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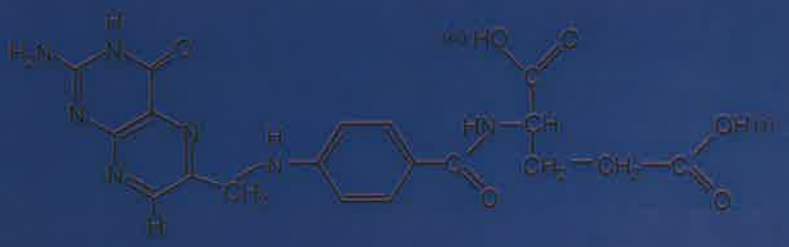
The effects of folic acid deficiency and defects in folate metabolism on chromosome damage



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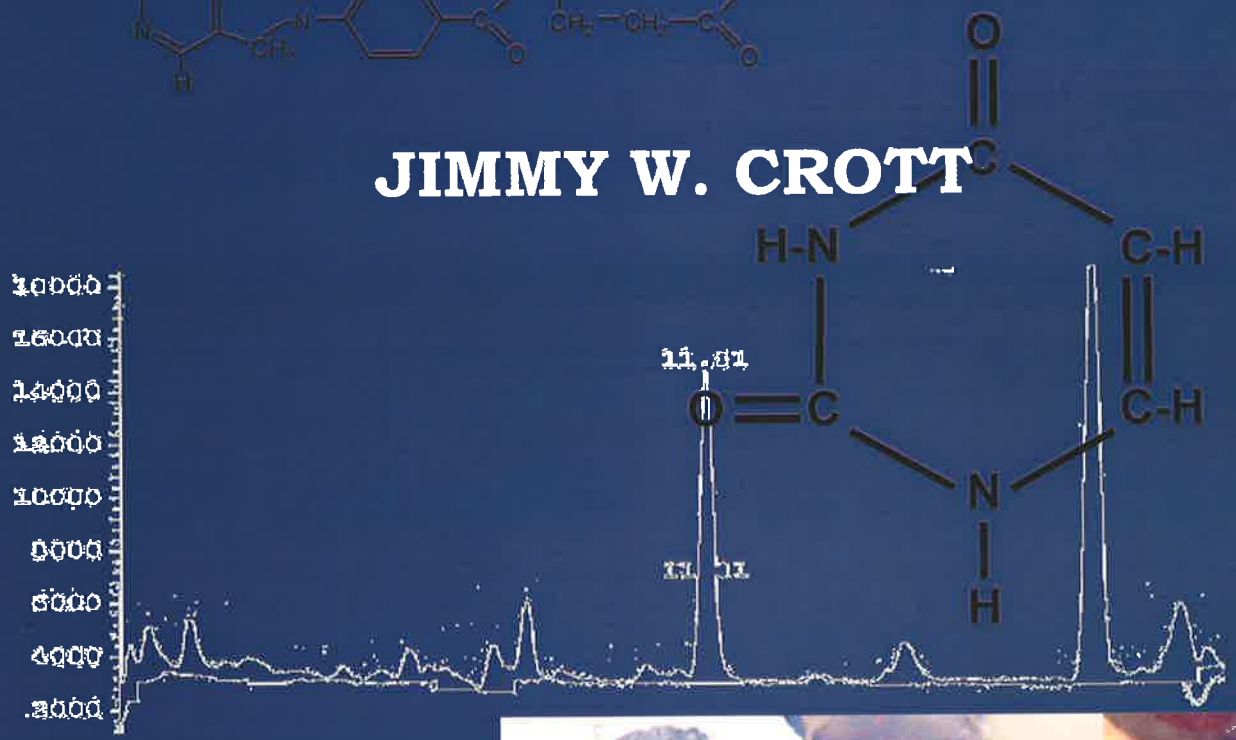

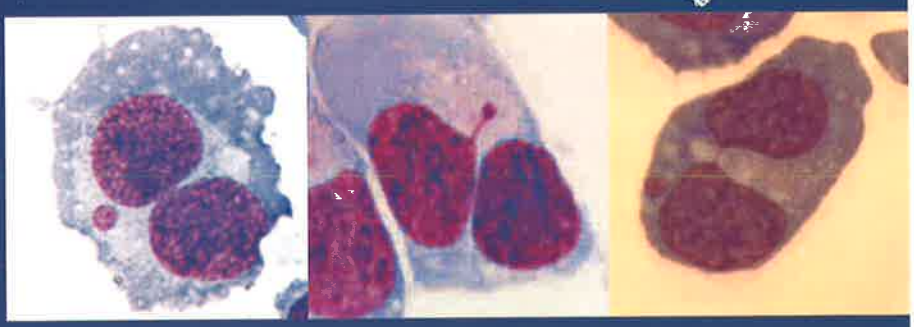

FOR MUM

The effects of folic acid deficiency and defects in folate metabolism on chromosome damage *in vitro*.

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Abstract

This thesis describes a series of experiments that aimed to investigate the effects of folic acid deficiency and defects in folate metabolism on chromosome damage rates in human lymphocytes. The accumulation of chromosome damage over time is an important issue because it is thought to contribute to the mechanism of ageing and the aetiology of diseases of old age such as cancer and Alzheimer's disease. Four main experimental directions were followed:

Potential genotoxicity of homocysteine

Homocysteine is a metabolite that increases in concentration during folate deficiency and is suggested to contribute to chromosome damage rates *in vivo* and the pathology of atherosclerosis and Alzheimer's disease. Lymphocytes from four male volunteers were repeatedly challenged with homocysteine (0- 400 μM) or similar concentrations of methionine (as control) and chromosome damage rates were assessed by scoring micronucleus (MN) induction in binucleated cells. Homocysteine concentration did not have a dose-dependent effect on MN induction, however analysis of combined data for all concentrations indicated that homocysteine induced a small but significant increase in MN frequency compared to methionine (P= 0.04). The physiological significance of this result is unclear and future studies are needed to verify these results with more physiological concentrations, various oxidised homocysteine derivatives, and culture medium containing both homocysteine and methionine.

Methionine dependence

Methionine-dependence, or the inability of cells to proliferate in medium where methionine is replaced by homocysteine, may be a phenotypic marker of methionine synthase deficiency and has previously been associated with the cancerous state. Methionine synthase deficiency is associated with an increased chromosome damage rate possibly due to a depletion of folate for thymidine synthesis. The frequency, cause and effects of methionine-dependence were studied in lymphocytes from 52 healthy volunteers. Methionine dependence was shown to be a common phenotype that is not related to baseline MN frequency and cannot be explained by three known polymorphisms in methylene tetrahydrofolate reductase and methionine synthase, however methionine-dependency was associated with increased cell proliferation in

methionine supplemented medium. Further experiments utilising longer cell culture durations and different folate sources, particularly 5-methyl tetrahydrofolate, are required to clarify the physiological relevance of methionine-dependence.

Folic acid deficiency

Folic acid deficiency is associated with increased cancer risk and is known to increase DNA uracil content and hypomethylation. It was aimed to thoroughly characterise the effects of folic acid deficiency on chromosome damage, to validate novel biomarkers of folic acid deficiency in binucleated lymphocytes and to identify folic acid concentrations that optimise *in vitro* genetic stability. Lymphocytes from 30 healthy volunteers were cultured in medium containing 12, 24, 60 and 120 nM folic acid for nine days. DNA uracil-content, MN, nucleoplasmic bridges (NPBs) and nuclear buds significantly and negatively correlated with folic acid concentration and were all minimised at 60 –120 nM. NPBs and buds are indicative of chromosome rearrangements and gene amplification respectively and this is the first report of their association with folic acid concentrations. Although further research using 5-methyl tetrahydrofolate is required to confirm these results, the data suggest that there may be some benefit, in terms of minimising chromosome damage rates, of raising average plasma folate levels in the community from current levels (10 – 30 nM) to approximately 60 nM. The importance of folic acid is highlighted by the fact that folic acid deficiency induced similar amounts of chromosome damage to those observed after exposure to significant doses of X-irradiation.

Methylene tetrahydrofolate reductase (MTHFR)

MTHFR converts 5,10-methylene tetrahydrofolate (5,10-MnTHF) to 5-methylTHF. The common C677T polymorphism is associated with reduced enzyme activity, hyperhomocysteinemia and reduced risk for blood and colorectal cancer. It was hypothesised that C677T reduces cancer risk by diverting folate towards thymidine synthesis, thereby minimising uracil-associated chromosome breakage. This hypothesis was tested within the above folic acid deficiency experiment where 10 people homozygous for C677T were compared to 20 matched controls. There was no significant difference in the markers of chromosome damage or DNA uracil-content between the two groups. This null result may be explained by high riboflavin concentrations in the medium, which may have increased MTHFR activity in mutants to a normal level.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any other university or other tertiary institution and, to the best of my knowledge and my belief, contains no material previously published or written by another person, except where due reference is made in the text.

I give consent to this copy of my thesis, when deposited in the University library, being available for loan and photocopying.

Signed Date....7/9/01.....

