-38 cells was inhibited by ± 80% at 5 mM butyrate, whilst neither transformed cell lines showed any DNA synthesis

FIG. 4.5 Determination of DNA (■—■), RNA (○—○) and protein (△—△) synthesis in the presence of various concentrations of sodium butyrate

Cells were seeded and allowed to attach and grow as described in the legend to Fig. 4.2. Treatment commenced by removal of the medium and replacement with fresh medium containing the indicated concentrations of sodium butyrate plus ^{3}H-thymidine, ^{3}H-uridine or ^{3}H-leucine where DNA, RNA or protein synthesis was monitored respectively. DNA, RNA and protein synthesis was expressed as the percentage of label incorporated into TCA insoluble material per 10^{6} cells as described in Materials and Methods (6.7).



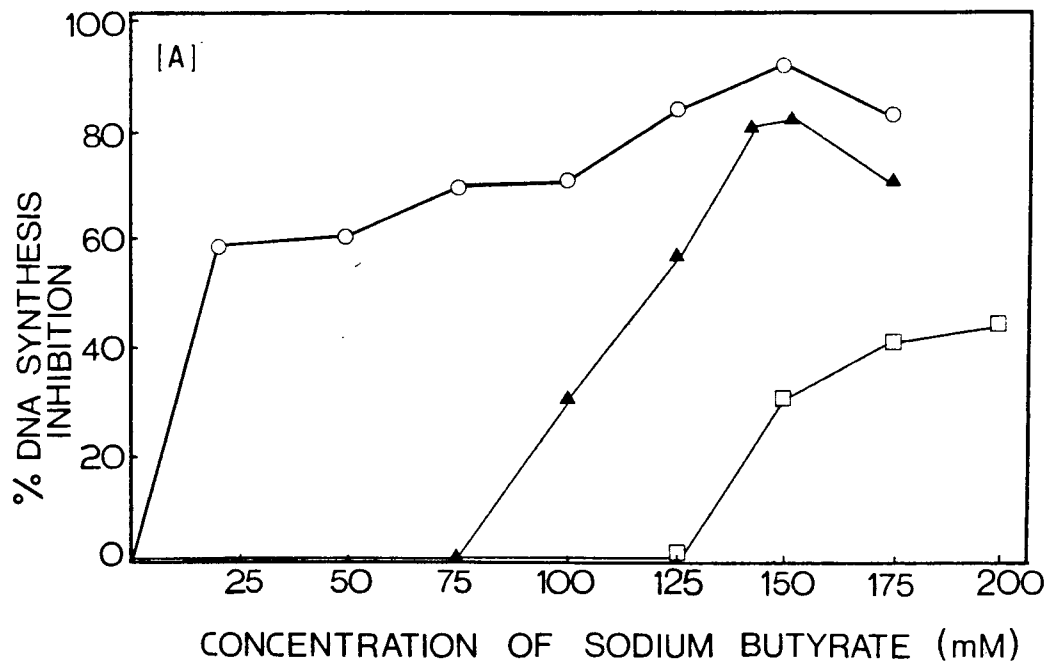
inhibition over this concentration range. DNA synthesis was unaffected in WI-38 cells treated with equivalent concentrations of sodium chloride (see Fig. 4.6B), thus implying that the observed inhibition was butyrate-specific and not due to medium hypertonicity.

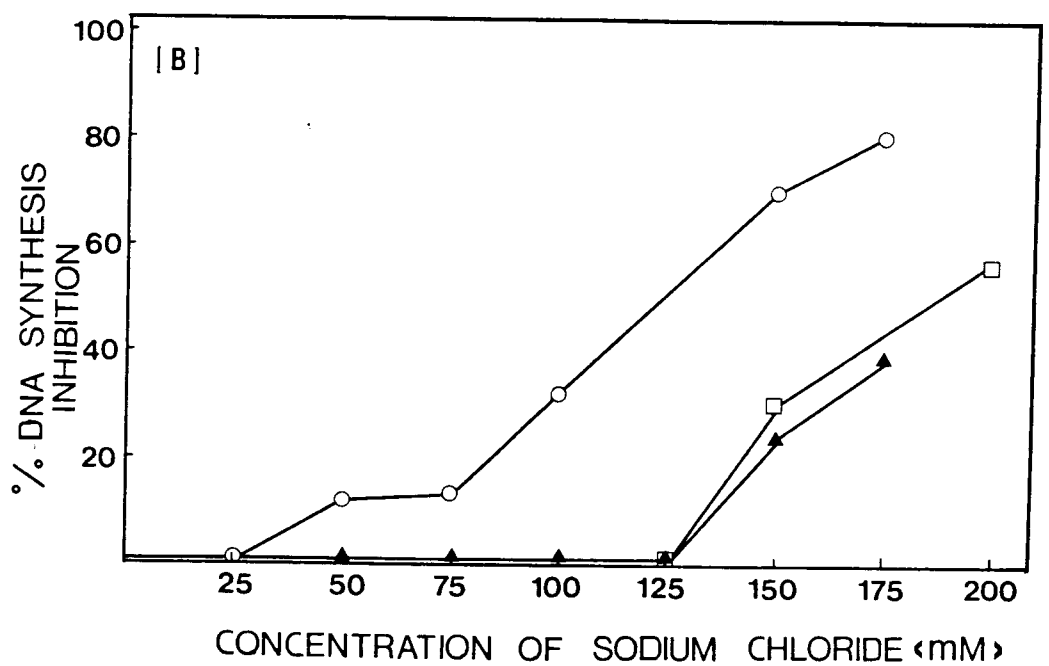
DNA synthesis in the transformed cells was only affected at higher concentrations of butyrate (Fig. 4.6A). SVWI-38 cells showed a 50% inhibition at 120 mM butyrate, while the CT-1 cells never even attained an inhibition value of 50%. A forty percent inhibition was observed at 150 mM butyrate and above for the CT-1 cells. Treatment with equivalent concentrations of sodium chloride (Fig. 4.6B) showed that the DNA synthesis inhibition observed in the case of the CT-1 cells was probably due to increased medium hypertonicity, for identical curves were obtained with both treatments. The inhibition of DNA synthesis in SVWI-38 cells was butyrate-specific, for an inhibition of only ±20% was observed at 150 mM and above.

That the observed inhibition at higher butyrate concentrations was indeed butyrate specific in WI-38 cells, was confirmed from the percent of DNA synthesis inhibition obtained after sodium chloride treatment. Less than 20% inhibition of DNA synthesis was induced by sodium chloride treatment within the 25 to 75 mM concentration range, while sodium butyrate treated cells showed an inhibition of 70-80%.

FIG. 4.6 Effects of sodium butyrate (A) and sodium chloride (B) on DNA synthesis in WI-38 (○—○), SVWI-38 (▲—▲) and CT-1 (□—□) fibroblasts

Cells were plated at $5x10^4$ cells per 30 mM^2 dish. After attachement and growth, the medium was replaced with fresh medium containing the indicated concentrations of either sodium butyrate (Fig. A) or sodium chloride (Fig. B) and ^3H-thymidine to monitor the extent of DNA synthesis, which was assessed as the amount of radioactivity in TCA insoluble material per 10^6 cells, and expressed as a percentage of the control.





It could therefore be deduced that DNA synthesis in the normal cell line (WI-38) was affected to a greater extent than that of its two transformed counterparts. Even in its susceptibility to the inhibition of DNA synthesis by sodium chloride, the normal cells were more affected.

4.2.3 The effect of butyrate on RNA and protein synthesis:

RNA and protein synthesis was monitored by the incorporation of either radiolabeled uridine or leucine into TCA insoluble material over a 14-16 hour time period as described in Materials and Methods (6.7).

Again normal and transformed cells responded differently in their RNA synthesizing ability in the presence of butyrate (see Fig. 4.5). WI-38 cells showed a ±20% inhibition of RNA synthesis at concentrations up to 20 mM butyrate, which contrasted strongly with that observed for both the transformed cells, where no inhibition was noted. This inhibition was dose-dependent and reached a maximum of ±80% at 100 mM butyrate. Only at 100 mM butyrate was RNA synthesis inhibited in both transformed lines, where SVWI-38 and CT-1 cells showed an inhibition of 50% and 40% respectively.

Protein synthesis was not affected in the WI-38 cells at concentrations up to 10 mM butyrate. At higher concen-

trations (i.e. 20 to 50 mM butyrate), a slight inhibition occurred (±10%), reaching a maximum of ±20% at 100 mM butyrate. No protein synthesis inhibition occurred in SVWI-38 cells even at concentrations as high as 100 mM butyrate. The CT-1 cells were also unaffected up to 50 mM butyrate and showed only minimal inhibition of ±20% at 100 mM butyrate.

4.2.4. SDS-PAGE Analysis of proteins synthesized in the presence of 10 mM Butyrate:

WI-38, SVWI-38 and CT-1 cells were treated with 10mM butyrate, together with 50 µCi/ml of ^{3}H-leucine for 24 hours. Proteins were analysed on SDS-PAGE, as detailed in Materials and Methods (6.8), and the radiolabelled bands visualised by flurography.

Fig. 4.7 compares the Coomassie stained gels (i.e. total cellular proteins that were synthesized both before and during 10 mM butyrate treatment) with an flurograph of the same gel (i.e. proteins synthesized during butyrate treatment only). The most dramatic change induced by 10 mM butyrate can be seen in the WI-38 cells. From the flurograph it appears that the synthesis of two high molecular weight proteins was inhibited by butyrate (Fig. 4.7B, lanes 7 & 8). From a plot of the log of the molecular weights of known marker proteins versus the distance of migration in the gel (see Fig. 4.8) it was

FIG. 4.7 Analysis of proteins synthesized in the presence of sodium butyrate

Cells were labelled with 5 µCi-ml of ^{3}H-leucine in the presence or absence of 10 mM butyrate (for 16-20 hours). Cells were processed as described in Materials and Methods (6.8) and electrophoresed on a 20 cm long 10% SDS polyacrylamide gel. Fig. 4.7A shows the Coomassie stained gel while Fig. 4.7B is a fluorograph of the same gel. Lanes 1 & 2, and 7 & 8 represent WI-38 cells, lanes 3 & 4, and 9 & 10 SVWI-38 cells and lanes 5 & 6, and 11 & 12 represent CT-1 cells. + and - indicates treatments with and without 10 mM butyrate respectively.

A.

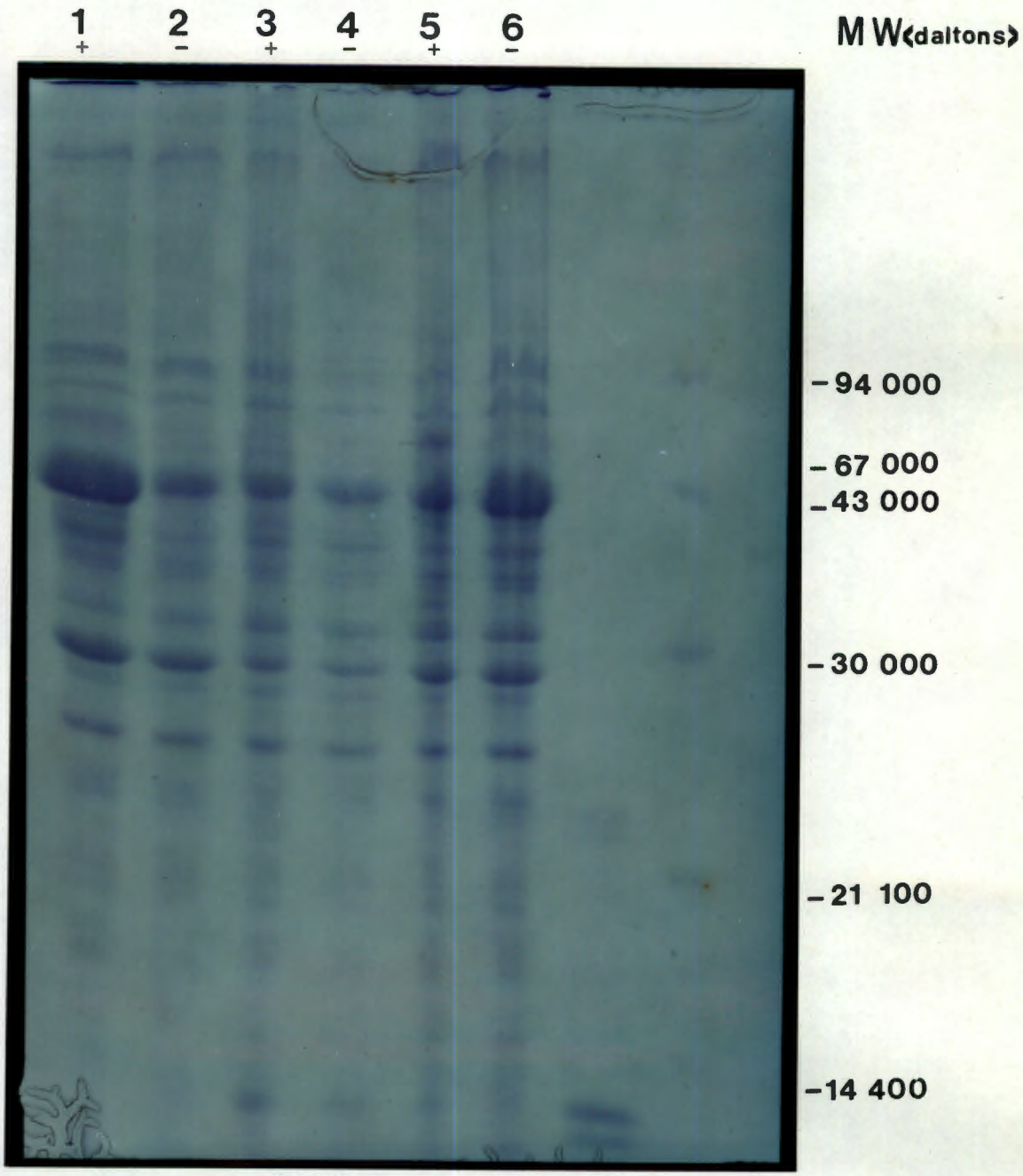


FIG. 4.7B

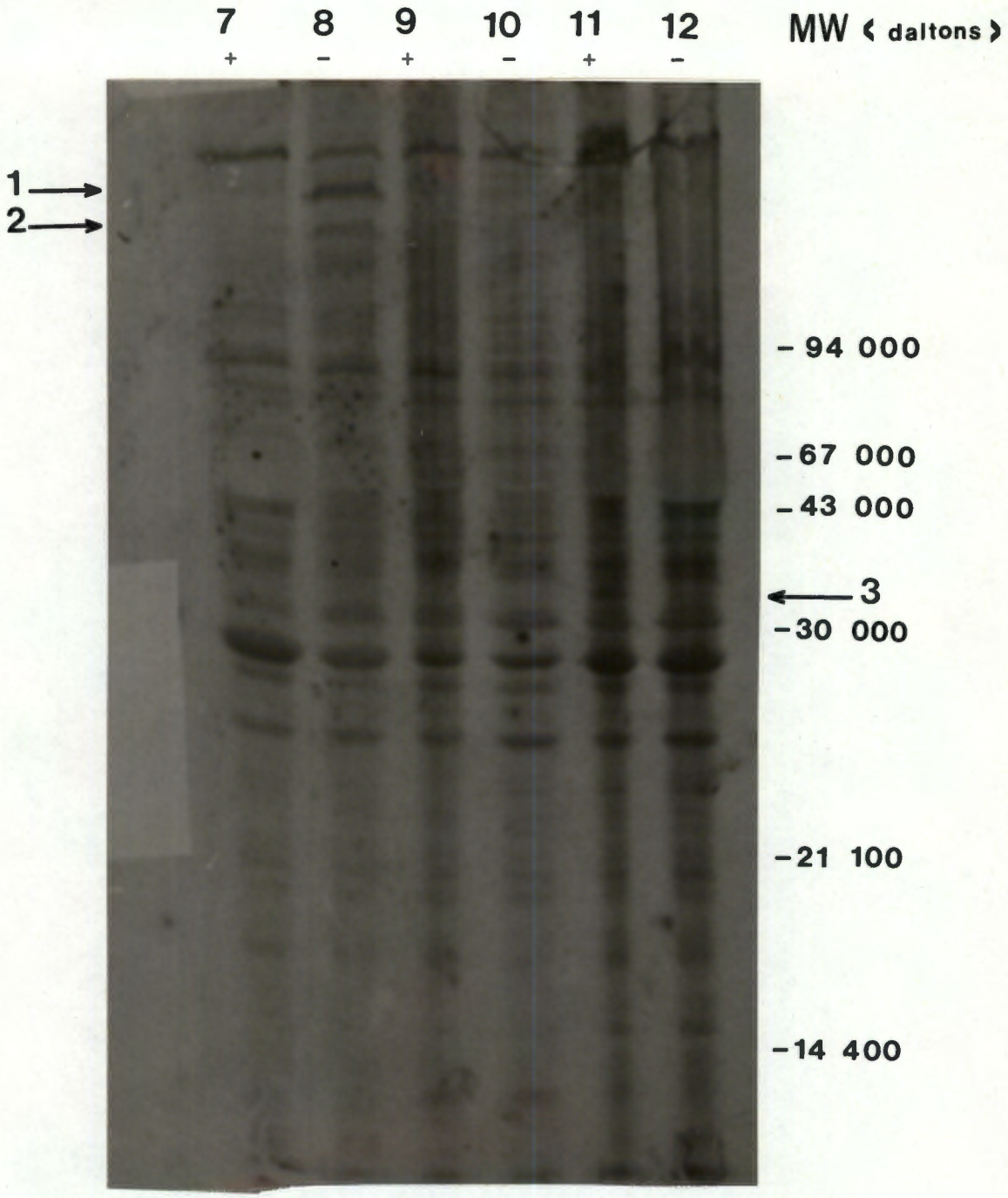
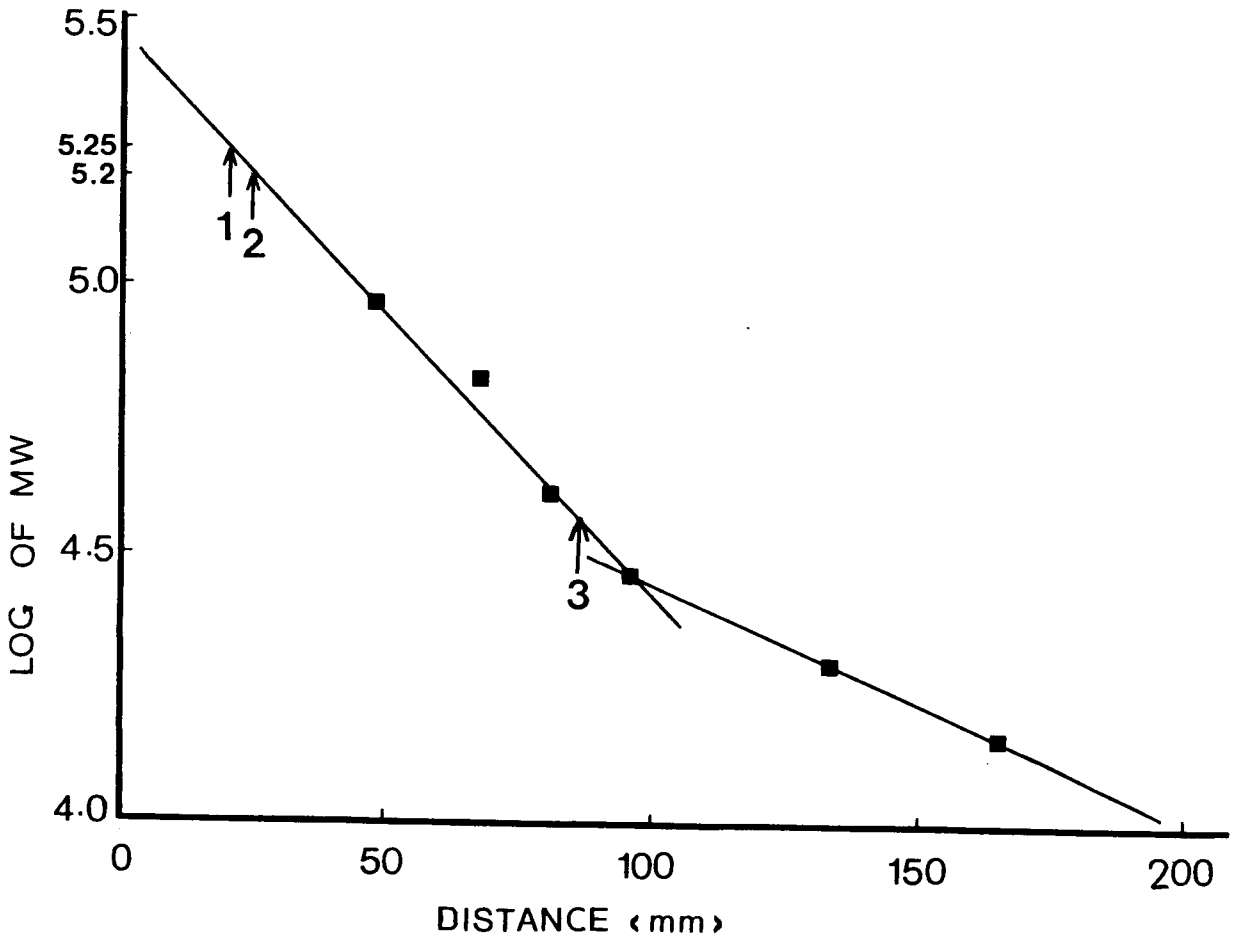

7
8
9
10
11
12
+
-
+
-
+
-
MW (daltons)
1
2
- 94 000
- 67 000
- 43 000
3
- 30 000
- 21 100
- 14 400

FIG. 4.8 Protein molecular weight determination

The distance of migration of the marker proteins in the gel was determined and plotted against a log of their respective molecular weights. The distance that the two unknown proteins in the WI-38 cells migrated was measured and their molecular weights determined as 178000 and 158000 respectively. Similarly the low MW protein induced by butyrate treatment was estimated to be ± 35000 daltons. ↑(1) indicates the 178000 d protein, ↑(2) the 158000 d protein and ↑(3) the 35000 d protein.



calculated that the bands corresponded to two high molecular weight proteins of approximately 158 000 and 178 000 daltons. Analysis of the Coomassie stained gel showed that the two bands also disappeared during butyrate treatment. Thus it is more likely that butyrate did not inhibit the synthesis of the two proteins in WI-38 cells, but rather that these proteins were modified during butyrate treatment. The inhibitory effects of butyrate were not observed on these two proteins in the two transformed lines.

One further change could be detected in the CT-1 cells that had been treated with butyrate. Butyrate induced the synthesis of a ±34000 dalton protein (see arrow 3, lanes 11 and 12 of Fig. 4.7B) which was not induced in either of the SVWI-38 or WI-38 cells (lanes 9 & 10; 7 & 8 of Fig. 4.7 B). This induction was confirmed on the Coomassie stained gel (lanes 5 and 6 of Fig. 4.7A). This protein was induced and not enhanced by butyrate, for no band was detected in untreated CT-1 cells (lane 6 , Fig. 4.7A).

Thus butyrate modified two high molecular weight proteins in the normal cell line and induced the synthesis of a lower molecular weight protein in the CT-1 cells only. Because so few changes were observed after butyrate treatment, it is understandable that no change in total protein synthesis was detected at 10 mM butyrate in the WI-38 and CT-1 cells (4.2.3. above).

4.2.5 ADPRT assay as a measure of DNA repair:

The adenosine-diphosphate ribosyl transferase (ADPRT) assay has been used as an indirect way of measuring DNA repair (165). This enzyme catalyses the transfer of adenosine-diphosphate (ADP) units onto protein substrates, through the use of nicotinamide-adenine diphosphate (NAD^+) as the ADP donating group. It was found that the DNA repair enzyme itself needed to be ADP-ribosylated in order to function efficiently. This allowed the development of an assay system that measures the amount of radioactive NAD^+ utilised, as a measure of DNA repair.

Treatment of cells with 20 mM butyrate for 48 hours causes a marked stimulation of DNA repair synthesis at early times after UV irradiation damage in normal fibroblasts (166). Furthermore, the initial rate of removal of the damaged sites is increased in butyrate treated cells, indicating that butyrate stimulates the initial rate of nucleotide excision repair. In this study it was decided to investigate whether butyrate could stimulate DNA repair in WI-38, SVWI-38 and CT-1 cells, and if so, whether these cells were differentially stimulated or not.

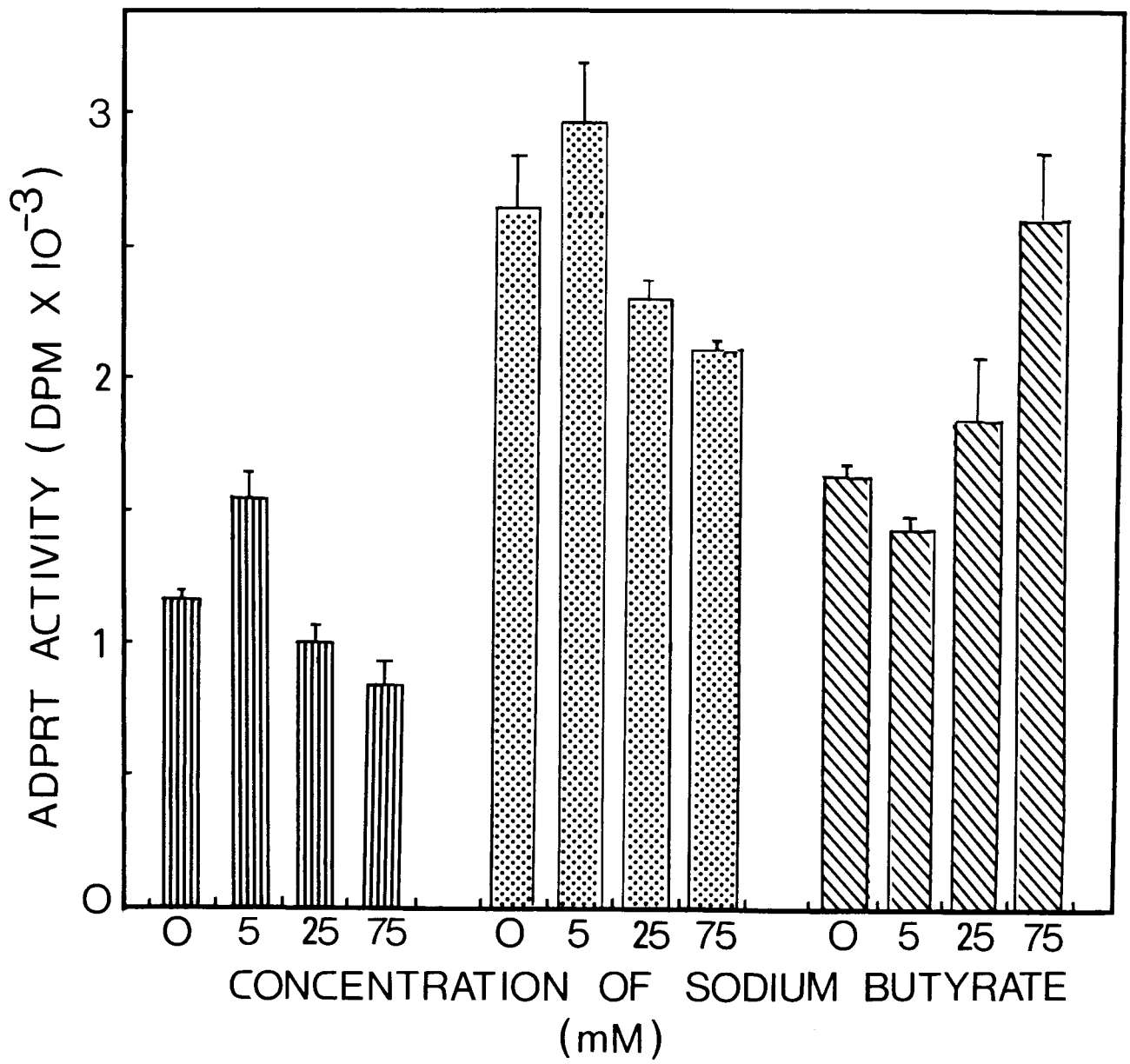
The ADPRT assay was performed on all 3 cell lines as described in Materials and Methods (6.13). Treatment with 5 mM butyrate resulted in a 28% increase in ADPRT activity in

the WI-38 cells. SVWI-38 cells also showed a ±23% increase in ADPRT activity. At higher concentrations (i.e. 25 mM and 75 mM butyrate) both the WI-38 and the SV-40 transformed cells showed enzyme activities that decreased to levels below that of the controls. In contrast to these two cell lines, the γ-radiation transformed cell line (CT-1) maintained its basal level of enzyme activity up to and including 25 mM butyrate, after which there was a 71% increase in ADPRT activity at 75 mM butyrate (see Fig. 4.9).