Jimmy W. Crott

Abbreviations

BFB	Breakage-fusion-bridge
BHMT	Betaine:homocysteine methyltransferase
BS	Bloom Syndrome
BN	Binucleate
BNed	Binucleated cell
Bud	Nuclear bud
CBMN	Cytokinesis-block micronucleus assay
CBS	Cystathione β-synthase
CC	Person without the MTHFR C677T polymorphism
CT	Person heterozygous for MTHFR C677T polymorphism
Cyto-B	Cytochalasin-B
DHF	Dihydrofolate
DHFR	Dihydrofolate reductase
DM	Double minute
DSB	Double-stranded (DNA) break
dTMP	deoxythymidine monophosphate
dUMP	deoxyuridine monophosphate
FAD	Flavin adenine dinucleotide
FCS	Foetal calf serum
HBSS	Hank's balanced salt solution
Hcy	Homocysteine
hr	hour
IL-2	Interleukin-2
Met	Methionine
5-MeTHF	5-methyl tetrahydrofolate
min	minute
MN	Micronucleus
MNed	Micronucleated
MNi	Micronuclei
5,10-MnTHF	5,10-methylene tetrahydrofolate
MS	Methionine synthase

MTHFR	Methylene tetrahydrofolate reductase
NDI	Nuclear division index
NPB	Nucleoplasmic bridge
PCR	Polymerase chain reaction
PHA	Phytohaemagglutinin
PLP	Pyridoxal 5'-phosphate
RDA	Recommended daily allowance
RFLP	Restriction fragment length polymorphism
SAH	S-adenosyl homocysteine
SAM	S-adenosyl methionine
THF	Tetrahydrofolate
TT	Person homozygous for MTHFR C677T polymorphism
yr	year

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Thesis amendments

1. *Page 3, Figure 1.1legend, line 8*: "extra" should be removed from point E.
2. *Page 4, 3rd paragraph, line 10*: "gluatmate" should read "glutamate".
3. *Page 10, Figure 1.2 legend, line 1*: " **dTMP**, deoxyuridylate" should read "**dTMP**, deoxthymidylate.
4. *Page 11, 2nd paragraph, line 3*: "9490" should read "9,490".
5. *Page 12, 3rd paragraph, line 6*: "biomarker or folate deficiency" should read "biomarker for folate deficiency".
6. *Page 12, 4th paragraph, line 6*: "(Jacob *et al.*, 1998; Kang *et al.*, 1987)" should read "(Kang *et al.*, 1987; Jacob *et al.*, 1998)".
7. *Page 15, 4th paragraph, line 3*: "(< 140 ng/ml)" should read "(Red cell folate < 140 ng/ml)".
8. *Page 16, 3rd paragraph, line 8*: "Pogribny *et al.*, 1997" should be removed from the reference.
9. *Page 19, 4th paragraph, line 4*: "show the that" should read "show that the" and "vitamin B_{12} young adults" should read "vitamin B_{12} in young adults".
10. *Page 20, 1st paragraph, line 2*: "700 mg" should read "700 μg".
11. *Page 24, 2nd paragraph, line 7*: "5-MnTHF" should read "5,10-MnTHF".
12. *Page 32, 4th paragraph, line 3*: "discovering that that gamma" should read "discovering that gamma" and "of MNi these cells." should read "of MNi in these cells.".
13. *Page 42, point 6*: "(HBBS), Trace Biosciences, Victoria, Australia)" should read "(HBBS, Trace Biosciences, Victoria, Australia)".
14. *Page 45, 2nd paragraph*: Points 3 and 4 should be replaced with " MNi must not be connected to or overlap the main nucleus but may touch the main nucleus only if the boundaries of the nucleus and the MN are clearly distinguishable."
15. *Page 51, Figure 3.1 legend, line 7*: "reductase" should be deleted.
16. *Page 52, 2nd paragraph, line 10*: "in humans approximately" should read "in humans is approximately".
17. *Page 57, paragraph 3, line 2*: "(Table 3.7)" should read "(Table 3.1)".
18. Page 65, 3rd paragraph, line 10: "during excision repair" should read "during base excision repair".
19. *Page 90, 1st paragraph, line 3*: "that while a there" should read "that while there".
20. *Page 117, Figure 6.1.C.II.*: "ga" should read "gap".
21. *Page 120, 4th paragraph, line 3*: "Figure 6.3" should read "Figure 6.4".
22. *Page 123,2nd paragraph, line 6*: "Figure 6.2" should read "Figure 6.3".

Chapter 1: General Introduction

Overview

The relationship between folate and the integrity of human DNA is explored in this thesis. **Folate is a vitamin that has a critical role in the synthesis, repair and methylation of DNA, processes which are central to the maintenance of DNA integrity. The health or integrity of our DNA is of importance because there is overwhelming evidence to suggest that many cancers and other diseases of old age are caused by the accumulation of damage to DNA over time** (Holliday, 1998; Fenech 1998).

The issue of folate and DNA stability is approached from two different angles, these being a) how folic acid deficiency and high homocysteine concentrations, an amino acid that accumulates during folate deficiency, directly affect genetic stability and b) how inborn errors of folate metabolism impact on genetic stability and how they interact with folic acid deficiency.

Underlying these issues was the aim to enhance the cytokinesis-block micronucleus assay, a robust measure of chromosome breakage and loss, to produce a comprehensive test for the simultaneous detection of multiple markers of genotoxicity and cytotoxicity.

1.1 Folate v folic acid

Folic acid (pteroylglutamic acid) is a vitamin that is essential to life. Its primary purpose is the transport and transfer of one-carbon units (methyl groups) between various intracellular reactions (Wagner, 1995). Folic acid was isolated in the nineteen thirties as the factor required to prevent anaemia (Stokstad and Manning, 1938) and was later termed folic acid (Mitchell *et al.*, 1941), derived from the Latin word *folium* meaning 'leaf', due to its abundance in green leafy vegetables.

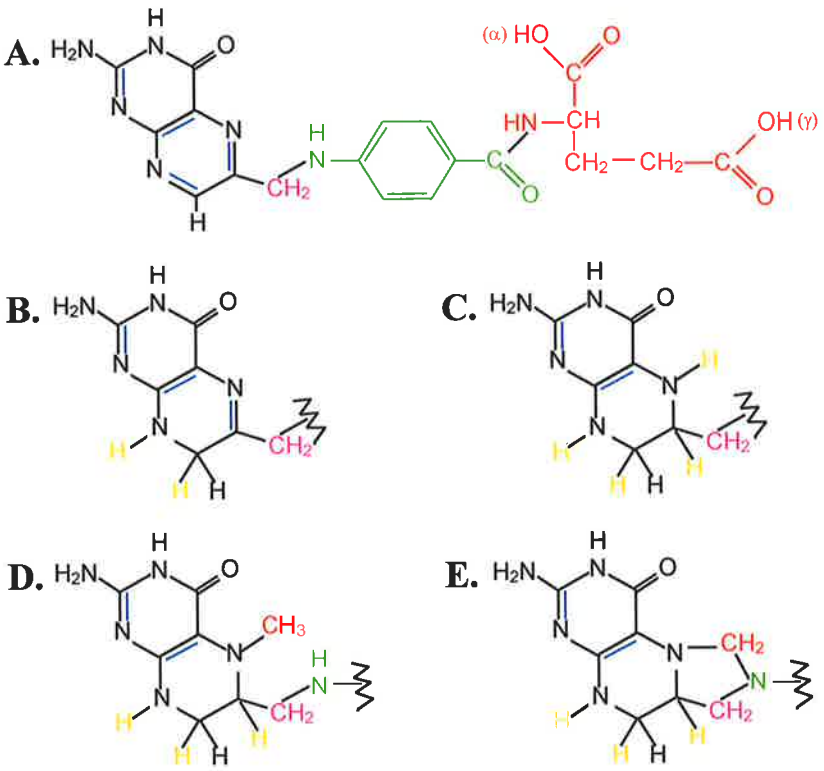
Folic acid consists of a pterin moiety linked via a methylene group to a *p*-aminobenzoyl-glutamate moiety. Folic acid refers to the molecule that has four double bonds in the pterin moiety and a single glutamic acid group (Figure 1.1 A). This parent compound is not naturally occurring but is found in supplements and fortified or oxidised foods. Folic

acid is converted (reduced) to the active dihydro- and terahydro- forms (Figure 1.1 A and B) by dihydrofolate reductase (Shane, 1995). These forms have an extra two or four hydrogen atoms than folic acid respectively.

Mammals cannot synthesise folate and therefore need to obtain it from the diet. Folate exists naturally in plants (or animals that eat them) as polyglutamates, which are hydrolysed to monoglutamates by brush border pteroylpolyglutamate hydrolase (conjugase) in the jejunum of our small intestine. These monoglutamates are absorbed from the jejunum into the portal blood system via saturable, pH-dependent transport and non-saturable passive diffusion at higher concentrations (> 10 μM) (Gregory, 1995). Monoglutamated folates are then taken up by the liver, polyglutamated by folylpolyglutamate synthetase and either stored in this form or re-released into the blood to supply the folate demands of various tissues. Since folylpolyglutamates have a greater affinity and lower K_m values for folate-dependent enzymes, they are better substrates for these enzymes than monoglutamates (Shane, 1995). The primary form of folate found in plasma is 5-methyl tetrahydrofolic acid (Figure 1.1 D). Low affinity protein binders, such as albumin and high affinity folate-binding protein, can bind folate in plasma. Folate-binding protein can be either found in plasma or attached to the plasma membrane, in which case it may be involved in the internalisation of folate by endocytosis. Mammalian tissues also possess a large number of folate transporters, which facilitate the uptake of folate into cells. It is believed that most of the intracellular folate is also protein-bound (reviewed in Shane, 1995).

Folic acid has numerous relatives, mono- or polyglutamated and with or with out the methyl groups which it donates to various intracellular reactions. All of these molecules are collectively termed 'folate'.

Figure 1.1 Structure of folic acid and related folates.