Of interest was the differential response in ADPRT activity in the three cell lines. This has also been observed by Smerdon et al (166) where different cell lines responded by varying degrees in their ability to repair DNA damaged sites. The CT-1 cells required a far greater sodium butyrate concentration to elicit any effect, compared with both the WI-38 and SV WI-38 cells. Furthermore, it should be noted that the de novo repair systems differ for the three cell lines investigated. Control levels of ADPRT activity were increased by 111% and 36% in SVWI-38 and CT-1 cells respectively, compared with the normal fibroblast cells. Chromosomal spreads of SVWI-38 cells have shown these to be highly irregular in shape with obvious deletions and insertions of stretches of DNA. The DNA is therefore in a state of disarray and repair processes would be expected to be fully operational. This could account for the large increase in ADPRT activity seen in the

FIG. 4.9 ADP-ribosyl-transferase assay as an indication of DNA repair

Cells seeded in 150 mm² flasks were treated with the indicated concentrations of sodium butyrate for 14 hours. The cells were trypsinised and the ADPRT assay performed as described in Materials and Methods (6.13). ADPRT activity was expressed as the amount of label incorporated into TCA insoluble material. ▥ , ▩ and ▨ represents WI-38, SVWI-38 and CT-1 cells respectively.



untreated SVWI-38 cells compared with its normal counterpart.

Because of the nature of the experiment (where butyrate is added to the cells for 14-16 hours prior to the assay, and is not present during the assay) butyrate probably acts by:

(1) having a direct damaging effect on the DNA, or by

(2) increasing the number of ADPRT molecules:

(a) possibly by causing DNA damage, the synthesis of ADPRT molecules is stimulated;

(b) the number of ADPRT molecules is increased by direct stimulation of gene expression by butyrate.

4.2.6 Hypermethylation of Parental and Daughter DNA strands:

DNA methylation was monitored by the incorporation of 6-^{3}H-uridine into DNA after conversion into deoxycytidine, and measuring the ratio of 5-methylcytosine to cytosine in hydrolysates of the isolated DNA (See section 6.10 of Materials and Methods). Cells were either labelled with 6-^{3}H-uridine for 24 hours, prior to exposure to millimolar concentrations of sodium butyrate, or during sodium butyrate treatment, as detailed in Materials and Methods (6.11). Analysis of prelabelled DNA enabled the determination of the 5-methylcytosine content of parental

DNA strands, while labelling of DNA concurrently with butyrate treatment detected the 5-methylcytosine content of daughter DNA strands.

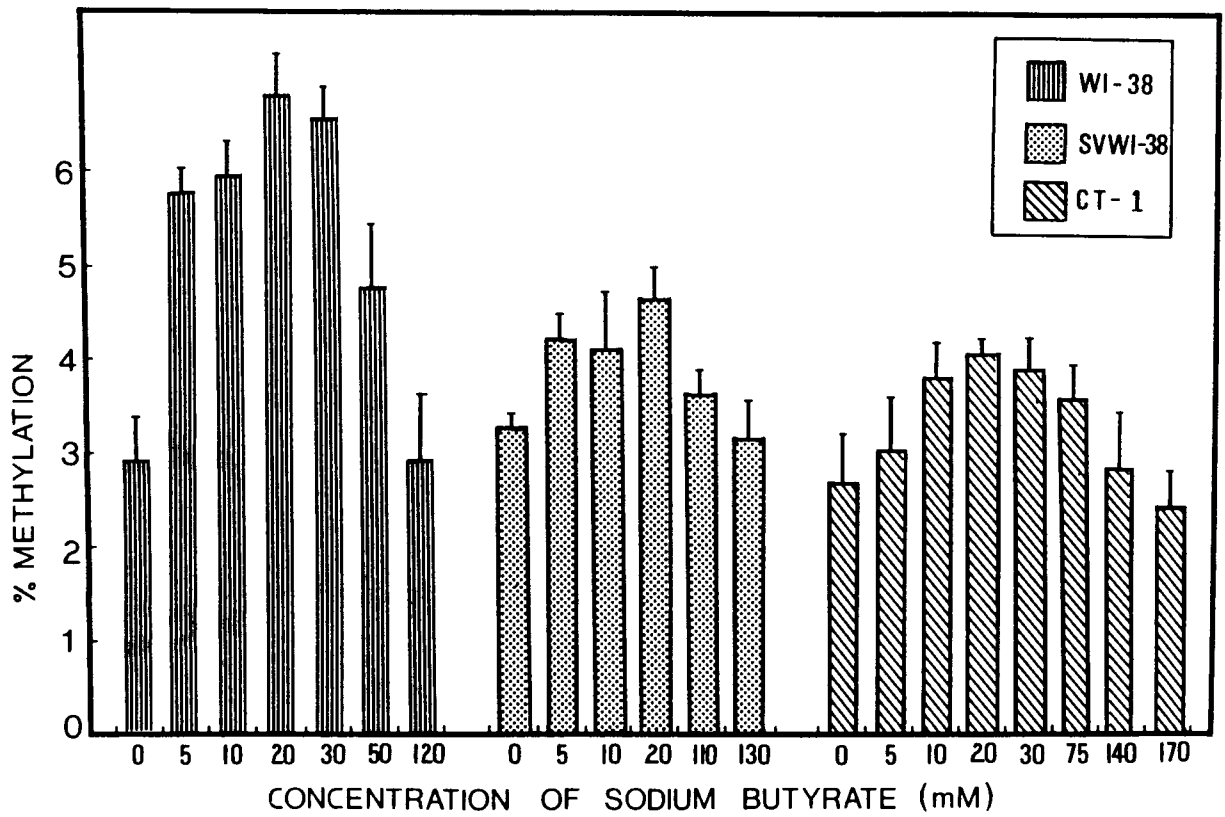
4.2.6.1 Analysis of Parental DNA Strands:

Low concentrations of butyrate (see Fig. 4.10) induced hypermethylation of parental DNA strands in all three cell lines tested. This hypermethylation was dose-dependent, and increased over the 5 to 20 mM range, after which it fell back to control values, giving a bell-shaped dosage curve for all three cell lines. Methylation was maximal at 20 mM butyrate for all three cell lines, but the extent of the induced methylation differed. Butyrate caused the greatest parental DNA strand hypermethylation in the normal cell line (6,8 ± 0,44), where an increase of 100% was observed at 20 mM butyrate. For the SVWI-38 and CT-1 cell lines, the induced hypermethylation was 41% and 51% respectively, at the same concentration, with actual methylation values of 4,62 ± 0,35 and 4,0 ± 0,16.

Butyrate thus induced hypermethylation of parental DNA strands in a dose-dependent manner in all three cell lines examined. The extent of this hypermethylation depended on the cell type and was most induced in the normal cell line. Both transformed lines showed similar responses to the butyrate induced hypermethylation of parental DNA strands.

FIG. 4.10 Hypermethylation of parental DNA strands in the presence of sodium butyrate

Cells were labelled with 1 μCi/ml of 6-^{3}H-uridine for 3 days after which the cell layers were rinsed and replaced with fresh medium containing the indicated concentrations of sodium butyrate for 16 hours. DNA was prepared and the 5-methylcytosine content determined by HPLC analysis as described in Materials and Methods (6.9.2 and 6.10).



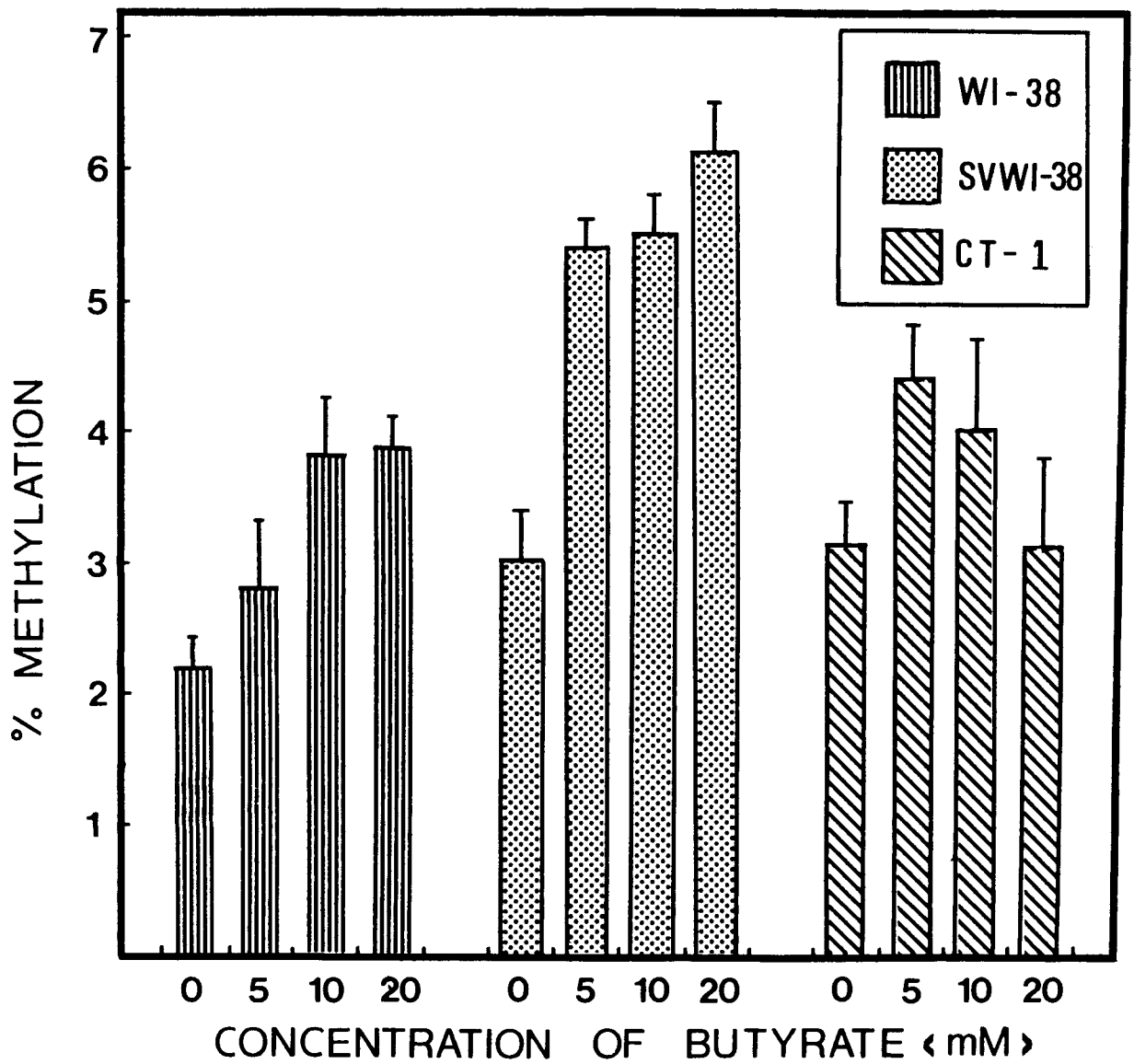
4.2.6.2 Analysis of Daughter DNA Strands:

Methylation of daughter DNA strands was analysed at low concentrations of butyrate only (0,5, 10 and 20 mM butyrate) (Fig. 4.11). Again, DNA hypermethylation was evident in all three cell lines. WI-38 and SVWI-38 cells showed a similar dose-dependent response to increasing concentrations of butyrate, where DNA methylation was maximal at 20 mM butyrate for both cell types, with an 81% and 102% induction of hypermethylation for WI-38 and SVWI-38 cells, respectively. The CT-1 cells responded differently to increasing concentrations of butyrate. DNA methylation was maximal at 5 mM butyrate (43% induction) and fell back to the control value at 20 mM butyrate. Analysis of the actual percent 5-methylcytosine showed that the SVWI-38 cells had a maximal induced methylation of 6,1 ± 0,39 while that of WI-38 and CT-1 cells was 3.84 ± 0,22 and 4,4 ± 0,39 respectively.

Butyrate therefore affected the methylation of newly replicated DNA differently, in the three cell lines. SVWI-38 cells showed the most hypermethylation which contrasted with the parental DNA strand hypermethylation, where the WI-38 cells showed maximum hypermethylation. Not only did butyrate therefore induce different degrees of hypermethylation in the three cell lines, but daughter and parental DNA strands were hypermethylated to varying extents within the same cell type.

FIG. 4.11 Hypermethylation of daughter DNA strands in the presence of sodium butyrate

Cells were plated at $5x10^5$ cells per 60 mm² dish. Before reaching confluency, cells were treated with sodium butyrate at the indicated concentrations, together with 6-^{3}H-uridine (1 µCi/ml) for 16 hours. DNA was prepared and the 5-methyl-cytosine content determined by HPLC analysis as described in Materials and Methods (6.9.2 and 6.10).



It is of interest to note that the percent 5-methylcytosine content obtained in these experiments for both WI-38 and SVWI-38 cells were roughly 2,9%. Thus no difference in the extent of methylation was observed between the normal cell and its transformed counterpart. These results are in agreement with those of Diala et al (68) where values of 2,9 ± 0,28 and 3,00 ± 0,28 were obtained for WI-38 and SV40 transformed WI-38 cells.

4.2.6.3 Use of restriction endonuclease digests to investigate methylation patterns:

Hpa II and MSp 1 are isoschisomers that can be used to probe the methylation status of DNA. These restriction enzymes cleave DNA at defined palindromic nucleotide sequences. Thus both Msp 1 and Hpa II recognise the 4 base sequence CCGG for cleavage. An additional feature which makes these two enzymes useful in probing methylation patterns of DNA is their ability to distinguish whether the internal cytosine is methylated or not. Msp 1 will cut the following sequence $C^{m}CGG$ while Hpa II will not.

Similarly, Hpa II will cut $^{m}CCGG$ but Msp 1 will not.