A, Folic acid or pteroylglutamic acid consisting of a pterin moiety (shown in black) with the four double bonds with the ring structure (shown in blue), a methylene group linkage (shown in purple), a para-aminobenzoic acid moiety (shown in green) and a glutamic acid moiety (shown in red). Additional glutamic acid residues are added to the γ-carboxyl group of glutamic acid moiety; **B**, Pterin moiety of dihydrofolate with three double bonds and two extra hydrogen atoms (shown in yellow); **C**, Pterin moiety of tetrahydrofolate with two double bonds and four extra hydrogen atoms; **D**, Pterin moiety of the 5-methyl tetrahydrofolate molecule showing the extra methyl group (shown in red); **E**, Pterin moiety of the 5,10-methylene tetrahydrofolate molecule showing extra the methylene group (shown in red).

1.2 Dietary sources of folate and its bioavailability

The Australian Recommended Daily Intake for folate is currently 200 μg for adults and 400 μg for pregnant females. These levels are based on intakes that prevent the occurrence of anaemia and neural tube defects in offspring.

Because of its role in folate storage, the mammalian liver is one of the best dietary sources of folate. Liver contains between 240 and 500 μg folate/100 g weight in cooked liver, depending on the type of animal. Fortified cereals typically contain 250 μg folate/100 g, boiled vegetables 51- 110 μg/100 g, fruit 1-31 μg/100 g, bread 29 – 30 μg/100 g, nuts 48 – 110 μg/100 g, meat 3- 17 μg/100 g and fish 6 – 29 μg/100g (Holland *et al.*, 1991). Significant losses of folate from food can be induced by leaching during preparation and oxidation and inactivation during heating, however only minor losses occur during frozen storage of foods (Gregory, 1995).

Over the past thirty years much work has been done to study the bioavailability of folate. Despite this, our knowledge of folate bioavailability is rather incomplete due to the inherent problems of this task. Bioavailability studies are confounded by the effect of volunteer folate status prior to the trial which is thought to influence both absorption and excretion of folate, the inability of studies to precisely and reproducibly detect changes in the area under the curve (a common measure of bioavailability) in response to doses of folate lower than 400 μg and the complex array of folate species that are present in food (Gregory, 1995; 2001). The bioavailability of folylpolyglutamates is also affected by the length of the glutamate chain, with absorption being limited by the ability of conjugase enzymes in the small intestine to deconjugate (remove gluatmate groups) folate (Gregory, 1995). To complicate folate bioavailability studies further, some foods contain conjugase inhibitors such as citrate, malate and ascorbate, which may slow the absorption of folates (Wei and Gregory, 1998).

During bioavailability studies, the ingestion of food may alter the outcome because eating risks the ingestion of additional folate (Prinz-Langenohl *et al.*, 1999) while fasting may interrupt enterohepatic circulation, an important pathway for the elimination of folate from the body (Pietrzik *et al.*, 1990). Other confounding factors include intestinal pH, gut

transit time and trapping of folate in the food matrix (Gregory, 1995). As discussed by Prinz-Langenohl and colleagues (1999), the bioavailability values derived from the ingestion of a single folate-containing food item are not indicative of the situation in free-living individuals who generally eat a more complex meal with numerous folate-containing items, all of which could affect parameters of digestive function such as transit time.

Because of the problems associated with determining the absolute bioavailability of folate from food, most research has focused on the bioavailability relative to synthetic (monoglutamyl) folic acid, which is usually ingested as an aqueous solution or tablet. The relative bioavailability of folic acid from fortified maize, rice and bread containing 1 mg/serve folic acid is reported to be approximately 56, 54 and 28% respectively (Colman *et al.*, 1975). Similarly, Sauberlich *et al.* (1987) report that the bioavailability of naturally occurring folate from various foods with a folate content of approximately 300 μg is no more that 50%. The results of Prinz-Langenohl *et al.* (1999) suggest that the relative bioavailability of folate from spinach (600 g containing approximately 480 μg folate) is far greater than of the above foods and is very similar to that of folic acid (400 μg) in an aqueous solution. In an attempt to identify novel, naturally rich sources of bioavailable folate, Fenech *et al.* (1999c), showed that the bioavailability of folate from aleurone flour (containing the nutrient-rich aleurone cells that are part of wheat bran) was not significantly different from synthetic folic acid in a tablet.

1.3 The importance of folate in humans

Folate is critical to the synthesis of purines and pyrimidines, the bases that constitute DNA. It provides two carbon atoms for the synthesis of the purine ring, the precursor for both adenylate (AMP) and guanylate (GMP), however the rate limiting step of DNA synthesis is the folate-dependent conversion of deoxyuridylate (dUMP) to deoxythymidylate (dTMP). Folate is also important in the synthesis of S-adenosyl methionine, the universal methyl group donor for the methylation of DNA.

In mammals, DNA methylation occurs at the fifth carbon position of cytosine residues within CpG dinucleotides. Approximately 70% of CpG dinucleotides are methylated

(Robertson and Jones, 2000). CpG dinucleotides exhibit a non-random distribution in the genome, with CpG being sparse in some regions and prevalent in others. As a whole, the genome is GpG-poor; with CpG being underrepresented approximately 5-fold relative to other dinucleotides.

In contrast, certain regions of the genome, known as 'CpG islands' contain the expected frequency of CpG dinucleotides (Robertson and Jones, 2000), i.e. similar to the frequency of other dinucleotides. CpG islands are generally between 0.5 and 2.0 kb in size and are commonly clustered at the 5' end or promoter region of genes (Laird and Jaenisch, 1994; Robertson and Jones, 2000). It is thought that DNA methylation may be a mechanism of gene silencing because the majority of tissue-specific genes exhibit a strong correlation between hypomethylation of the promoter region and gene activity (Laird and Jaenisch, 1994). Furthermore, it is thought that changes in DNA methylation, such as the hypomethylation of *c-fos, c-myc*, Ha-*ras*, Ki-*ras*, *Erb*-A1 and *bcl*-2 proto-oncogenes may be the cause of some cancers (reviewed in Laird and Jaenisch, 1994).

Recently it has also been suggested that the methylation of lysine residues within histones is another important epigenetic phenomenon that may be linked to gene expression and silencing (Rice and Allis, 2001).

As described later in this chapter, the role of folate in the synthesis and methylation of DNA underlies its requirement in protecting against anaemia, cancer and (probably) neural tube defects in humans (Figure 1.2).

1.3.1 Intracellular folate metabolism

Folate enters our cells from the bloodstream mainly in the form of 5-methyl tetrahydrofolic acid (a monoglutamate), but also in various other oxidation states, and is polyglutamated by folylpolyglutamate synthetase. These derivatives are converted to dihydrofolate (DHF) and tetrahydrofolate (THF) as described in section 1.1.

As mentioned above, the primary function of folate in the folate cycle is to provide one-carbon units to various intracellular reactions. This will be discussed in three parts, namely the entry, the conversion and the removal (utilisation) of one-carbon units from the hypothetical 'pool'. This topic is thoroughly reviewed in Wagner (1995). The term 'one-carbon pool' is commonly used to describe this cycle, which donates and accepts carbon atoms to and from various intracellular reactions. One-carbon units enter and exit the pool at various levels of oxidation, namely formate (HCOOH), formaldehyde (CH_2=O) and methanol (CH_3-OH) states, with methanol being the most reduced and formate being the most oxidised state.

A diagram detailing the aspects of folate metabolism that are most relevant to this project is given in Figure 1.2. Note that some folate derivatives and other molecules and cofactors; particularly those that introduce one-carbon units into the cycle have been omitted from the diagram for ease of understanding. These folates are however discussed below.

A: Entry of one-carbon units:

One-carbon units enter the folate cycle at four main points at two different levels of oxidation.

Folate derivatives that carry one carbon units at the formate level of oxidation enter the cycle at three points; 1) **via 10-formylTHF** (10-HCO-THF) which is produced from THF and formic acid (HCOOH) by 10-formylTHF synthetase. 10-formylTHF can then be cycled back to THF directly or via 5,10-methenylTHF and 5,10-methyleneTHF, 2) **via 5-formiminoTHF** (5-NH=CH-THF) which is produced from THF and formimino glutamic acid (a breakdown product of the amino acid histidine) by glutamate formimino transferase. 5-formiminoTHF is then converted to 5,10-methenylTHF and 3) **via 5-formylTHF** (5-HCO-THF) which is produced from 5,10-methenylTHF by serine hydroxy methyltransferase.

The primary point of entry for folate carrying one-carbon units in the formaldehyde level of oxidation is **via 5,10-methyleneTHF** (5,10-CH_2-THF or 5,10-MnTHF). 5,10-methyleneTHF is mainly formed from THF and serine by serine hydroxymethylatransferase. However, a more complex multi-step process involving the

cleavage of glycine as well as reactions involving the transfer of one-carbon units from dimethyl glycine and sarcosine to THF also occur in the mitochondria.

B: Interconversion of one-carbon-carrying folates:

Folate in the form of 5-formyl and 10-formylTHF is converted to 5,10-methenylTHF by 5,10-methenylTHF synthetase and cyclohydrolase respectively. 5,10-methenylTHF is converted to 5,10-methyleneTHF (5,10-MnTHF) by 5,10-methyleneTHF dehydrogenase. This latter folate species is very important because it is the substrate for both methylene tetrahydrofolate reductase (MTHFR) and thymidine synthase as described below. MTHFR is of great interest because several mutations have been identified in the gene encoding this enzyme, some of which reduce its activity and alter the distribution of folates and related metabolites as discussed in section 1.5. Methylene tetrahydrofolate reductase converts 5,10-methyleneTHF to 5-methylTHF, in an irreversible reaction, which is then converted by vitamin B_{12}-dependent methionine synthase to THF in order to accept more one-carbon units as described above. Thymidine synthase also converts 5,10-methyleneTHF to DHF, which must then be converted to THF by dihydrofolate reductase before it can accept another one-carbon unit.

C: Exit of one-carbon units from the folate cycle/pool:

Just as one-carbon units enter the folate pool in various states of oxidation, they exit the pool in different oxidation states.

In addition to the stepwise conversion of 10-formylTHF to 5,10-methenylTHF, 5,10-methylene, 5-methylTHF and finally THF, this conversion may occur in two one-step reactions that donate carbons in the formate oxidation state for the synthesis of the purine ring.

Carbons in the formaldehyde oxidation state from 5,10-methyleneTHF are donated to the thymidine synthase-mediated conversion of uracil to thymidine. During this process, the carbon unit must be reduced from the formaldehyde state to the methanol state. The electrons for this reduction are provided by the THF ring, which is consequently oxidised to DHF. This process is critical and is the rate-limiting step of DNA synthesis. As described in section 1.4.2, low folate levels can impair the synthesis of thymidine and

result in a build up of its precursor, uracil, which can subsequently be incorporated into DNA.

One-carbon units in the methanol oxidation state, from 5-methylTHF, exit the pool when donated to the methionine-synthase mediated recycling of homocysteine to methionine.

D: Other important reactions:

In addition to the methionine synthase-mediated reaction, the conversion of homocysteine to methionine may also be facilitated by betaine:homocysteine methyltransferase (BHMT), using methyl groups not originating from the one-carbon pool, in hepatic and renal cells. This reaction converts homocysteine and betaine, a metabolite of choline, to methionine and dimethylglycine. Possibly because of its limited tissue distribution, BHMT has only a limited capacity to process excess homocysteine, a fact demonstrated by the accumulation of homocysteine when a defect is present in the folate-dependent remethylation pathway (Green and Jacobsen, 1995).

Although homocysteine can be recycled to methionine, approximately half of the homocysteine produced is committed to cysteine synthesis (Finkelstein *et al.*, 1984). The conversion of homocysteine to cysteine begins with vitamin B_6-dependent cystathionine β-synthase in a reaction that requires serine. Methionine, as well as being an essential amino acid used in protein synthesis, is the precursor to S-adenosyl methionine (SAM), the universal methyl group donor for the methylation of DNA and proteins. Besides its function as a methyl group donor, SAM is important in the regulation of various folate-metabolising enzymes. For example, SAM exerts negative inhibition on MTHFR (Kutzbach and Stokstad, 1971) and BHMT (Green and Jacobsen, 1995).

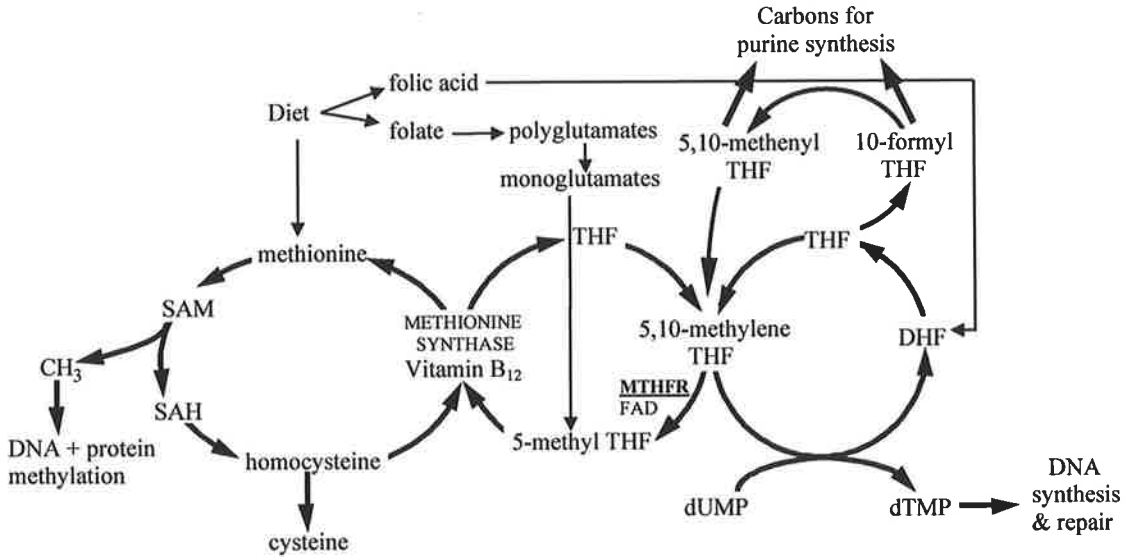


Figure 1.2 Metabolism of folate

CBS, cystathione β synthase; **DHF**, dihydrofolate; **dTMP**, deoxyuridylate; **dUMP**, deoxyuridylate; **FAD**, flavin adenine dinucleotide; **MTHFR**, methylene tetrahydrofolate reductase; **SAH/SAM**, S-adenosyl homocysteine/methionine; **THF**, tetrahydrofolate.

1.3.2 Folate in health and disease

It is well established that folate deficiency causes megaloblastic changes in the bone marrow as well as changes in the peripheral blood, which include increased mean cell volume and neutrophil hypersegmentation. Eventually, folate deficiency leads to anaemia or a reduction in the red blood cell count (Lindenbaum and Allen, 1995). Although there is not a definitive cut-off serum folate concentration that corresponds with the development of anaemia, megaloblastic changes in the bone marrow are present in 50% of people who have serum folate concentrations of 2.1 ng/ml (4.7 nM) or lower (Lindenbaum and Allen, 1995).

Low blood or red cell folate concentrations have been associated with hyperhomocysteinemia (Kang *et al.*, 1987), Spina Bifida (reviewed in Eskes, 2000), Alzheimer's disease (Clarke *et al.*, 1998) and probably also with the genetic abnormality, Down syndrome (James *et al.*, 1999).

Low folate status has also been repeatedly associated with an elevated risk for cancer. It is thought that folate deficiency is a mutagenic situation because it results in aberrant

DNA methylation patterns and chromosome breaks, the accumulation of which is known to increase the risk for developing cancer. Because rapidly dividing tissues have a high demand for thymidine bases and methyl groups, which are required to maintain the integrity of DNA, it should be expected that these tissues be worst-affected, in terms of the accumulation of mutagenic lesions, by low folate availability. Indeed, it is in the fast-dividing tissue of the colorectum where the strongest evidence for a link between low folate and cancer exists (reviewed in Mason, 1995).

Perhaps the best evidence on the link between folate and cancer comes from a large prospective study on colorectal cancer performed by Giovannucci and colleagues (1993). Volunteers (15,984 women and 9490 men) who had received a colonoscopy or sigmoidoscopy were selected from two much larger ongoing cohorts, namely The Nurse's Health Study and the Health Professionals Follow-up cohort, which began in 1976 and 1986 respectively. From detailed semi-quantitative dietary questionnaires it was revealed that those in the highest quintile of folate intake had a significantly lower risk of colorectal adenoma compared to those in the lowest quintile (relative risk 0.65; 95% confidence interval= 0.49 - 0.85; P= 0.01). When carotene and vitamins A, C, D and E were included in the multiple logistic model, the inverse relation between folate intake and adenoma persisted while, none of the other vitamins were significantly associated with adenoma (Giovanucci *et al.*, 1993).

Because folate and methionine are intricately related in their role in donating and transporting methyl groups, it is difficult to dissect out effects on disease risk due to one of these nutrients alone. Furthermore, because alcohol is an inhibitor of pteroylpolyglutamate hydrolysis and pteroylmonoglutamate absorption (Halsted, 1995) it also complicates the relationship between folate deficiency and cancer risk. In other words, the beneficial effect of a high folate or methionine intake may be reduced or abolished by the consumption of alcohol. Indeed, it is the low methionine- low folate-high alcohol diets (low-methyl diet), which carry the highest risk for colorectal cancer. In the above-mentioned study, Giovanucci and colleagues (1993) report that people consuming a low-methyl diet had a relative risk of 3.3 (95% CI= 1.69 – 5.59; P= 0.0003) for colorectal adenoma compared to people consuming a high methionine- high folate-low alcohol diet (high-methyl diet). This trend was confirmed in a later study of 47,931 men from the Health Professionals Follow-up study in which 205 new cases of colorectal

cancers were identified (Giovannucci *et al.*, 1995). It was reported that men who consumed a low-methyl diet were at a markedly higher risk of developing colon cancer with a relative risk of 7.44 (95% CI= 1.72 – 32.1; P< 0.01) compared to men who consumed a high-methyl diet. In this study there was only a weak trend for increased cancer risk with a low folate diet when considering folate intake alone and not methionine or alcohol. This result highlights the complexity of the system by which one-carbon units are supplied to the synthesis and methylation of DNA. Indeed it seems possible that people may be protected against the effects of a low methionine intake by a high intake of folate, and vice-versa.

The effect of low folate concentrations on genetic stability and hence cancer risk is discussed in the following section. Although it is difficult to assign a disease risk to folate alone in epidemiological studies, experimental studies have repeatedly shown us that folate deficiency causes a wide range of deleterious changes to DNA.

1.4 Molecular and physiological effects of folate deficiency

As described in Section 1.3, dietary folate deficiency causes anaemia and is associated with an increased risk for cancer and neural tube defects in offspring. The central mechanism in the aetiology of these diseases involves the crucial role of folate in maintaining DNA integrity. This will be discussed below, together with the possible role of homocysteine in causing genetic damage. Although homocysteine is considered as a biomarker or folate deficiency, it is becoming apparent that homocysteine itself may be involved in the pathology of some diseases.