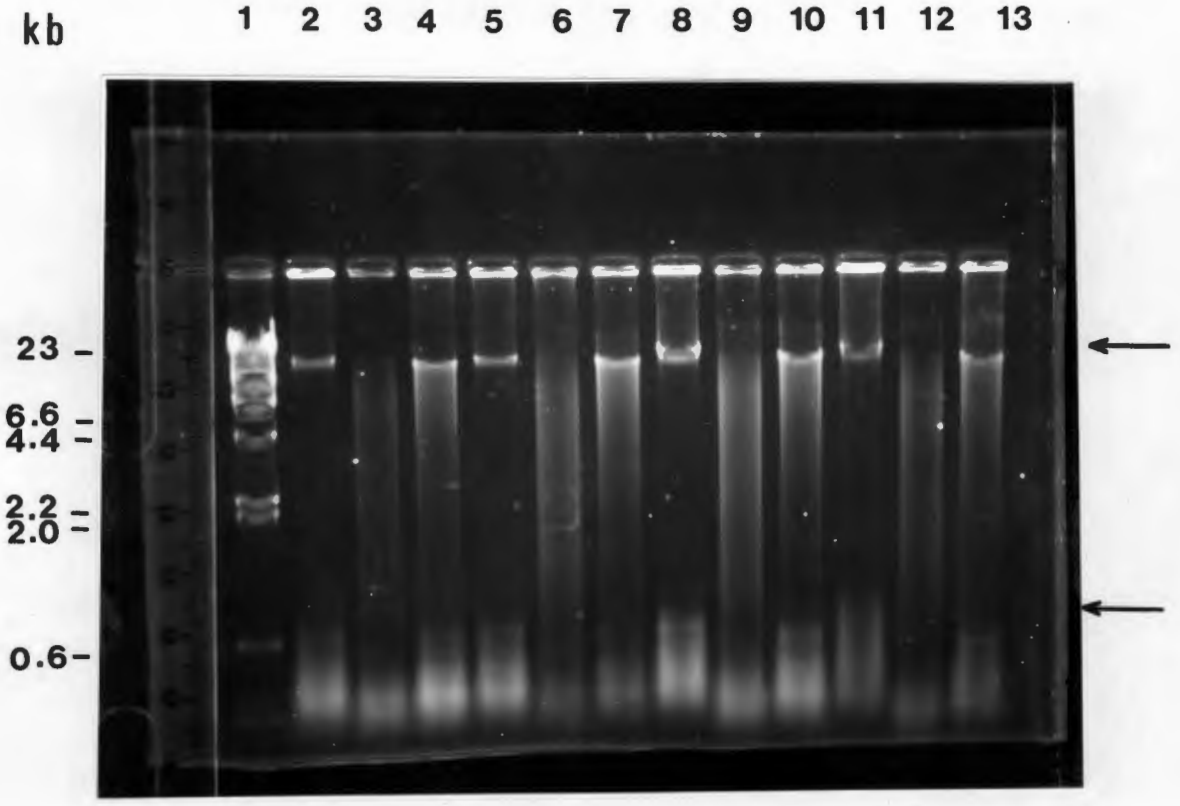
However, both will not cut the sequence where both cytosines are methylated (i.e.: $^{m}C^{m}CGG$).

WI-38 cells were treated with the indicated concentrations of butyrate (0 to 20 mM butyrate) for 16 hours. The DNA was isolated, digested with Msp 1 and Hpa II, electrophoresed on 1% agarose gels, and the extent of methylation determined by scanning a negative of the agarose gel (For detailed methods, see Section 6.14).

Cleavage of WI-38 DNA with either Msp 1 or Hpa II resulted in a smear of DNA that could easily be integrated using a cliniscan densitomeric scanner (Fig. 4.12). Fig. 4.13 shows the scans obtained after integration of the agarose gel. Both Msp 1 and Hpa II digestions showed similar responses to increasing butyrate concentrations, namely a shift towards larger sized DNA fragments on the gel. This is only possible if the DNA was hypermethylated during butyrate treatment, for neither Msp 1 nor Hpa II will cleave the palindromic sequence CCGG/GGCC if both cytosines are methylated. Both 5 mM and 10 mM butyrate caused a sequential shift towards higher molecular weight in both Msp 1 and Hpa II digested DNA, with 5mM butyrate causing the larger shift. 20 mM Butyrate also caused a shift towards larger sized DNA fragments, but this shift was not as dramatic as that induced by lower butyrate concentrations. However, HPLC analysis of parental and daughter DNA strands showed that 20 mM butyrate induced the most hypermethylation (see Fig. 4.14).

FIG. 4.12 Agarose gel of WI-38 DNA restricted with Msp 1 and Hpa II

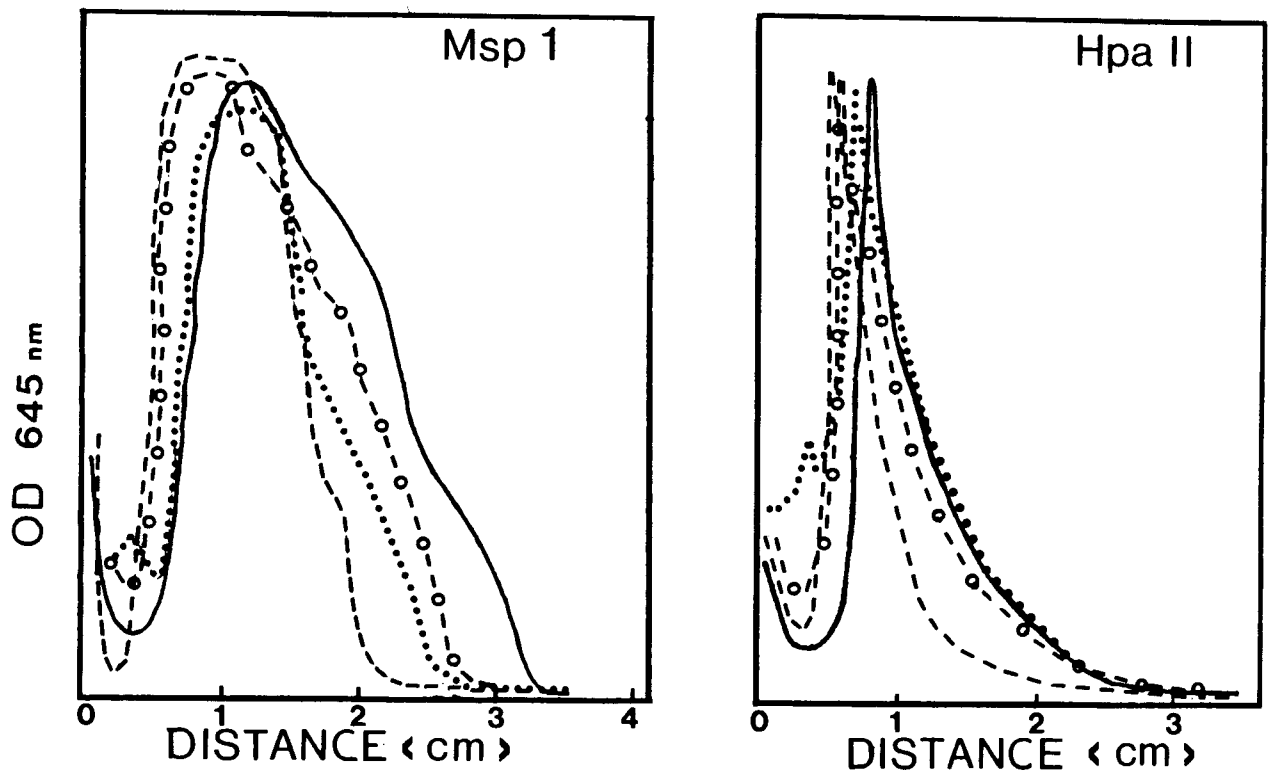
WI-38 cells were treated with 5, 10 or 20 mM butyrate for 20 hours after which DNA was prepared as described in Materials and Methods (6.9.1). DNA was restricted with either Msp 1 or Hpa II at 2 units/ µg of DNA. The DNA was electrophoresed on a 0,8% horizontal agarose slab gel at 150 mA for ± 4 hours, and visualised by staining with ethidium bromide as described in Materials and Methods (6.14). Lane 1 represents a λ-Hind III cut marker while lanes 2-4, 5-7, 8-10 and 11-13 represent DNA that was treated with 0, 5, 10 and 20 mM butyrate. Lanes 2, 5, 8 and 11 represent DNA that was not restricted while lanes 3, 6, 9 and 12 represent DNA that has been Msp 1 digested, and lanes 4, 7, 10 and 13 are Hpa II digested DNA.



The agarose gel was scanned between the indicated arrows.

FIG. 4.13 Methylation analysis of WI-38 DNA from control and butyrate treated cells digested with either Msp 1 or Hpa II

Cells were treated with the indicated concentrations of butyrate and the DNA prepared as described in the legend to Fig. 4.12. After electrophoresis on a 0.8% agarose gel and visualisation of the DNA by ethidium bromide staining a negative of the gel was scanned using a Cliniscan densitometer (see Materials and Methods (6.14). Fig. A represents Msp 1 restricted DNA while Fig. B represents Hpa II restricted DNA. (——) untreated; (---) 5 mM, (····) 10 mM and (O---O) represents 20 mM sodium butyrate.



One possible explanation for this difference could be that at lower butyrate concentrations (i.e. 5 and 10 mM butyrate) the CG dinucleotides were preferentially hypermethylated, while at higher butyrate concentrations, the hypermethylation was more random. The results of the restriction digests therefore confirm that butyrate was indeed capable of inducing hypermethylation of DNA.

4.2.6.4 Maintenance of the hypermethylated state:

Because butyrate is able to induce hypermethylation of parental and daughter DNA strands, it is possible to use butyrate as a tool to investigate the methylation event itself.

Growth of cells in millimolar concentrations of butyrate results in hypermethylation of both parental and daughter DNA strands. The question that is posed, is whether this hypermethylation is maintained during subsequent rounds of replication after removal of butyrate, or whether the presence of butyrate is required to maintain the hypermethylated state?

For the analysis of parental DNA strands, WI-38 cells were prelabelled for 72 hours with 6-^{3}H-uridine, the label was removed and the cells incubated overnight (16 hours) with 10 mM butyrate. In a parallel experiment, other cells were treated in the same manner, except that after butyrate treatment, they were allowed a further round of replication in fresh medium without butyrate. For the analysis of daughter DNA strands, 10 mM butyrate and 6-^{3}H-uridine was added to WI-38 cells simultaneously for 16 hours. Again, parallel cultures were allowed a further round of replication in fresh medium without butyrate (for detailed methods, see 6.12.1). The DNA was isolated and analysed as detailed in Materials and Methods (6.9.2 and 6.10). It was decided to use 10 mM butyrate for further analysis, as this concentration induces sufficient hypermethylation in both parental and daughter DNA strands and would be less toxic to the cells (see Fig. 4.14 for a comparison of the induced hypermethylation of parental and daughter DNA strands).

Fig. 4.15 shows the results obtained in the experiment detailed above. 10 mM butyrate treatment resulted in the expected hypermethylation of both parental and daughter DNA strands. After release from the butyrate treatment, the 5- methylcytosine content of the parental DNA strands decreased to that of control values. However, the elevated 5-methylcytosine levels of the daughter DNA strands were maintained.

FIG. 4.14 A comparison of parental and daughter DNA strand hypermethylation in WI-38 cells treated with various sodium butyrate concentrations

Cells were either prelabelled with 6-^{3}H-uridine followed by butyrate treatment (parental strand) or labelled concurrently with butyrate treatment (daughter strand), see Materials and Methods (6.11). After 16-20 hours of butyrate treatment at the indicated concentrations, DNA was isolated and the 5-methylcytosine content determined as described in Materials and Methods (6.9.2 and 6.10).