1.4.1 Altered distribution of metabolites: homocysteine

Low dietary folate intake not only causes a depletion of blood and intracellular folates but also causes an altered distribution of related metabolites. Blood homocysteine concentrations are sensitive to changes in folate availability because 5-MeTHF is the methyl group donor for the methionine synthase-mediated conversion of homocysteine to methionine (see Figure 1.2). It is well established that plasma homocysteine concentrations increase in response to folate deficiency (Jacob *et al.*, 1998; Kang *et al.*, 1987). Under folate replete conditions, plasma homocysteine concentrations generally

range from 6 – 9 μM (Bronstrup *et al.*, 1998; Fenech *et al.*, 1998; Green and Jacobsen, 1995). During severe folate depletion (serum folate < 2 ng/ml) serum homocysteine concentrations can reach up to 18 μM (Kang *et al.*, 1997).

In addition to folate, vitamin B_{12} is an important determinant of plasma homocysteine concentrations. Vitamin B_{12}, in the form of methylcobalamin, is the essential cofactor of methionine synthase and facilitates the transfer of methyl groups from 5-MeTHF to homocysteine to form methionine. As such, low serum B_{12} concentrations (10- 100 pM) have been associated with plasma homocysteine concentrations of up to 23 μM (Brattstrom *et al.*, 1988).

Besides being a biomarker for folate and vitamin B_{12} status, homocysteine has gained a great deal of research interest because of its association with various disease states. Following the pioneering work by McCully in the late nineteen sixties, homocysteine is now considered a significant risk factor for cardiovascular disease (McCully, 1969). Even more interesting was the finding of the OPTIMA (Oxford Project to Investigate Memory and Ageing) project, which showed significant positive correlations between serum homocysteine concentrations and the frequency of histologically confirmed Alzheimer's disease (Clarke *et al.*, 1998). Furthermore, people in the highest tertile of serum homocysteine concentrations, exhibited accelerated degeneration of their medial temporal lobe, the region of the brain associated with dementia, compared to those in the lower tertiles over the study period (Clarke *et al.*, 1998). Because of the fact that both folate and vitamin B_{12} levels were also associated with Alzheimer's disease (in the opposite direction to homocysteine), it is difficult to dissect out the relative contribution of homocysteine to pathology. Indeed, it seems entirely possible that homocysteine could simply be a biomarker for underlying folate and vitamin B_{12} deficiency in those who develop Alzheimer's disease.

Studies have shown us that homocysteine can be cytotoxic to cells in culture (Wall *et al.*, 1980; Starkebaum and Harlan, 1986) and this could possibly be one of the mechanisms in its role in the pathology of disease. Recently the novel observation was made that homocysteine concentrations also positively correlate with micronucleus frequency, a measure of chromosome breakage (see Chapter 2) in humans (Fenech *et al.*, 1997a; 1998),

but again the question remains of whether homocysteine is simply a reflection of folate and vitamin B_{12} status.

Because it is unclear whether or not homocysteine can be directly genotoxic and thereby directly contribute to the pathology of diseases such as cancer, one of the aims of this thesis was to investigate the genotoxic potential of homocysteine in human cells under controlled folate, vitamin B_{12} and methionine conditions. This work is detailed in Chapter 3.

1.4.2 Genetic instability: Uracil, DNA breakage and hypomethylation

Uracil in DNA

It has been known for many years that folate deficiency causes damage to DNA. One of the first reports of this described how culture medium without folic acid caused an increase in the frequency of chromosome gaps and breaks (in metaphase analyses) in human lymphocytes compared to folic acid-supplemented medium (1 μg/ml or 2.26 μM) (Reidy *et al.*, 1983).

Prior to this work, Goulian and colleagues (1980) showed that treatment of human lymphoid cells with methotrexate, an inhibitor of dihydrofolate reductase, resulted in the incorporation of uracil into DNA. In their experiment, the incorporation of radiolabelled uracil ([^{3}H]dUTP) into cellular DNA was traced. DNA isolated from these cells was digested to deoxynucleosides. By doing this they were able to show that, in cells not treated with methotrexate, the radioactive fraction was thymidine. This indicated that the labelled uracil was being effectively methylated to form thymidine. In contrast, when cells were treated with methotrexate, the most radioactive fraction was the one containing uracil, implying that methotrexate inhibited the conversion of uracil to thymidine and that the uracil was subsequently being incorporated into DNA.

Reidy (1987) later provided evidence that the incorporation of uracil into DNA caused DNA breakage in a simple but highly significant experiment. He showed that culturing whole blood cells in medium devoid of folic acid resulted in a much larger number of chromosome breaks, as measured by metaphase analysis, than when cells were grown with added folic acid (1 mg/L). Furthermore, addition of deoxyuridine to the medium

increased the number of chromosome breaks in folic acid deficient cultures by approximately two-fold. This elevation in chromosome breakage due to folic acid deficiency and the presence of deoxyuridine was abolished by the addition of thymidine to the cultures.

Because 5,10-MnTHF is the methyl group donor for the conversion of dUMP to dTMP (see figure 1.2), folate deficiency causes an increase in the intracellular ratio of uracil: thymidine and thereby increases the amount of uracil that is incorporated into DNA by DNA polymerase enzymes. In fact, studies measuring the deoxynucleotide pool of Chinese hamster cells show that after 20 passages in medium without folic acid, the dUTP: dTTP ratio was greater than 4.5, compared to a ratio of 0.5 in medium with folic acid (James *et al.*, 1994). Likewise, Melnyk *et al.* (1999) report that the dUTP: dTTP ratio in folate-sensitive Chinese hamster cells (CHO-UV5) increased from <1 to approximately 11 when cultured in medium without folic acid for 11 days.

Studies that have quantitatively measured, by gas chromatography-mass spectrometry (GC-MS), the amount of uracil in DNA report values of 24,300 – 104,100 uracils/diploid cell (7 – 30 pg/μg DNA) in rat liver DNA (Blount and Ames, 1994), 62,220 – 256,200 uracils/diploid cell (17 – 70 uracils/10^6 thymidines) in primary murine erythroblasts (Koury *et al.*, 1997) and 500,000 – 4,000,000 uracils/diploid cell in human DNA from whole blood (Blount *et al.*, 1997). The data presented in Chapter 6 for primary human lymphocytes are in good agreement with the range of the first two studies above (10 – 48 pg/10 mg DNA or 34,600 – 167,400 uracils/diploid cell.

In all of the above studies, the DNA uracil-content of cells was sensitive to folic acid concentrations in culture medium/blood. For example, in the study by Blount and colleagues (1997) it was shown that folate deficient individuals (< 140 ng/ml) had eight- (P= 0.003) and nine-fold (P= 0.004) higher DNA uracil-contents in whole blood and bone marrow cells respectively. Furthermore, eight weeks of folic acid supplementation (5 mg/day) resulted in a 22-fold reduction in DNA uracil-content (3,960,000 to 186,000 uracils/diploid cell; P= 0.003) in the folate-deficient group and a 3.7-fold reduction (498,000 – 134,000; P= 0.001) in the normal-folate group. In conjunction with these changes in uracil levels, folate supplementation induced a 2.7-fold reduction (P= 0.03) in

the frequency of micronucleated reticulocytes in the folate-deficient group but not in the normal-folate group. Prior to supplementation, the micronucleus frequency in the folate deficient group was elevated 3-fold (P= 0.012) in reticulocytes and elevated 3.3-fold (P= 0.047) in erythrocytes compared to the replete group (Blount *et al.*, 1997).

Similarly, Koury and colleagues (1997) reported that primary erythroblasts taken from folate deficient mice showed a significant accumulation of uracil in DNA when cultured in folate deficient medium (5 nM), compared to control medium (6.4 μM folate), for only two days (Koury *et al.*, 1997). Furthermore, (prior to their 1997 study) Blount and Ames (1994) showed the DNA uracil-content of rat liver was significantly increased in response to methotrexate and partial hepatectomy. Methotrexate is a folate antagonist and chemotherapy agent which inhibits dihydrofolate reductase and causes a depletion of THF and subsequently 5,10-MnTHF, the methyl donor for thymidine synthesis (Rang *et al.*, 1995). Partial hepatectomy (70%) stimulates cell proliferation and therefore increases the demand for nucleotides such as thymidine.

The detection of uracil by GC-MS provides an absolute estimate of DNA-uracil content, however some researchers use a simpler semi-quantitative method for detecting changes in uracil levels. This method involves the single-cell gel electrophoresis (Comet) assay in which DNA fragmentation, in the form of a comet-like tail of cells subjected to electrophoresis, is compared between cells that have been treated with uracil-DNA glycosylase (to create single-stranded breaks in the DNA) and cells that have not. This method also clearly shows that the DNA-uracil content of various cells is sensitive to medium folic acid concentrations (Pogribny *et al.*, 1997; Duthie and Hawdon, 1998; Duthie *et al.*, 2000). One limitation common to all of these studies is the fact that only very small dose-ranges are tested, which often include supra-physiological folate concentrations.

Although it was established that uracil could be incorporated into DNA and that this was associated with chromosome breaks, it was not known how the uracil caused double stranded breakage. In an elegant experiment, Dianov and colleagues (1991) used plasmid DNA to show that the simultaneous removal of two uracil bases from opposite DNA strands could lead to double-stranded DNA breakage. They inserted a polylinker sequence

flanked by 165 bp direct repeats into the tetracycline resistance gene of pBR327, thereby inactivating it. The plasmids were constructed with two uracil residues on opposite stands (12 base pairs apart) within the polylinker sequence. It was shown that, in a cell-free system, the circular plasmid was linearised upon exposure to a crude cellular extract (containing uracil glycoslyase) more frequently when two uracils were present compared to when only one or no uracils were present. Furthermore, in a cellular system, they showed that *E.coli* containing the plasmid with two uracils were more likely to produce transformants resistant to tetracycline than cells with plasmids containing one or no uracils. This was because uracil excision caused double-stranded DNA breaks that sometimes lead to recombination between the 165 bp repeats, thus restoring the *tet* gene sequence and the cell's resistance to tetracycline.