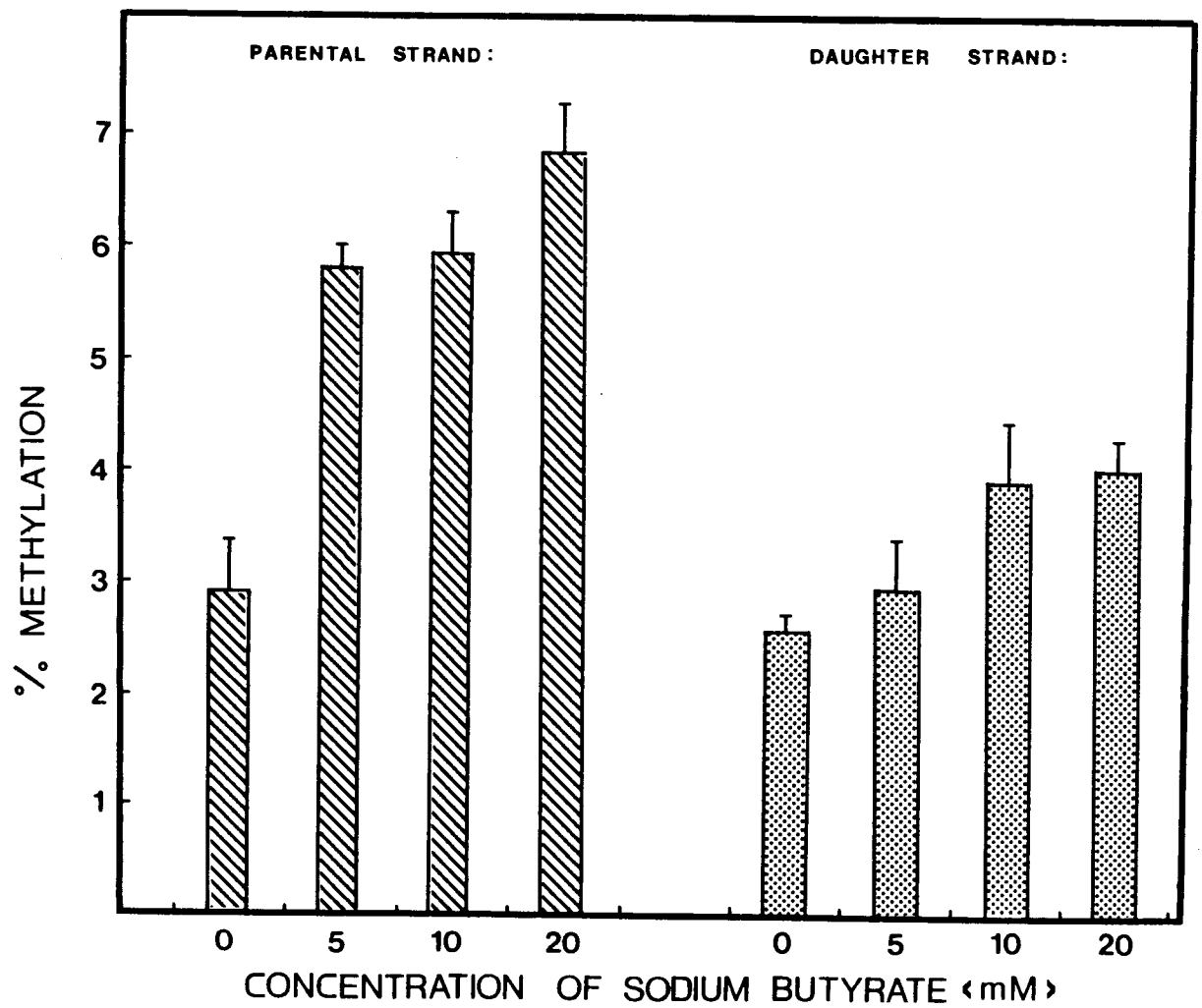
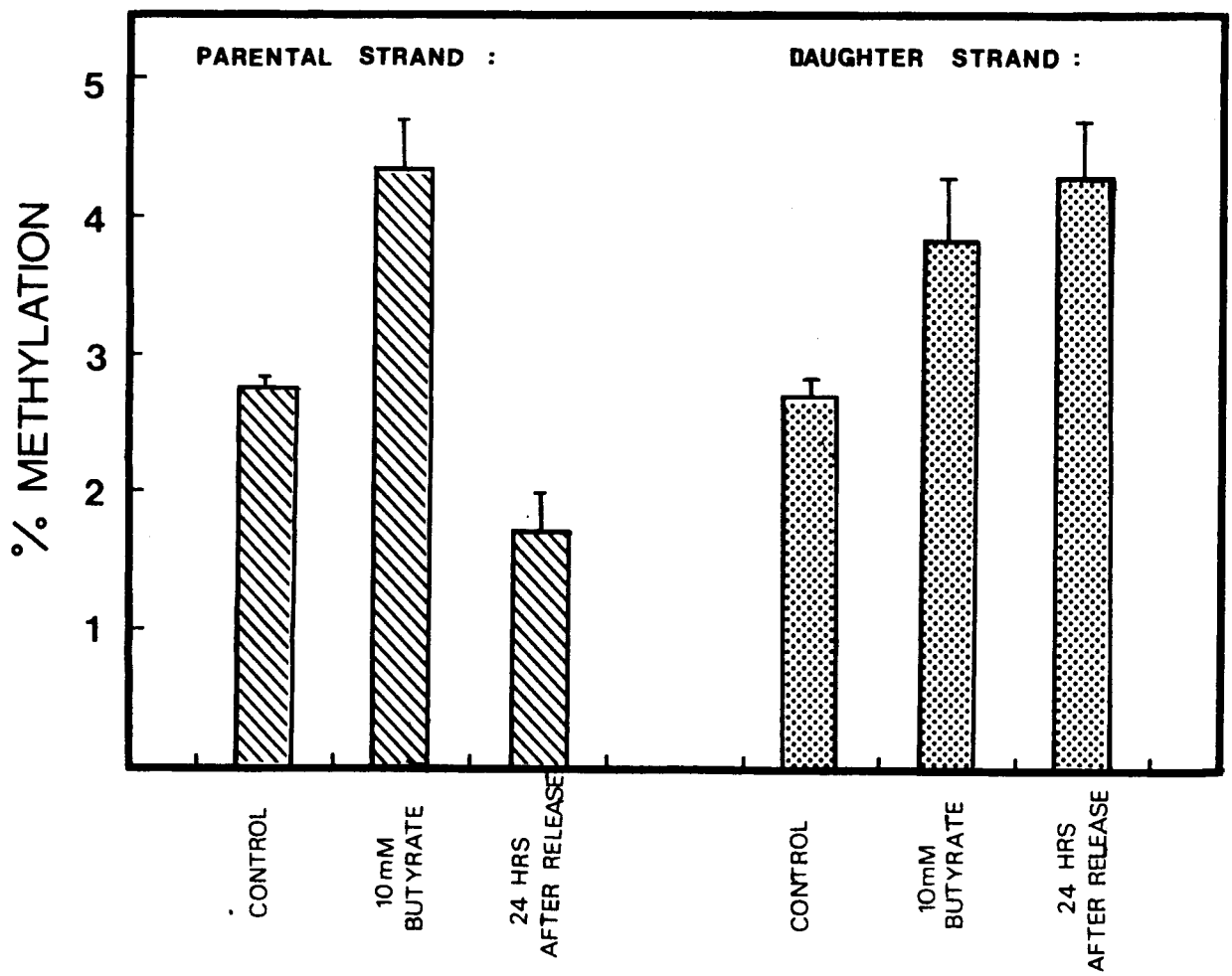


FIG. 4.15 Heritability of the hypermethylation status

WI-38 cells were either prelabelled with 6-^{3}H-uridine (1 μCi/ml) for 3 days which detects parental DNA strands, or labelled concurrently (5 μCi/ml) during 10 mM butyrate treatment, thus detecting daughter DNA strands. Half of the dishes were harvested immediately after 16-20 hour butyrate treatment, while the other half were changed to fresh medium for a further 24 hours (see Materials and Methods (6.12.1)). Both sets were analysed for 5-methylcytosine content as described in Materials and Methods (6.9.2 and 6.10). Number of determinations per experiment = 5.



To confirm these results, a dual label experiment was performed, where WI-38 cells were prelabelled for 72 hours with ^{14}C-uridine, the label removed and then treated with either:

(1) 6-^{3}H-uridine for 16 hours
(2) 6-^{3}H-uridine plus 10 mM butyrate for 16 hours
(3) 10 mM butyrate for 16 hours; then ^{3}H-uridine for a further 16 hours (see Materials and Methods, Section 6.12.2). The 5-methylcytosine content was analysed as described in Materials and Methods (6.10). Fig. 4.16 shows the results obtained in the dual labelling experiment. Again, after a further round of replication, the 5-methylcytosine content of the parental DNA strand returned to control values (i.e. lane c), while that of the daughter DNA strand was maintained, thus confirming the results obtained previously.

From the two experiments detailed above, it appears that in order for the induced hypermethylation to be maintained, the methylation event must be coupled to DNA synthesis i.e. it must occur in newly replicating DNA. Furthermore, parental DNA strands lose the induced 5-methylcytosine after removal of butyrate, which is only possible through the action of a demethylase.

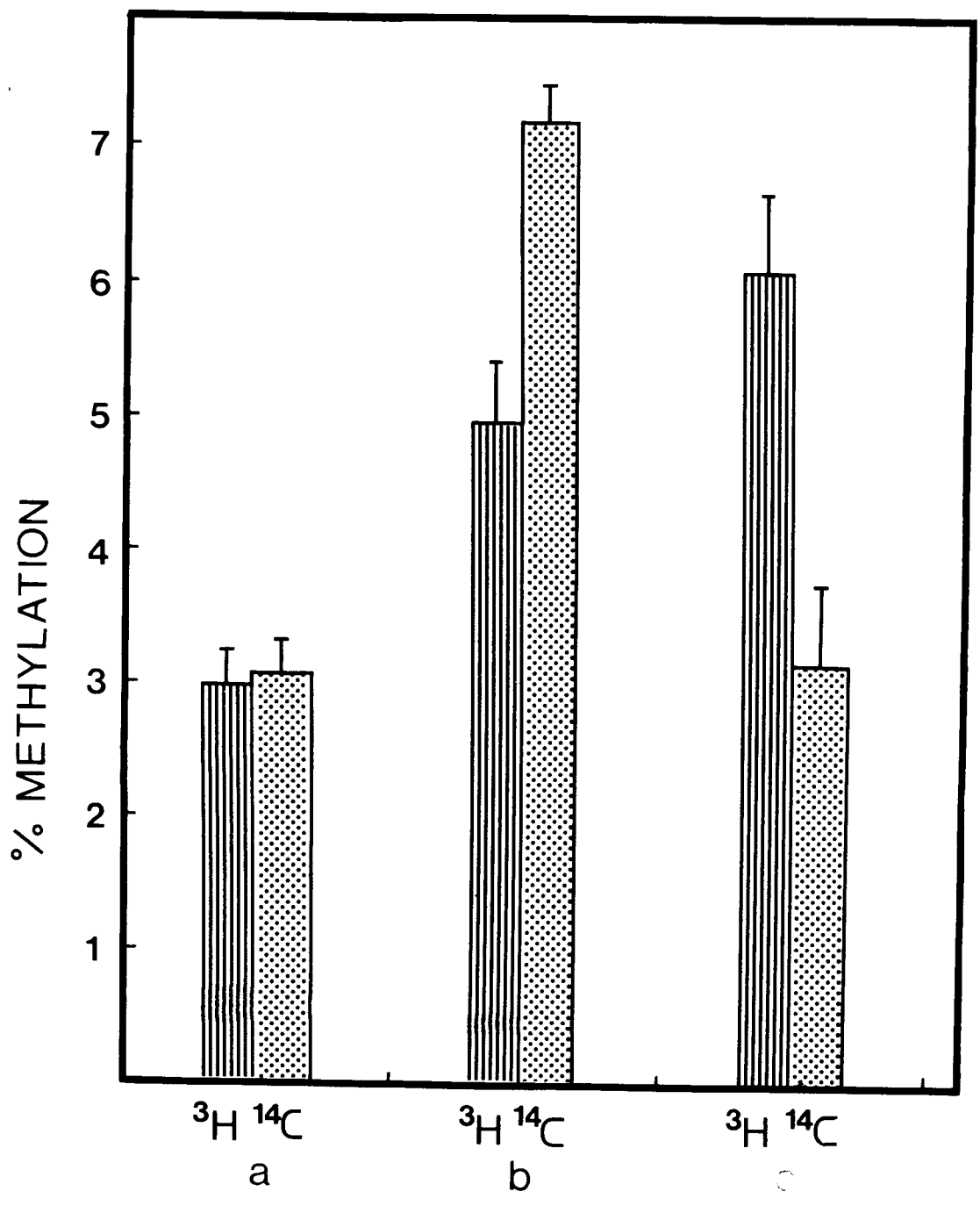
FIG. 4.16 Dual label analysis of hypermethyla status

WI-38 cells were prelabelled with ^{14}C-uridine for 3 days. After extensive washing of the cell layer, medium containing 6-^{3}H-uridine plus 10 mM butyrate was added. The 5-methyl-cytosine content of the DNA samples was determined at the following times:

(a) 16 hours after addition of 6-^{3}H-uridine to the ^{14}C prelabelled cells (i.e. no butyrate treatment)

(b) As in (a) above, except that 10 mM butyrate and 6-^{3}H-uridine were added simultaneously during the 16 hour pulse

(c) As in (b) above, except that the cells were allowed to grow in fresh medium for an additional 16 hours after butyrate treatment (see Materials and Methods 6.12.2).



4.4 DISCUSSION

Most studies on the biochemical and morphological effects of butyrate have focussed on specific tumor cell lines (150, 152, 154, 162, 163). In many cases, the loss of malignant properties induced by butyrate treatment has been accompanied by the concomitant appearance of more differentiated states (e.g. in mouse neuroblastoma cells (57)). The lack of a suitable normal cell line for comparison has been a problem in many studies. A tumor may not have originated in the tissue in which it is found (metastatic) or normal cells may comprise only a small percentage of the cells present in a tissue e.g. pancreatic cancer where the tumor is thought to originate in the ductal cells of the organ, which make up only 4% of the pancreas (152). The use of a normal cell line as well as its transformed counterparts allowed us to compare directly the effects of butyrate on normal and transformed cells in culture.

Butyrate has been suggested as a useful chemotherapeutic agent for the treatment of certain leukemias, due to its relatively low toxicity and its ability to induce gene expression in a number of systems (57). In particular, butyrate was able to induce terminal differentiation in human promyelocytic leukemic cells (148), and its clinical use was shown to be feasible, *in vitro* and *in vivo*, in a patient with acute myelogenous leukemia (55). One

possible drawback to its clinical acceptance is its rapid half-life in plasma. A steady state level of 0.28 mM butyrate is rapidly achieved with an intravenous infusion of 2 g/kg/24 hours as a 3% solution (55). Following cessation of the infusion, the plasma half-life of butyrate is extremely rapid, and estimated to be less than five minutes. Nevertheless, study into its biochemical mode of action is important if it is to have significant clinical application. Possibly synergistic use with other drugs will prove more feasible in clinical trials. A recent report has shown that exposure of Murine embryonal carcinoma cells to butyrate resulted in modest levels of differentiation, whereas simultaneous exposure to butyrate plus retinoic acid led to extensive differentiation (167).

This study has clearly shown the different responses of normal and transformed cells to sodium butyrate. In particular, DNA synthesis in normal cells was maximally inhibited at 5-10 mM butyrate, while the transformed counterparts showed no inhibition at these concentrations. Our results agree with those of Wintersberger et al (133), where DNA synthesis was shown to be inhibited by 90% in 3T6 mouse fibroblast cells and by only 10% in SV-40 transformed cells grown in medium containing 2 mM butyrate. The following explanation was given by these authors to account for this effect: Eukaryotic cells must accumulate a labile protein(s) before they can pass the restriction point (R) in the G1 phase of the cell cycle (168). This labile 'R'

protein(s) acquires increased stability in transformed cells, thereby allowing continued growth under conditions that would arrest untransformed cells. A possible candidate for this "R" protein(s) is a 53 K dalton protein that has been isolated and found to bind to T-antigen in SV-40 transformed cells (169). The lack of DNA synthesis inhibition could therefore be a consequence of the production of larger concentrations of T-antigen in the SV-40 - transformed cells, which could stabilize "restriction point" protein(s).

Although our results support those of Wintersberger et al (133), in that DNA synthesis was less inhibited in the SV40-transformed cells (and may thus have been a consequence of increased T-antigen production), the γ-irradiation transformed CT-1 cells, which do not produce T-antigen, also show reduced DNA synthesis inhibition in the presence of butyrate. Possibly the CT-1 cells synthesize other protein(s) that are similar to the T-antigen, which could then stabilise the "R"-protein. Analysis of total cellular proteins on SDS-PAGE did indeed show one protein that was stimulated by 10 mM butyrate in the CT-1 cells and not in the SVWI-38 cells. Furthermore, this protein was induced and not enhanced by butyrate treatment. Possibly it is this protein that stabilises the "R"-protein and is analogous to the T-antigen.