Although uracil can pair with adenine in DNA, just as thymidine does, uracil is removed from the code by glycosylases because it can also be formed by the deamination of cytosine. If a deaminated cytosine (uracil) were allowed to persist in DNA, the uracil would pair with adenine in a successive cell division and result in the transition from G-C in the parent chromosome to A-U in one of the daughter chromosomes (Stryer, 1995). This mutagenic potential is highlighted by the fact that of eight known glycosylases, four remove uracil from the DNA (Lindahl and Wood, 1999).

Folate deficiency-induced chromosome breakage is an important health issue because the accumulation of chromosome aberrations, such as double-stranded breaks, is an established risk factor for the development of cancer (Hagmar *et al.*, 1994; Hagmar *et al.*, 1998; Bonassi *et al.*, 2000). For example, Bonassi and colleagues (2000) report that, compared to people in the lowest tertile of chromosome aberrations, people who are in the highest tertile of chromosome aberrations have a 2.35 and 2.66 fold increased risk of developing (any) cancer in Nordic and Italian cohorts, respectively.

DNA methylation

Because folate is involved in the synthesis of S-adenosyl methionine (SAM)(see figure 1.2), the universal methyl group donor for DNA methylation, folate depletion causes a reduction in SAM pools (Pogribny *et al.*, 1997), which induces global DNA hypomethylation. Folate deficiency-induced global hypomethylation has been observed in human lymphocytes (Jacob *et al.*, 1998) and in rat liver (Pogribny *et al.*, 1995) although

some rat studies do not report global DNA hypomethylation but p53 gene specific hypomethylation (Kim *et al.*, 1997).

In the study by Jacob and colleagues (1998), ten post-menopausal women were housed in a metabolic unit and underwent a five-week folate depletion period (56 μg folate/day) followed by folate repletion for a further seven weeks in which folate intake was progressively increased up to 516 μg/day. The results showed that global DNA methylation was significantly reduced due to folate depletion, as shown by a more than two-fold increase in DNA methyl acceptance ($P<0.05$), and failed to reach baseline levels before the completion of the study. Corresponding with the global DNA hypomethylation was a significant 35% increase ($P< 0.05$) in the ratio of uracil: thymidine in DNA (Jacob *et al.*, 1998).

Global DNA hypomethylation is believed to be an important risk factor for cancer (Laird and Jaenisch, 1994; Jones, 1996). Human studies have shown that DNA in colorectal carcinomas is significantly less methylated than adenomas and that normal-appearing colonic mucosa is significantly more methylated than carcinomas from the same individual (Cravo *et al.*, 1994). Furthermore, cancerous gastric tissue is reported to be significantly less methylated than in para-cancerous tissue (3 – 5 cm from cancerous margin) which, in turn, was less methylated than non-cancerous tissue from the same patient (Fang *et al.*, 1997). DNA Hypomethylation has also been shown to be greater in dysplastic/cancerous cervical tissue compared to healthy cervical tissue and also to increase with the severity of neoplastic cervical lesions (Kim *et al.*, 1994).

Experimentally induced DNA hypomethylation has been shown to cause the loss of chromosomes 1, 9, 15, 16 and Y during mitosis due to the under-condensation of peri-centromeric heterochromatin (Guttenbach and Schmid, 1994). Chromosome loss leads to aneuploidy, a recognised risk factor for cancer (Rasnick and Duesberg, 1999). Furthermore, because most genes are silenced by methylation of the promoter region, hypomethylation of specific growth-promoting genes such as proto-oncogenes may confer a selective advantage to cells, which may lead to transformation (Laird and Jaenisch, 1994).

DNA methylation is also thought to play a role in a host defence system that suppresses parasitic sequences or transposons, which make up at least 35% of the human genome (Yoder *et al.*, 1997). Transcripts of these transposons are known to be more abundant in tumour cells compared to normal cells. Hypomethylation-induced transcription of transposons is thought to initiate a series of mutagenic events that can lead to cancer (Yoder *et al.*, 1997).

Micronucleus induction

As described in Chapter 2, micronuclei (MNi) are a practical and robust measure of chromosome damage that are sensitive to the effects of radiation and micronutrient deficiencies. In collaboration with the abovementioned study by Jacob and colleagues (1998) in which the effect of folate depletion and repletion on DNA methylation and uracil-content was studied in ten women, Titenko-Holland and colleagues (1998) measured the micronucleus (MN) frequency in exfoliated buccal cells taken from these women. It was shown that the frequency of micronucleated (MNed) cells increased by approximately 30% (14.8 to 19.5 MNed cells/1000 cells) due to folate depletion and declined back to baseline levels during the repletion period. Through the use of anti-kinetochore antibody staining it was determined that this increase in MN frequency was mainly due to the loss of whole chromosomes, rather that chromosome fragments, into MNi (Titenko-Holland *et al.*, 1998).

A decade earlier, Everson and colleagues (1988) reported that of 20 individuals studied following splenectomy, one individual exhibited an erythrocyte (immature and mature) MN frequency approximately 20-fold higher than the other 19 individuals. After only eight days of folinic acid (5-formyl THF) supplementation, the volunteer's MN frequency decreased from 72 MNed cells per 1000 cells to 12 per 1000 in immature (RNA-positive) erythrocytes while declining more gradually in the mature (RNA-negative) erythrocytes (Everson *et al.*, 1988).

Studies have also repeatedly shown that MN frequency correlates negatively with plasma or red cell folate concentrations. For example, MacGregor and colleagues (1997) report that both plasma and red cell folate are inversely related to MN frequency in erythrocytes. Furthermore, data from this laboratory show the that lymphocyte MN frequency correlates negatively with plasma folate and vitamin B_{12} young adults (Fenech and Rinaldi, 1995)

and that the MN frequency is decreased by 25% in lymphocytes following supplementation with 700 mg folic acid and 7 mg vitamin B_{12} in those with an above average baseline MN frequency (Fenech *et al.*, 1998).

Although folic acid deficiency has repeatedly been shown to cause an increase in DNA-uracil content, strand breakage and global hypomethylation, many of the studies involved the detection of only a small subset of endpoints over a small or non-physiological folic acid concentration range. Human studies on folate deficiency have, to date, been small and lack detail in the characterisation of cellular response to folate deficiency. One of the aims of this project was to thoroughly characterise the cytotoxic, cytostatic and genotoxic effects of folic acid deficiency on human lymphocytes over a broad physiological dose range with a novel comprehensive *in vitro* test system. This work is described in Chapter 6.

Recently the concept was introduced that the RDI (RDA) of certain vitamins and nutrients, particularly folate and vitamin B_{12}, should be revised from levels that prevent deficiency disease (e.g. anaemia) to levels that maximise the stability of the genome and thereby reduce the risk for diseases such as cancer that are associated with a degeneration of DNA (Fenech, 2001). This work described in Chapter 6 may represent an important pre-clinical step in this process and identifies folic acid concentrations that minimise various markers of chromosome damage in human cells *in vitro*.

1.5 Methylene tetrahydrofolate reductase

Methylenetetrahydrofolate reductase (MTHFR) is the enzyme which catalyses the irreversible reduction of 5,10-MeTHF to 5-MeTHF, the predominant folate species in plasma (see Figure 1.2). The cofactor for MTHFR is flavin adenine dinucleotide (FAD), which is a metabolite of vitamin B_2 (riboflavin). A number of polymorphisms have been identified in the gene encoding MTHFR (Goyette *et al.*, 1994, 1995, 1996; Frosst *et al.*, 1995; Kluijtmans *et al.*, 1998), with the C to T base transition at position 677 of the gene (Frosst *et al.*, 1995) being the most common and researched. The allele frequency of this polymorphism ranges from 4.5% to 44.9% depending on the population studied

(Schneider *et al.*, 1998). Data from this laboratory show that approximately 10% of South Australians are homozygous for this polymorphism.

The C677T polymorphism results in an amino acid change in the catalytic domain of the enzyme and causes MTHFR to have a reduced affinity for its cofactor, FAD, compared to the wildtype variant (Guenther *et al.*, 1999). Individuals who are heterozygous (CT) and homozygous (TT) for this mutation have an *in vitro* enzyme activity of 65% and 30% of wildtypes respectively (Frosst *et al.*, 1995). Because plasma homocysteine concentrations are significantly elevated in TTs the *in vivo* enzyme activity is also assumed to be reduced compared to CCs (Frosst *et al.*, 1995; Zittoun *et al* 1998; Kluijtmans *et al.*, 1997). Interestingly, high levels of folate were shown to decrease the rate of FAD release and activity loss (Guenther *et al.*, 1999). Furthermore, Hustad and colleagues (2000), report that plasma riboflavin concentrations were negatively correlated with plasma homocysteine, but only in TTs. This indicates that high riboflavin concentrations can improve MTHFR activity, probably by increasing the availability of FAD for the enzyme (Hustad *et al.*, 2000).

Studies have shown that the TT genotype is associated with an elevated risk for cardiovascular disease (Kluijtmans *et al.*, 1997; Gallagher *et al.*, 1996), which may be linked to elevated blood homocysteine concentrations. In addition, TTs are reported to have a 2.8-fold higher risk for endometrial cancer (Esteller *et al.*, 1997), a 2.7- and 2.8-fold higher risk for Crohn's disease and ulcerative colitis respectively (Mahmud *et al.*, 1999) and a 2.6- (Hobbs *et al.*, 2000) to 3.2- (James *et al.*, 1999) fold higher risk for Down syndrome in offspring. Various reports also show that TT babies have a 1.6- (Sheilds *et al.*, 1999), 1.8- (van der Put *et al.*, 1997) or 2.2- (Christensen *et al.*, 1999) fold higher risk for neural tube defects.