Of interest is the fact that although the normal cells showed maximal DNA synthesis inhibition at low butyrate concentrations, these doses were non-toxic, while conversely DNA synthesis was not affected in the transformed cells, yet they showed greater susceptibility to butyrate. Thus butyrate affected the viability of transformed cells to a greater extent than that of the normal cells in a time and concentration dependent manner. However, care should be taken if sodium butyrate is to be administered clinically, because the normal cells are also affected by the drug, in that DNA synthesis was strongly inhibited. This could explain the results of Novogrodsky et al (55) when sodium butyrate was administered intravenously for ten days to a patient with acute myelogenous leukemia, for they observed elimination of myeloblasts from the peripheral blood, with the appearance of mature myeloid cells. They also reported a reduction of ^{3}H-thymidine uptake by the peripheral blood cells and this was taken to indicate partial reversion to the normal state. Although butyrate appeared to reduce the number of leukemic cells, the observed decrease in ^{3}H-thymidine uptake may have been due to effects of sodium butyrate on the DNA synthesizing ability of the mature myeloid cells.

Only at higher butyrate concentrations did DNA synthesis inhibition occur in the transformed cells, and furthermore, this inhibition was shown to be partly due to medium hypertonicity. RNA synthesis was slightly inhibited in

the normal cells (± 20%), while RNA synthesis in both transformed cells was unaffected by butyrate. Both the normal and the transformed cells showed no inhibition of protein synthesis up to 10 mM butyrate, and only WI-38 cells showed a slight inhibition at higher butyrate concentrations. Butyrate thus specifically inhibited the DNA synthesizing ability of the normal cells without affecting any of the other fundamental macromolecular synthesizing processes of the cell. This indicates that butyrate acts through a specific action on the DNA synthesizing machinery of normal cells, possibly by direct action on the DNA polymerase. Our results agree with those of Kawasaki et al (170) and Butt et al (171) where butyrate was shown to have no effect on RNA or protein synthesis.

Low concentrations of butyrate (5 mM) stimulated ADPRT activity in both the normal and the SVWI-38 cells. Butyrate was suggested to act either specifically on the DNA, possibly by causing single or double stranded DNA breaks, or by increasing the number of ADPRT molecules in a variety of ways (see section 4.2.5). If butyrate does indeed introduce breaks into the DNA, then it is possible for gene rearrangements to occur with a higher frequency, and a gene may become inserted near a promotor region making read-through of that gene possible. This could account for the induced gene expression observed after butyrate treatment in many systems. Of importance is the

differential stimulation of ADPRT activity in the normal and the transformed cells at the same butyrate concentrations. The CT-1 cells required higher butyrate concentrations to stimulate ADPRT activity. Possibly, high concentrations of butyrate caused more DNA damage in these cells. A further possibility to account for these results is that butyrate may have inhibited the ADPRT enzyme itself in WI-38 and SV-40 transformed cells, such that the ADPRT activity was inhibited at high butyrate concentrations, but that the ADPRT activity of the CT-1 cells was not affected. This could explain why ADPRT activity of both the WI-38 and the SVWI-38 cell lines declines to below that of the control at higher butyrate concentrations (75 mM)(see Fig. 4.9). Butt et al (171) showed that 5 mM butyrate inhibits the activity of the poly(ADP-ribose) synthetase in Swiss 3T3 cells. In our system we are unable to show inhibition at 5 mM, however inhibition is apparent at higher concentrations in both WI-38 and SVWI-38 cells. The ADPRT assay itself leaves many unanswered questions as to butyrate's effect on DNA repair. At this stage it is impossible to speculate which of the above mechanisms occur in the cell. Direct analysis of single stranded DNA breaks on sucrose gradients would be one way of determining whether butyrate has a direct effect on the DNA. It can however be concluded from this study that butyrate enhanced ADPRT activity at low butyrate concentrations in the WI-38 and SVWI-38 cells, and that

higher doses were required to stimulate ADPRT activity in the CT-1 cells.

SDS-PAGE analysis of proteins synthesized in the presence of 10 mM butyrate showed the differential response of normal and transformed cells. Most noticeable was the disappearance of two high molecular weight proteins in the WI-38 cells. This was suggested to be due to a post-synthetic modification of the proteins which was induced by butyrate. A lower molecular weight protein was induced by butyrate in the CT-1 cells only. Thus butyrate was able to induce and modify individual proteins in different cell lines. These results are in agreement with other workers, where both an induction and protein modifications of specific proteins have been reported (153,159,172,173).

Methylation of DNA is thought to be a controlling factor in gene expression. Increased methylation is generally correlated with decreased gene expression, while conversely decreased methylation is associated with increased gene activity. Because butyrate is capable of stimulating or inhibiting the expression of genes in many systems (which accords well with the SDS-PAGE data obtained in this study), it was decided to investigate the effects of sodium butyrate on total cellular DNA methylation in both the normal and transformed cells. In both normal and transformed cells, parental and daughter DNA strands showed increased methylation after treatment with butyrate,

although the extent of this methylation varied for the different cell lines. Within the same cell type (e.g. the WI-38 cells), the extent of hypermethylation of parental and daughter DNA strands differed. Furthermore, the extent of hypermethylation differed in the three cell lines, where WI-38 cells showed the most hypermethylation of parental DNA strands, while the SVWI-38 cells showed the most hypermethylation of daughter DNA strands. This variation reflects the ability of butyrate to differentially affect the methylases in normal and transformed cells, as well as having a differential effect on daughter and parental DNA strands within the same cell. Furthermore, the induced DNA hypermethylation is dose-dependent up to 20 mM butyrate, after which it returns to control levels.

Because the methylation status of a cell is maintained by "maintenance methylases" that ensure symmetrical methylation of daughter DNA strands after synthesis (2.17.18), the observed hypermethylation of daughter strand DNA after butyrate treatment could have resulted from:
(1) Activation of maintenance methylases: Butyrate may function as an activator of the enzyme, or it may have removed a repressor molecule,
(2) An increase in the number of maintenance methylase molecules: Possibly butyrate stimulated the expression of the maintenance methylase genes, resulting in an increased number of enzyme molecules per cell,

(3) Exposure of more methylatable sites in the DNA to the enzyme: Possibly butyrate induced conformational changes in the DNA, which would then have exposed additional sites not normally accessible to the methylases, or
(4) Any combination of the above.

It must be remembered that whatever the mechanism, the SVWI-38 cells were the most responsive to the butyrate induced hypermethylation of daughter DNA strands.

Boehm and Drahovsky (54) have shown that when a nucleoside analogue, 1-β-D-arabinofuranosylcytosine (ara-C) is given to mouse p815 mastocytoma cells at 0,1 μg/ml, DNA synthesis is retarded by 50% and hypermethylation of the DNA results. Although the normal cells were the only cells to show a butyrate-specific inhibition of DNA synthesis in our system, all three cell lines showed hypermethylation of parental DNA strands, although to varying degrees. Butyrate may have acted in a similar manner to ara-C in WI-38 cells, in slowing down DNA synthesis and thereby causing hypermethylation of DNA. This may also explain why parental DNA strands were preferentially methylated in WI-38 cells, for DNA synthesis inhibition was maximal compared with the two transformed lines, where DNA synthesis was unaffected by butyrate. Two possible mechanisms that may result in the selective hypermethylation of parental strand DNA are postulated:

(1) Because of the inhibition of DNA synthesis by butyrate, maintenance methylases could have "back-tracked" along parental DNA strands and methylated sites which were not normally methylated, or

(2) Butyrate might have stimulated the de novo methylating enzymes which then randomly methylated non-replicating DNA. Two recent reports have shown that an isolated methyltransferase performed both "maintenance" and "de novo" methylation in *in vitro* experiments (33). These authors suggested that one enzyme was therefore capable of performing both functions. From our results, the fact that both parental and daughter DNA strands in the same cell line showed hypermethylation suggests that this could be feasible. However, the extent of hypermethylation differed, which indicates that possibly two separate enzymes may be stimulated by butyrate.

Again it should be emphasized that whatever the mechanism of action stimulated by butyrate, the parental DNA strands of the WI-38 cells were the most susceptible, resulting in the largest induced hypermethylation.

In all cases, DNA hypermethylation was concentration-dependent and occurred maximally at 5 to 10 mM butyrate. At 20 to 50 mM butyrate and above, the percent 5-methylcytosine returned to control values. This could have been due to inhibition of the methylating enzymes by butyrate,

preventing any further increase in methylation. It was postulated that a low level of methylation exists below which abnormal patterns of gene expression would result in cell death (74). Possibly at higher butyrate concentrations, methylation levels decrease to below that which is capable of supporting life, resulting in the reduced cell viability. Aza-C (AzaCdR) has been shown to reduce 5-methylcytosine levels (175) due to its incorporation into DNA and because of its triazine ring structure (where a nitrogen is substituted in place of a carbon atom in the 5th position of the ring), methylation is prevented (176). This is thought to be due to a suicide complex-formation with methylase enzymes (see Section 1.0). Butyrate, however, does not become incorporated into the DNA, but possibly decreases the 5-methylcytosine content by inhibiting the methylase directly, either before the methylase attaches to the DNA, or once attached. Alternately, if an enzyme(s) exists that can remove methyl groups (and already evidence is accumulating to show its existence in some systems (37,38)), it is possible that butyrate increases the level of methylation by inhibiting the action of the "demethylase" enzyme. The observed methylation level therefore becomes an average value of methyl group "turnover". Data presented in this study supports the idea of a demethylase in these cells.

WI-38 cells that became hypermethylated after exposure to 10 mM butyrate showed different responses to the maintenance of this hypermethylated state after removal of the drug. Hypermethylation of daughter DNA strands was maintained during a subsequent round of replication after removal of butyrate. However, hypermethylation of parental DNA strands was not maintained and returned to control values. This implies that:

(1) Hypermethylated parental DNA strands must have undergone a demethylation event after removal of butyrate. The conventional way that DNA becomes hypomethylated is thought to result from the failure of newly synthesized daughter DNA strands to be methylated at appropriate sites. This mechanism cannot apply to parental DNA strands, because the loss of methyl groups in this case occurred in DNA that was not newly synthesized. Our data thus suggest the existence of a demethylase enzyme that is capable of removing methyl groups from non-replicating DNA.

(2) If a demethylase does exist, then butyrate must be capable of regulating its activity. By postulating the existence of a demethylase, it is immediately apparent that methyl-group "turn-over" must occur, and maintenance of the status quo is only possible if the activity of these two enzymes is controlled. Because hypermethylation was induced at low butyrate concentrations in both normal and transformed cells, butyrate must either stimulate the

activity of the methylase or inhibit the activity of the demethylase, thus resulting in the overall hypermethylation. It is now possible to envisage why normal and transformed cells showed differential hypermethylation in response to butyrate treatment. Butyrate may affect the extent of methylase stimulation and demethylase inhibition to varying degrees in normal and transformed cells.

These results also show that in order to maintain the altered methylated state, DNA replication was required; i.e. permanent methylation of DNA was only possible if the methylation occured on newly synthesised DNA, and only then was it hereditable. An interesting model for the maintenance of the altered genotype can thus be postulated. If during the life span of a cell, random incorrect methylation events occur, the cell has a way of controlling these errors, as any methylation event that occurs in non-replicating DNA is removed, possibly by the demethylase. The demethylase must therefore be able to distinguish between random methylation events and methylation that occurred during strand synthesis. Methylation that occurred during DNA synthesis would be symmetrical, while random methylation events presumably would not be. Possibly the demethylase recognises the asymmetric methylation event and removes it for this reason. However, if the random methylation occurs during DNA synthesis it is not recognised as incorrect, and it will

become part of the daughter cell's heriditable genome. Presumably during DNA synthesis, the random event will be symmetrically duplicated in the opposite strand of the DNA helix, by the maintenance methylases and therefore not be recognised as incorrect by the demethylase(s).

Thus it can be seen that random methylation events may only affect gene expression and maintenance if the methylation occured in stretches of replicating DNA. This is interesting in the light of findings that butyrate is able to induce a more differentiated state in a number of transformed systems (57,148,167). We and others (133) have shown that DNA synthesis is more inhibited during butyrate treatment in normal cells than in transformed cells. Because DNA synthesis is required for the maintenance of the induced methylation status, this could explain why butyrate affects transformed cells more than their normal counterparts. (e.g. in SVWI-38 cells, no inhibition of DNA synthesis occurred at low butyrate concentrations, therefore the hypermethylation of daughter DNA strands could be maintained.) The proposal that DNA synthesis is required for gene activation was put forward by a number of workers at least 15 years ago (177,178,179), where they suggested that at least two rounds of mitosis are required for erythroid differentiation in the presence of inducers of differentiation; or that the inducer must be present during or immediately after DNA synthesis. Subsequently, other data (180) have shown that DNA

synthesis is not required for differentiation. Our data therefore agree with earlier work, where DNA synthesis is required for the maintenance of the altered methylation pattern.

Although butyrate caused hypermethylation in all three cell lines examined in this study, it has been reported that 2 mM butyrate caused hypomethylation of DNA in Friend cells (181). This discrepancy with our results could be due to the use of a different system. We examined human lung embryonic fibroblasts while Christman et al (181) used Friend erythroleukemia cells. It has been shown that butyrate elicits different effects in different cell lines (113).

In this study the following effects were observed when normal and transformed cells were exposed to millimolar concentrations of sodium butyrate:

(1) Sodium butyrate caused a greater toxic response in the transformed cells compared with their normal counterpart, this being in a time and concentration dependent manner.

(2) DNA synthesis of the normal cells was maximally inhibited by low and high concentrations of butyrate, while the transformed cells showed no inhibition at low butyrate concentrations. Inhibition occurred only at higher concentrations which was probably due to medium

hypertonicity. RNA and protein synthesis was not affected in the transformed cells, whilst only minimal inhibition of RNA synthesis was observed in the normal cell line, with only a ±20% inhibition of protein synthesis noted at higher butyrate concentrations.

(3) Butyrate induced the formation of a specific low molecular weight protein in the γ-irradiation transformed cells (which was suggested to be similar to the T-antigen in its ability to stabilise 'R' proteins), while it modified two high molecular weight proteins in the WI-38 cell line.