In contrast to these reports of increased disease risk associated with the C677T polymorphism, various reports document that TTs have a 1.2- (Slattery *et al.*, 1999), 1.7- (Chen *et al.*, 1996) and 3.0- (Ma *et al.*, 1997) fold reduced risk for colorectal cancer (carcinoma) compared to CCs. Despite this evidence relating to carcinomas, the TT genotype does not seem to afford any protection against the formation of colorectal adenomas (Chen *et al.*, 1998; Marugame *et al.*, 2000; Ulrich *et al.*, 1999). It is suggested that the MTHFR polymorphism may only play a part in the conversion of adenoma to

carcinoma when the rapidly dividing epithelial cells have a large thymidine demand which may overwhelm supply in CCs but be efficiently met in TTs due to diversion of folate to thymidine synthesis (Chen *et al.*, 1998) as described below. In this situation, reduced MTHFR activity may serve to minimize the accumulation of DNA damage and the increase in genetic instability that is needed for tumour progression to occur. Recently, the TT genotype has also been associated with a 4.3-fold reduction in the risk for developing acute lymphocytic leukaemia (Skibola *et al.*, 1999). Interestingly, Wiemels and colleagues (2001) show that the TT genotype is also associated with a 1.9-fold reduced risk for infant lymphoblastic or myeloblastic leukaemia with *MLL* translocations but not for childhood lymphoblastic leukaemias with *TEL-AML1* fusions or hyperdiploid karyotype. This is interesting because *TEL-AML1* is the most common translocation that occurs in acute lymphocytic leukaemia, the subtype of leukaemia for which Skibola and colleagues (1999) demonstrated a protective effect of the TT genotype.

Although the TT genotype is associated with a decreased risk for some cancers, it is clear that this effect is highly dependent on diet. Indeed, it is likely that the risk for cancer in TTs may be equal to or greater than that of CCs when dietary folate is low and alcohol intake is high. Because the reduced MTHFR activity associated with the C677T polymorphism is thought to cause a diversion of 5,10-MnTHF towards thymidine synthesis at the expense of 5-MeTHF, it is likely that, under some conditions, DNA methylation may be compromised due to a diminished availability of methyl groups. To support this theory there is already preliminary evidence to suggest that the DNA of TTs is relatively hypomethylated compared to CCs (Stern *et al.*, 2000). In practice, this means that TTs may only be protected against cancer under folate-replete conditions when thymidine synthesis is optimised. When folate or methyl supply is low, TTs may be unable to meet methyl group demands for DNA methylation and their reduced risk for cancer would be abolished due to the increased chance of hypomethylation-associated mutagenesis. Indeed, results from the above mentioned studies show that the protective effect of the polymorphism is only apparent in those who have an adequate or high folate status (Chen *et al.*, 1996; Ma *et al.*, 1997; Marugame *et al.*, 2000) and that the TTs may even be at increased risk for cancer compared to CCs when intake of folate, vitamin B_6, vitamin B_{12} or methionine are low (Ulrich *et al.*, 1999).

As mentioned above, irrespective of folate intake, there is evidence that the TT genotype increases the risk for some cancers. For example, Piyathilake and colleagues (2000) report that both CTs and TTs have an increased risk (Odds Ratio = 2.8 and 3.5 respectively; P trend= 0.03) for cervical intraepithelial neoplasia. In women who have had a child, this risk is markedly increased to 23-fold above that of nulliparous wildtypes (P= 0.02). A possible explanation for this massive increase in risk is that, during pregnancy, the demand for folate to supply methyl groups for nucleotide synthesis and *de novo* DNA methylation increases dramatically (because of the rapid formation of the foetus) and may approach the methyl delivery capacity of the folate-metabolising pathway. At this time, the possibility may exist for DNA methylation reactions being compromised, especially in TTs who already divert folate away from DNA methylation.

Esteller and colleagues (1997) also report that the T allele (CTs and TTs combined) was associated with a 2.8-fold increased risk of endometrial carcinoma (P= 0.03). Unfortunately, this study did not consider the reproductive history of its subjects as this may have significantly altered the risk associated with the T allele as seen in the above-mentioned study by Piyathilake *et al.* (2000). It seems that the C677T polymorphism not only increases the risk of female reproductive tract cancers as a recent survey of a Chinese population reports a significantly increased risk (OR = 6.8) of oesophageal squamous cell carcinomas in TTs compared to CCs, although no data on folate intake was collected (Song *et al.*, 2001).

As is evident from the differing cancer risk depending on cancer site and diet, the relationship between the MTHFR C677T polymorphism and carcinogenesis is rather complex. In general, the polymorphism seems to be protective against cancers of the colorectum when folate is adequate and a risk factor for cancers of the female reproductive tract and oesophagus irrespective of folate status. Much work is still needed to tease out the effect of the T allele in various tissues, folate states, and in combination with other polymorphisms, especially in MTHFR, methionine synthase and thymidine synthase.

It has been hypothesised that TTs are protected against some cancers because reduced MTHFR activity causes a diversion of 5,10-MnTHF towards thymidine synthesis (Blount *et al.*, 1997). This altered folate distribution is thought to minimise intracellular

dUMP:dTMP ratios, therefore minimising the chance of uracil being incorporated into DNA and thus the chance for DNA strand breakage. Consistent with this hypothesis is the finding that the red blood cells of CCs contain only 5-MeTHF polyglutamates while the RBCs of TTs also contain formylated THF polyglutamates (Bagley and Selhub, 1998). This altered folate distribution indicates that in immature RBCs, the multi-step conversion of formylated folate to 5-MeTHF involving MTHFR (see Figure 1.2) is impaired in TTs. One of the aims of this thesis was to test the hypothesis that TTs are protected against uracil incorporation into DNA relative to CCs. This work is described in Chapter 6.

1.6 Methionine Synthase

Vitamin B_{12}-dependent methionine synthase is the major enzyme responsible for the conversion of homocysteine to methionine. A reduction in methionine synthase activity, due to either dysfunctional enzyme or methylcobalamin deficiency, not only restricts the recycling of homocysteine but causes an accumulation of folate in the 5-MeTHF form, a situation known as '**folate trapping**' (see Figure 1.2). The resulting decrease in S-adenosyl methionine (SAM) concentrations enhances the 'trapping' because SAM inhibits MTHFR, and a decrease in SAM causes more 5-MnTHF to be irreversibly converted to 5-MeTHF by MTHFR (Kutzbach and Stokstad, 1971).

Because the anaesthetic gas nitrous oxide (N_2O) irreversibly oxidises methylcobalamin, the essential cofactor for methionine synthase, exposure to N_2O causes significant reductions in methylcobalamin and concomitant reductions in methionine synthase activity as tested in a human glioma cell line (Reidel *et al.*, 1999). Furthermore, exposing rats to 80/20 nitrous oxide/oxygen for eighteen hours is reported to cause a doubling of 5-MeTHF (folate trapping) and halving of THF concentrations compared to control rats, which was associated with a seven-fold reduction in methionine synthase activity of pancreatic tissue compared to control rats (Horne and Holloway, 1997).

Folate trapping can impact on genetic stability because less folate, specifically 5,10-MnTHF, is available for thymidine synthesis. As described in section 1.4.2, this situation results in the excessive incorporation of uracil into DNA. The relationship between folate

trapping and genetic stability is supported by evidence that occupational exposure of anaesthetic nurses to N_2O causes a four-fold elevation in micronucleus frequency, a measure of chromosome breakage and loss (see Chapter 2), compared to non-exposed nurses from the same hospital (Chang *et al.*, 1996).

People who have a severe methionine synthase deficiency normally suffer from pernicious/megaloblastic anaemia. Of great interest, therefore, is the report describing a person with severe methionine synthase deficiency who did not suffer from anaemia (Kvittingen *et al.*, 1997). Because the person was also homozygous for the C677T polymorphism in MTHFR (see section 1.5), it is thought that a reduced MTHFR activity prevented the trapping of folate as 5-MeTHF because of a diversion of folate to thymidine synthesis.

Many researchers believe that, because methionine synthase is responsible for the synthesis of methionine, the inability of cells to grow in the absence of methionine but with in the presence of homocysteine, may be a phenotypic expression of methionine synthase deficiency (Judde *et al.*, 1989; Fiskerstrand *et al.*, 1997). If this is the case, then it is reasonable to hypothesise that 'methionine-dependent' cells may experience some 'folate trapping' which in turn may impair thymidine synthesis as described above. In light of evidence that methionine synthase deficiency may underlie methionine-dependency it was hypothesised that methionine-dependence would be associated with an elevated level of DNA breakage and micronuclei caused by excessive incorporation of uracil into DNA. It is interesting to note that the methionine-dependence phenotype is common in cancer cells (Hoffman, 1985) and may be pre-existing in people who develop certain types of cancer (Mikol and Lipkin, 1984). One of the aims of this thesis was to investigate a) the relative prevalence of methionine-dependency in human cells, b) the relationship between methionine-dependency and micronucleus frequency and c) whether methionine dependency is associated with polymorphisms in two key folate-metabolising enzymes, namely methionine synthase and MTHFR. This work is described in Chapter 4.