(4) ADPRT activity was stimulated at low butyrate concentrations (5 mM) in both the WI-38 and SVWI-38 cells but only at much higher concentrations (75 mM) in the CT-1 cell line.

(5) Butyrate caused marked hypermethylation of both parental and daughter DNA strands in the normal and transformed cells, but the extent of this hypermethylation varied for the different cell types.

(6) Maintenance of the hypermethylated state was only possible if the methylation event occurred in DNA that was replicating. Evidence is also provided for the existence of a demethylase enzyme(s) that removes random 5-methyl-cytosines in parental DNA strands.

Normal and transformed cells were therefore differentially affected when exposed to millimolar concentrations of sodium butyrate. It is hoped that this study has provided a further insight into the mechanism of action of this 4-carbon polar fatty acid, and furthermore that it has shown the usefulness of butyrate to study the mechanism of gene regulation.

CHAPTER 5

DISCUSSION

Malignant transformations have been correlated with decreased methylation in many tumor lines (182,183,184). Furthermore, oncogenic transformation often leads to activation or inactivation of genes (185,186,187,188,189), the former being correlated with decreased methylation at specific sites within a gene. Possibly it is the demethylation at important regulatory sites, or the demethylation of oncogenes, that leads to the transformed state. Normal cells contain sequences within their DNA that are homologous to the transforming genes (v-onc) of retroviruses. To date, several human cellular oncogenes (c-onc) have been identified (13) and furthermore it has been established that cellular oncogenes from tumor cells differ from their normal counterpart. One study (190) has shown that the cellular homologue of the Moloney murine sarcoma sequence (c-mos) is hypermethylated and transcriptionally silent in a variety of normal rodent cell lines, which contrasts with a Moloney sarcoma virus-transformed cell line in which the integrated viral sequence (v-mos) is hypomethylated and transcriptionally active. Thus if demethylation of DNA is an important carcinogenic event, then the possibility of remethylating the DNA and reversing the oncogenic state arises.

In this study it could be shown that agents that specifically inhibit DNA synthesis caused hypermethylation

of the DNA. Ara-C and aphidicolin (two inhibitors that affect the γ-polymerase) specifically caused the hypermethylation of transformed cell DNA. Hydroxyurea which is also an inhibitor of DNA synthesis (it elicits its effect by inhibiting ribonucleotide reductase) also induced hypermethylation of DNA, this induction being more pronounced in the normal cells in our study. Thus these agents were capable of methylating sites within the DNA which were not normally methylated. Various mechanisms were postulated to account for the induced hyper-methylation. Possibly the "maintenance methylase" is stimulated by the drug itself or due to the slowing down of DNA synthesis, the enzyme becomes a more efficient methylase. A further possibility is the production of more maintenance methylases by the cell. Furthermore, possibly "de novo" methylase(s) are stimulated by the drugs. Thus part of the anti-cancer effects of these drugs may be that of reversing the oncogenic state of the cell back to normality by causing remethylation of oncogenes (191). Future work should involve the isolation of the enzymes responsible for the induced hypermethylation to try to establish whether these agents do stimulate the enzyme directly, and if so, why methylases of transformed cells (in this study) are preferentially stimulated.

Furthermore, this study has shown that in order for the induced hypermethylation to be maintained, methylation must be coupled to DNA replication. Altered methylation levels

were only heritable in daughter DNA strands, while the increased methylation was lost during subsequent rounds of replication in parental DNA strands. Sodium butyrate has been suggested as a useful chemotherapeutic drug, hence it was decided in this study to investigate its effect on normal and transformed cells. Butyrate induced preferential hypermethylation of daughter DNA strands in a transformed cell line, and this state was maintained during subsequent rounds of replication. Possibly this is how butyrate selectively decreases myelogenous cells and increases the number of myeloid cells in clinical trials (55). By inducing preferential hypermethylation of transformed cell DNA, oncogenes are probably "silenced" and reversion to the differentiated myeloid state is possible. Future work in this field should try to correlate agents that increase methylation with the reversion of the cells to a more differentiated state. Agents that preferentially hypermethylate daughter DNA strands of transformed cells (e.g. ara-C, butyrate and aphidicolin) should be examined in more detail, because induced methylation events are maintained in subsequent rounds of replication.

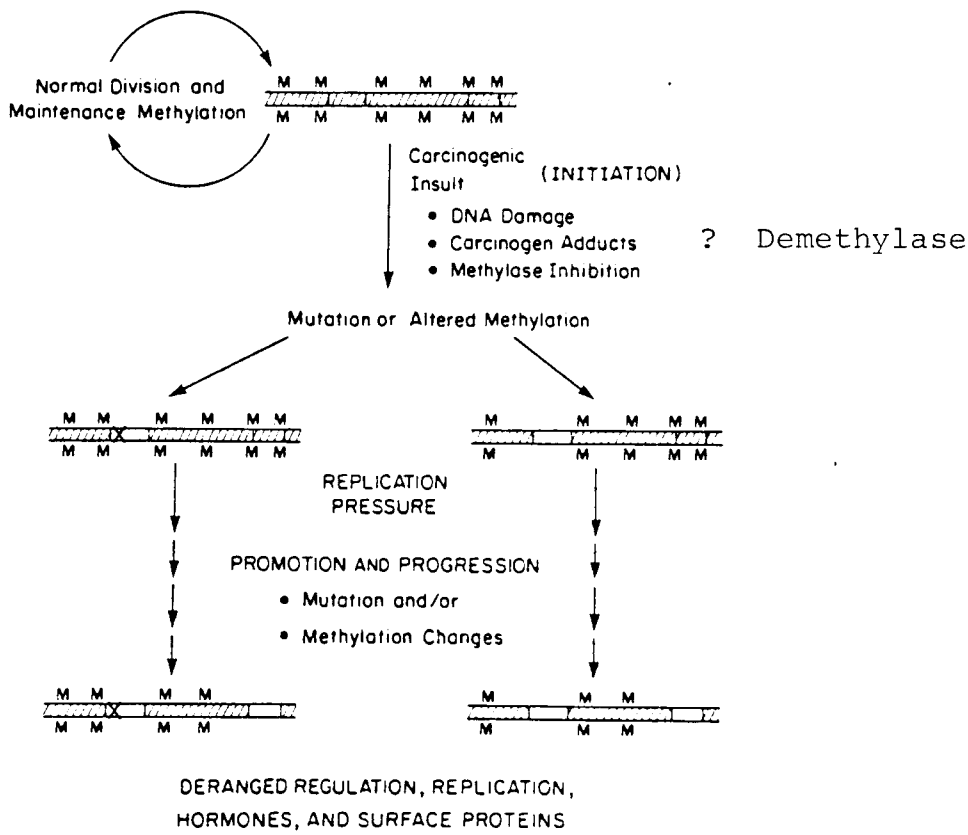
The results obtained in this study also suggest the existence of a "demethylase" that distinguishes between demethylation events that are coupled with DNA replication and those that are not. Methyl groups at non-heritable sites are excised from the DNA by this enzyme. If the

existence of a demethylase is proved, then it is feasible to consider methyl-group turnover, because the regulation of this turnover would have important implications in carcinogenesis. Possibly the demethylase has a "correcting" function in that it could recognise random incorrect methylation events in the DNA and remove them. Further complications might arise if the demethylation event is defective, and random demethylation occurs, which could ultimately lead to malignant transformation of that cell.

In conclusion, the current working model proposed for cancer is shown in Fig. 5.1 as outlined by Riggs and Jones (13) in their review on 5-methylcytosine, gene regulation and cancer. Of importance is the fact that aberrant methylation patterns induced by carcinogenic exposure is propagated in the absence of further carcinogenic treatment, due to the hereditability of "demethylated" sites. These authors propose that demethylation is initiated by an inhibition of the maintenance methylase(s). From the study reported in this thesis, the existence of a demethylase must also be considered, and its possible mode of action incorporated into their model, as detailed above. They emphasize the advantages of postulating a "demethylation" for the initiation of the cancerous state, in that the transformed state might be truly reversible through the remethylation of specific sites. Evidence is provided in our study for agents that selectively enhance

FIG. 5.1 Model proposed to account for the induction of the carcinogenic state

The initiation of the cancerous state is thought to be due to (1) DNA damage (2) Carcinogen adduct formation in the DNA or (3) Inhibition of methylases. Demethylases should also be considered in this model, as incorrect demethylation events could have detrimental effects on cellular function (results presented in this study indicate the existence of such enzymes) (From Riggs and Jones (13)).



the methylation status of transformed cells and may therefore play an important role as chemotherapeutic drugs in the future. It is hoped that this study has provided further insight into the complex biochemical mechanisms that govern malignant transformations of cells.

CHAPTER 6

MATERIALS AND METHODS

6.1. Cell culture:

Cells were maintained in Eagle's Basal Medium (BME) containing 10% heat-inactivated foetal calf serum, 100 µg/ml of penicillin and 100 units/ml streptomycin. At confluence, the normal and transformed cells were split at a ratio of 1:2 and 1:3 respectively. The cells were trypsinized with 0.05% trypsin in Ca^{2+} and Mg^{2+} free phosphate buffered saline (PBS) containing 10 mM EDTA. WI-38 cells (human embryonic lung fibroblasts) were obtained from the American Type Culture Collection (ATCC CL-75). The two transformed cell lines were
(i) a SV-40 transformed counterpart (SVWI-38), and
(ii) a γ-irradiation transformed counterpart (CT-1, a gift from Dr M. Namba, Tokyo).
The cells were incubated at 37^{0}C in a humidified 95% air-5% CO_2 incubator.

6.2. Cell Synchrony: Thymidine Block

Thymidine was filter-sterilized using a 0.45 µM millipore filter and was added to the cells at a final concentration of 2 mM, for 16 hours, after which the medium was removed by suction, and the cells rinsed twice with PBS to remove any excess thymidine. The cells were then given fresh

medium for a further 9 hours after which thymidine at a final concentration of 2 mM was again added to the cells for a further 16 hours. Cells were released from the block by removal of the medium, rinsing twice with PBS and replacing with fresh medium to commence with the experiments described in section 2.2.

6.3. DNA determination using Hoescht H 33258:

1 ml of Hoescht H 33258 dye was dissolved in 10 mls of sterile distilled water. This was stored at 4^0C for up to 1 week (100 µg/ml stock solution). For the assay, the stock solution was further diluted 1:100, giving a 1 µg/ml "assay solution". Each assay was performed in triplicate according to the following protocol:

To glass test tubes the following were added:
2 mls of 1 x SSC (Standard saline citrate: 0.15 M NaCl, 0.015 M citrate)
50 µl of DNA to be assayed
1 ml of "assay solution" (the final Hoescht dye solution now being 333 ng/ml)

The mixture was vortexed and incubated in the dark for 10 minutes. The fluorescence was measured using a Perkin-Elmer LS-5 luminescence spectrometer, at an excitation wavelength of 356 nm and an emission wavelength of 458 nm.

6.4. Inhibitors of DNA synthesis:

The three inhibitors used in this study (see Ch. 3) were

(1) 1-β-D-arbinofuranosylcytosine (ara-C)

(2) hydroxyurea (HU)

(3) aphidicolin

Ara-C and HU were both dissolved in PBS, while aphidicolin was dissolved in dimethylsulfoxide (DMSO). Stock solutions of each were prepared and sterilised by passing the solutions through a 0.45 μm millipore filter, and stored at -2 ^{0}C. The stock solution concentrations were:

(1) 15 mM for ara-C

(2) 100 mM for HU

(3) 1 mg/ml for aphidicolin.

The final concentrations used in the experiments were:

(1) 1.5 mM for ara-C

(2) 10 mM for HU, and

(3) 100 μg/ml for aphidicolin.

These concentrations resulted in DNA synthesis inhibition values of between 95 to 98%.

Sodium butyrate was dissolved in BME and stored at 4 ^{0}C as a 1 M stock solution after it had been filter-sterilised by passage through a 0.45 μm millipore filter.

6.5. Growth Curves:

Cells were plated at a density of $5x10^4$ cells per dish in 30 mm^2 plastic petri-dishes in BME containing penicillin and streptomycin (see 6.1.1). The cells were allowed to attach to the plastic surface before commencing treatment. All treatments were for 16 hours, unless a time-course experiment was performed, in which case the treatment was as indicated in the text. Treatment was terminated by removal of the medium, the cell layer was rinsed with PBS and the cells trypsinised in 0,05% trypsin-EDTA (see section 6.1).

6.6. Cell viability:

Trypan blue exclusion tests were performed as follows: To 500 µl of trypsinised cells was added 50 µl of a 0.4% trypan blue stock solution, and the tubes left to stand for 2 minutes. 10 µl of cell suspension was then viewed under a light microscope using a hemocytometer to determine the number of viable cells in the solution, the viable cells being those that have not taken up the dye.

6.7. DNA, RNA and protein synthesis:

Cells were seeded at $5x10^4$ cells per 30 mm dish. Twenty hours later (i.e. after attachment and growth), ^{3}H-thymidine, ^{3}H-uridine or ^{3}H-leucine (all from Amersham, 40 Ci/mol,

43 Ci/mol and 50 Ci/mol respectively) was added at 1 μCi/ml, together with either ara-C, HU, aphidicolin or butyrate at concentrations indicated in the text. After the indicated time periods, the cells were trypsinised and an aliquot used for cell number determination using a Coulter counter (Coulter Electronics, Inc. Hialeah, Florida). Another aliquot was precipitated with 10% Trichloroacetic acid (TCA) for 1 hour at 4 ^{0}C on ice, filtered onto Whatman GFC filters, and the filtrate was washed thoroughly with 5% TCA, followed by 96% ethanol. The radioactivity was determined in a Beckman LS-1800 counter.

The amount of DNA, RNA or protein synthesised was expressed as the amount of label incorporated per 10^{6} cells. When RNA synthesis was monitored, 10 μM each of deoxycytidine and thymidine was added simultaneously with the labelled uridine to the cells, in order to reduce incorporation of label into DNA via the salvage pathways. Where ^{3}H-deoxyadenosine and 5,6 ^{3}H-deoxyuridine were used as alternate markers for DNA synthesis, 5 μCi was added per dish. (The specific activities were 480 mCi/mmol and 43 Ci/mmol for dAde and deoxyuridine respectively.

6.8. SDS Polyacrylamide Gel Electrophoresis (PAGE) analysis of proteins

Cells were grown in 150 mm^2 flasks in BME as described in section 6.1. ^{3}H-Leucine (50 Ci/mol) was added to the cells at a final concentration of 5 µCi/mol either with or without 10 mM butyrate. The cells were grown in this medium for ±20 hours after which the medium was removed, the cell layer rinsed twice with PBS and the cells trypsinised using 0.05% trypsin-EDTA. 1 mM phenylmethyl sulphonyl fluoride (PMSF) was added immediately after trypsinisation to prevent protein degradation, and was present in all subsequent steps. The cells were lysed in 1% SDS, 10 mM EDTA and 1.5 mM β-mercaptoethanol, and heated to 90 ^{0}C for 3 minutes. To each sample, 2 µl of gel marker dye buffer was added. (Gel marker dye buffer: 0.025% bromophenol blue, 1 mM EDTA, 0.5% SDS, 50% glycerol).

The proteins were separated on a 20 cm long 10% polyacrylamide gel at 100 volts for 20 hours. The gel was prepared as follows:

(1) The separating gel: (10%)

30% Acrylamide/0.8% Bis	17,1 mls
1.5 M Tris pH 8.8	9,0 mls
100 mM EDTA	1,8 mls
and H_2O was mixed in a flask	19,4 mls

The mixture was degassed and to this the following were added:

20% SDS	250 µl
Temed	50 µl
10% ammonium persulfate	500 µl

The gel was poured and left to set.

(2) The stacking gel: (3%)

30% Acrylamide/0,8% Bis	1 ml
0.5 M Tris pH 6.8	2.5 ml
100 mM EDTA	500 µl
and H_2O was mixed in a flask	5,8 ml

The mixture was degassed and to this the following were added:

20% SDS	50 µl
Temed	10 µl
and ammonium persulfate	100 µl

The stacking gel was poured above the separating gel and allowed to set for 1 hour.

After the samples had electrophoresed for 20 hours, the gel was stained with Coomassie blue dye (25% isopropanol, 10% acetic acid, 0.25% Coomassie), destained in 10% acetic acid and prepared for flurography by soaking in 1M sodium salicylate until formation of crystals occurred. The gel

was dried and placed against X-ray film (Dupont Chronex-4) for 2 weeks.

6.9. Preparation of DNA:

DNA was isolated by two different procedures, depending on whether it was to be used for restriction endonuclease digestion and subsequent agarose gel electrophoresis, or for HPLC analysis.

6.9.1. Preparation of DNA for restriction endonuclease digestion and agarose gel electrophoresis:

Cells that had been treated with either butyrate (see section 4.2.6.3) or ^{3}H-methionine (see section 2.2.1) were collected by trypsinisation, pelleted by centrifugation and lysed in 1% SDS, 1 mM EDTA. The proteins were digested by incubating the lysed cells with 100 μg/ml Proteinase K for 4 hours at 50^{0}C. The samples were then extracted with an equal volume of phenol saturated with TE (TE = 10 mM Tris pH 7.5, 1 mM EDTA) until no protein interphase was visible. The aqueous phase was then further extracted twice with chloroform:isoamylalcohol (24:1). The DNA was precipitated by the addition of 1/10 volume of 5 M NaCl and 2 volumes of 96% ethanol and left at -20^{0}C for 1 hour. The DNA was spooled onto a glass rod and washed twice in 70% ethanol. The remainder was left to precipitate for 24 hours to obtain the RNA. After evaporating off the

ethanol, the DNA was dissolved in TE buffer. The RNA was obtained after centrifugation, drying of the ethanol and resuspending in TE buffer.

6.9.2. HPLC analysis:

DNA for HPLC analysis was isolated as described by Wilson & Jones (192). Cells were grown in 60 mm^2 flasks. After treatment with either the inhibitors or butyrate at the indicated concentrations and 5 µCi of 6-^{3}H-uridine (15 Ci/mmol), the medium was removed, the cells washed with PBS, lysed in 0.5% SDS, 0.3 N NaOH and incubated at 37^{0}C for 16 hours. After alkaline hydrolysis of the RNA, the incubate was neutralised by adding Tris HCl pH 7.5 to a final concentration of 50 mM Tris and 0,3 N HCl. Protein removal was achieved by the addition of 50 µg/ml proteinase K, and incubated for a further 24 hours at 37^0 C. The DNA was precipitated by the addition of an equal volume of 10% TCA and left at 4^{0}C overnight. The DNA was pelleted by centrifugation, rinsed in 70% ethanol and dried under vacuum.

6.10. Analysis of the 5-methylcytosine content (HPLC analysis):

DNA was hydrolysed by the addition of 30 µl of 88% formic acid to the dried DNA. The mixture was sealed in

capillary tubes and hydrolysed at 180^{0}C for 25 minutes. The tubes were frozen in liquid nitrogen (formic acid is highly explosive when heated), before opening. The formic acid was evaporated under vacuum and the hydrolysate resuspended in 0.1 N HCl. The resuspended bases were separated on a Beckman Ultracil TM-Cx column and eluted with sodium acetate buffer, pH 3.35 (see below for composition). A flow rate of 0.5 ml/min and constant pressure of 70 bars gave an elution profile shown in Fig. 6.1. The eluted bases were collected at 30 second intervals, using an automatic fraction collector, into scintillation vials for β-counting. The 5-methylcytosine content was determined as follows:

$$\% \text{ mC} = \frac{\text{dpm in mC} \times 100}{\text{dpm in (mC + C)}}$$

Sodium Acetate buffer was prepared according to the following protocol:

100 mM Na Acetate

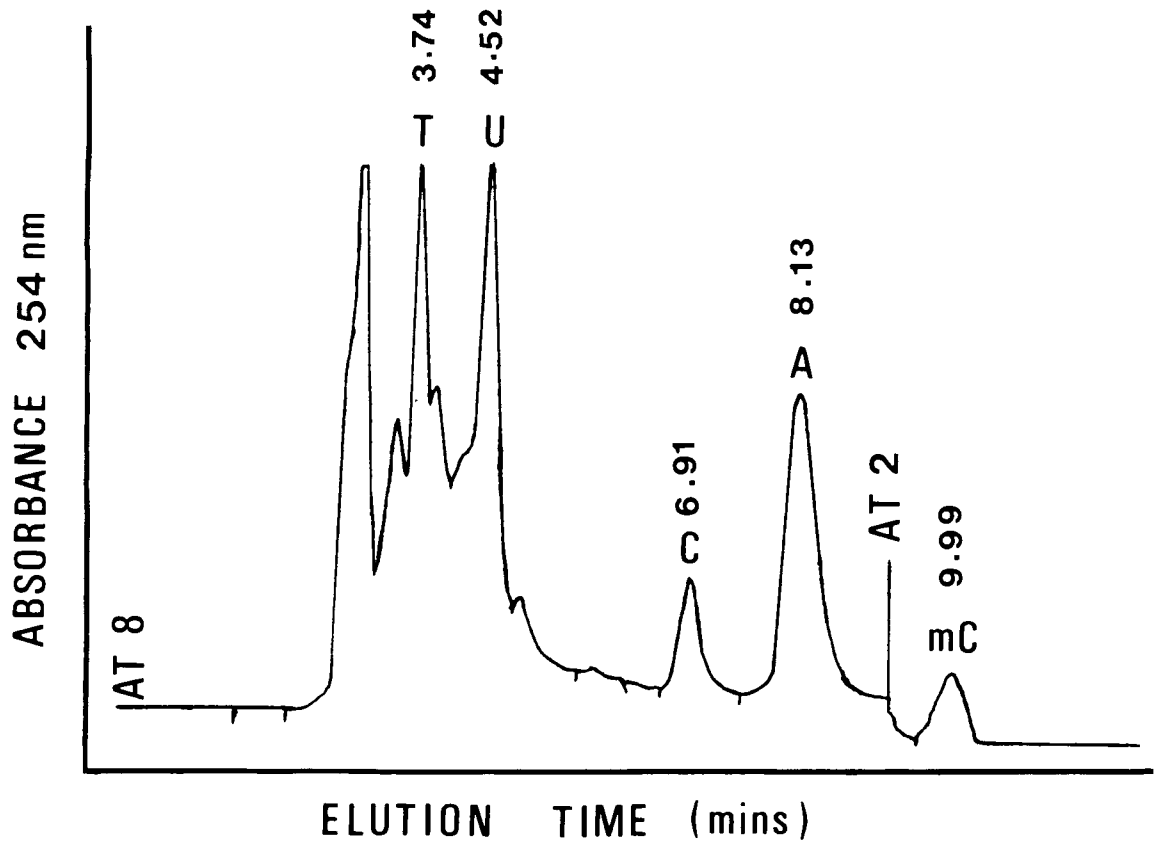
5% Methanol, pH 3.35 with glacial acetic acid.

This solution was filter sterilised and stored at 4^{0}C. Before use, the buffer was heated to 37^{0}C, and de-aerated by bubbling with Helium.

FIG. 6.1 HPLC elution profile of DNA after acid hydrolysis

DNA was hydrolysed in formic acid and treated as described in Materials and Methods (6.10). The bases were separated on a Beckman TM-CX ultracil column using 100 mM sodium acetate pH 3.35 containing 5% methanol, at a flow rate of 0.5 ml/min and constant pressure of 70 bars. Fractions were collected at 30 second intervals and the % mC determined as indicated in the text.

At 8 = attenuation of 8
At 2 = attenuation of 2 (more sensitive scale)
T = thymine
U = uracil
C = cytosine
A = adenosine
mC = 5-methylcytosine



6.11. Labelling procedure for determining parental and daughter DNA strand methylation:

Cells were seeded at $2x10^5$ cells per 60 mm^2 dish. After attachment of the cells to the dish, 5 μCi of 6-^{3}H uridine (15 Ci/mmol) was added to the medium, and the cells allowed to grow in this medium for 72 hours. The medium was then removed, the cell layer rinsed with PBS and treated with sodium butyrate at the indicated concentrations for 16 hours in the absence of label. This detected parental DNA strand methylation. Where daughter DNA strand methylation was monitored, 5 μCi of 6-^{3}H-uridine (15 Ci/mmol) was administered simultaneously with the DNA synthesis inhibitor or butyrate for 16 hours. Treatment commenced after removal of the medium and replacement with fresh medium containing either butyrate at the indicated concentrations or one of the three DNA synthesis inhibitors discussed in section 6.4. Treatment was terminated by removal of the medium, rinsing of the cell layer with PBS and the addition of 0,5% SDS, 0,3 N NaOH. The DNA was isolated as detailed in section 6.9.2.

6.12. Maintenance of Methylation:

This was done by both single isotope labelling or dual isotope labelling.

6.12.1. Single Isotope labelling:

WI-38 cells were seeded at $2x10^5$ cells in 60 mm^2 dishes. After attachment, the cells were either given 5 µCi of 6-^{3}H-uridine (15 Ci/mMol) for 72 hours, the medium was removed and the cells treated with 10 mM butyrate for an additional 16 hours (this detects parental DNA strand methylation), or 10 mM butyrate plus label was administered simultaneously for 16 hours, thus detecting daughter DNA strand methylation. The former treatment was either terminated after butyrate treatment (and the 5-mC content determined as detailed in section 6.10) or the cells were allowed to grow for an additional 16 hours in fresh medium and then analysed for their 5-mC content. In parallel, cells were also treated with 10 mM butyrate for 16 hours, the medium removed after treatment, and 5 µCi of 6-^{3}H-uridine was added.

6.12.2. Dual Isotope labelling:

WI-38 cells were seeded at $2x10^5$ cells per 60 mm^2 dishes in BME medium. After attachment, 1 µCi of ^{14}C-uridine (488 mCi/mMol) was added to the cells for 72 hours. The medium was removed and the cell layer rinsed twice with PBS. Cells were either given

(1) 6-^{3}H-uridine for 16 hours

(2) 6-^{3}H-uridine plus 10 mM butyrate for 16 hours

(3) 6-^{3}H-uridine plus 10 mM butyrate for 16 hours, the medium removed, the cells rinsed twice with PBS, fresh medium added and the cells incubated for a further 16 hours.

All treatments were terminated by the removal of the medium, followed by two rinses with PBS and the cells were lysed with 0.5% SDS, 0.3 N NaOH. The DNA was isolated as detailed in section 6.9.2.

6.13. ADPRT Assay:

The indicated concentrations of butyrate were added to cells seeded in 150 mm^{2} flasks and incubated for 14 hours. The cells were harvested by trypsinisation, centrifuged and resuspended in 5 ml of BME. The ADPRT assay was performed as described by Cleaver et al (165). The cells were centrifuged and resuspended in permeabilisation buffer (10 mM Tris pH 7.8, 1 mM EDTA, 4 mM MgCl , 30 mM β-mercapto-ethanol, 0.05% Triton X-100) and left on ice for 30 minutes. The cells were pelleted and resuspended in assay buffer (30 mM Tris pH 7.8, 1 mM EDTA, 40 mM MgCl , 20 mM -mercapto-ethanol, 0,05% Triton X-100), and aliquoted at 5x10^{5} cells per tube containing 0.5 µCi of |adenine-2,8-^{3}H| -NAD^{+} (New England Nuclear, 25,0 Ci/mmol) and 4 mM cold NAD^{+} in a final volume of 200 µl. The tubes were incubated at 37^{0}C for 1 hour, followed by the addition of 1 ml of 20% TCA. The tubes were left on ice for 1 hour, filtered onto

Whatman GFC filter paper and the radioactivity determined by scintillation counting, as described in 6.7.

6.14. Restriction enzyme digestion of DNA with Isoschisomers Msp 1 and Hpa II:

DNA was prepared as described in 6.9.1 and digested with either Msp 1 or Hpa II by dissolving in the buffer recommended by the suppliers and adding enzyme at 2 units/µg DNA. The reaction was incubated at 37°C for 3 hours, and was terminated by the addition of stop buffer (0.05% Bromophenol blue in 0.5% SDS, 1 mM EDTA, 5% glycerol). DNA was electrophoresed for 4 hours at 150 mA on a 0,8% horizontal agarose gel in 40 mM Tris-acetate pH 7.8, 50 mM Na-acetate, 10 mM EDTA. The gel was stained with 0.1 mg/ml ethidium bromide and visualised under ultraviolet light. A negative of the gel was scanned using a Cliniscan densitometer (Helena Laboratories, Texas).

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