1.6.1 Vitamin B_{12}

As discussed above, vitamin B_{12}, or more specifically methylcobalamin, is the cofactor for methionine synthase and facilitates the transfer of methyl groups from 5-MeTHF to homocysteine. Deficiency in vitamin B_{12} results in pernicious/megaloblastic anaemia (Shane, 1995) and also in hyperhomocysteinemia (Brattsrom *et al.*, 1988). Furthermore, plasma vitamin B_{12} concentrations have been shown to correlate negatively with homocysteine concentrations in humans (Fenech and Rinaldi, 1995; Fenech *et al.*, 1998).

Vitamin B_{12} is also required by another enzyme, methyl-malonyl CoA mutase, which requires deoxyadenosyl cobalamin. This requirement is demonstrated by results which show that the methyl-malonyl CoA mutase activity of a human glioma cell line declines in parallel to adenosyl cobalamin levels in response to nitric oxide exposure (Riedel *et al.*, 1999). This enzyme is responsible for the conversion of L-methyl-malonyl CoA to succinyl CoA. In vitamin B_{12} deficiency, methyl-malonyl CoA and its precursor proprionyl CoA accumulate and are substituted for malonyl CoA and acetyl CoA during fatty acid synthesis, respectively. This ultimately results in the incorporation of defective fatty acids, which have an odd and branched chains, into the myelin sheath that covers nerve axons and causes neuropathy (Savage and Lindenbaum, 1995).

As described above (section 1.6), reduced methionine synthase activity due to inactivation or deficiency of vitamin B_{12} causes the accumulation of folate in the form of 5-MeTHF. Hoffbrand and Jackson (1993) confirm this by showing that the DNA synthesis defect (as measured by the suppression of ^{3}H-thymidine incorporation into DNA by uracil) in bone marrow taken from people with anaemia caused by vitamin B_{12} deficiency is corrected by the addition of tetrahydrofolate. This result also highlights why caution should be used when treating anaemia. Because anaemia can be corrected by folic acid supplementation regardless of whether it is caused by deficiency in vitamin B_{12} or folate, vitamin B_{12} deficiency can be 'masked' in terms of haematological symptoms by folate supplementation while the neuropathy will proceed to develop due to a continued vitamin B_{12} deficiency (Savage and Lindenbaum, 1995).

The relationship between folate trapping and DNA damage is described above (section 1.6) with reference to studies describing the effects of nitrous oxide-induced micronucleus

formation (Chang *et al.*, 1996). In support of these results, it has been repeatedly shown that plasma vitamin B_{12} concentrations correlate negatively with lymphocyte micronucleus frequency in humans (Fenech and Rinaldi, 1995; Fenech *et al.*, 1997a; Fenech *et al.*, 1998). This data on micronuclei, a biomarker for chromosome damage- the accumulation of which is a risk factor for cancer (see Chapter 2), fits well with the findings that plasma B_{12} concentrations are, on average, lower in women with breast cancer than controls (Wu *et al.*, 1999).

1.7 Vitamin B_6

Vitamin B_6 is the cofactor for a number of enzymes, here however, it is its role as a cofactor for cystathione β-synthase and serine hydroxymethylatransferase that will be focussed on. Vitamin B_6 exists in a number of different forms, namely pyridoxal 5'-phosphate (PLP), 4-pyridoxic acid, pyridoxamine phosphate, pyridoxal, pyridoxine and pyridoxamine with PLP being the biologically active and most abundant form found in plasma (Kang-Yoon and Kirksey, 1992).

Approximately half of the homocysteine produced in the body is converted to cytathionine by vitamin B_6-dependent cystathione β-synthase while the other half is recycled back to methionine (Green and Jacobsen, 1995). The importance of vitamin B_6 in homocysteine metabolism is demonstrated by a report that people in the highest quartile of vitamin B_6 levels had significantly lower plasma homocysteine levels (2.36 μM less) than those in the lowest quartile.

Vitamin B_6-dependent serine hydroxymethylatransferase converts THF and serine to 5,10-MnTHF and glycine. It is thought that because vitamin B_6 is important in producing the folate required for thymidine synthesis, vitamin B_6 deficiency may cause a depletion of 5,10-MnTHF which inturn may promote uracil incorporation into DNA (Ames, 2001). To date, the only evidence that vitamin B_6 deficiency is related to genetic instability is that low plasma vitamin B_6 concentrations are associated with an increased risk for lung (Hartman *et al.*, 2001) and prostate (Key *et al.*, 1997) cancer. Chapter 5 describes a series of experiments that attempted to provide further information on whether vitamin B_6 status influences genetic stability.

1.8 Aims and hypotheses of this thesis

As described in this chapter, folate deficiency or defects in folate metabolism have been shown to cause damage to our DNA. The aim of the work described in this thesis was to investigate this relationship and more specifically to:

1. test the hypothesis that homocysteine is genotoxic to human lymphocytes.

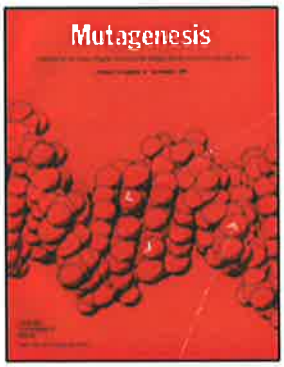
2. test the hypothesis that the methionine-dependence phenotype is related to increased MN frequencies, can be explained in part by common polymorphisms in MTHFR and MS and is a risk factor for genetic instability in human lymphocytes.

3. test the hypothesis that the MTHFR C677T polymorphism protects against folic acid-deficiency induced incorporation of uracil into DNA and chromosome damage in human lymphocytes.

4. characterise thoroughly the effect of folic acid deficiency, over a broad physiological concentration range, on DNA uracil-content and various markers of chromosome and cellular health in human lymphocytes with the aim to identify optimal concentrations for the prevention of genomic instability.

5. improve the cytokinesis-block micronucleus assay and validate new biomarkers of folate deficiency to produce a practical, versatile and comprehensive test for the genotoxic and cytotoxic effects of folic acid deficiency.

1.9 Publications arising from this thesis



Crott,J.W. and Fenech,M. (2001) Preliminary study of the genotoxic potential of homocysteine in human lymphocytes *in vitro*. *Mutagenesis*, **16(3)**, 213-217.

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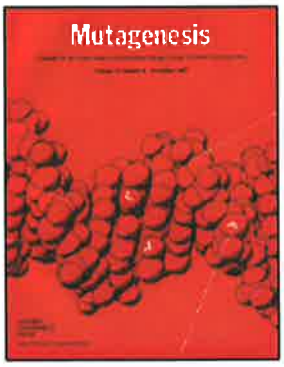

Crott,J., Thomas,P. and Fenech,M. (2001) Normal human lymphocytes exhibit a wide range of methionine-dependency which is related to altered cell division but not micronucleus frequency. *Mutagenesis*, **16(4)**, pp 317-322.

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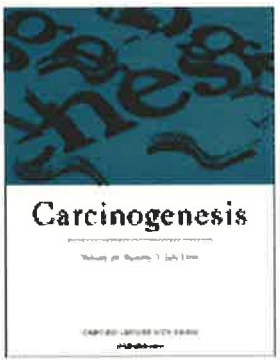
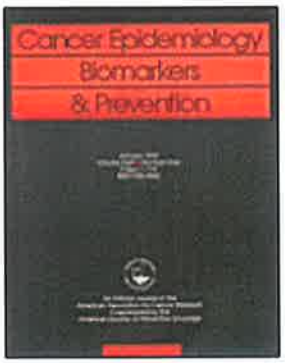


Crott,JW., Mashiyama,S.T. Ames,B.N. and Fenech,M.F. (2001) MTHFR C677T polymorphism does not alter folic acid deficiency-induced uracil incorporation into primary human lymphocyte DNA *in vitro* **[Accelerated Paper[2]]**. *Carcinogenesis*, 22(7), 1019-1025.

Impact factor[1]: 4.118

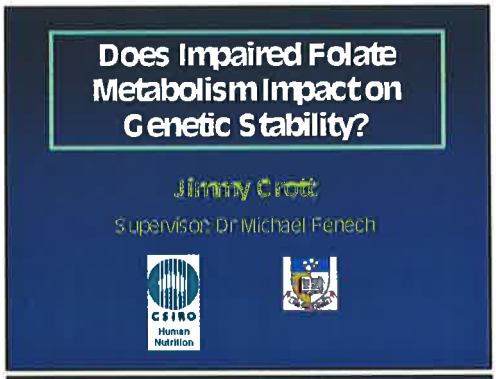


Crott,J.W. and Fenech,J.W. (2001) Folic acid deficiency increases chromosome breakage and rearrangement, gene amplification and DNA uracil-content in human lymphocytes *in vitro*: effect of the MTHFR C677T polymorphism. *Cancer Epidemiology Biomarkers and Prevention*. In Press

Impact factor[1]: 3.572

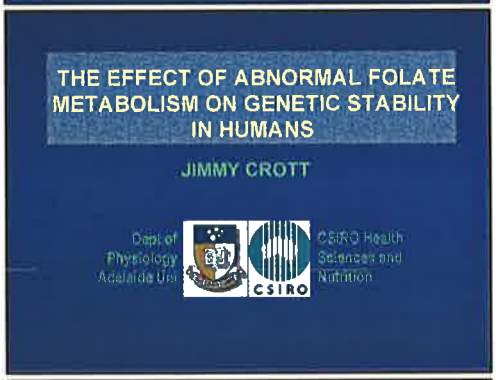
[1] Journal impact factors from 1999 ISI Journal Citation Reports.
[2] 'Accelerated Papers' are those deemed by the journal's editors to be "particularly timely and important". These papers are published in approximately half the time of a normal paper and appear at the front of the journal.

1.10 Presentations arising from this thesis



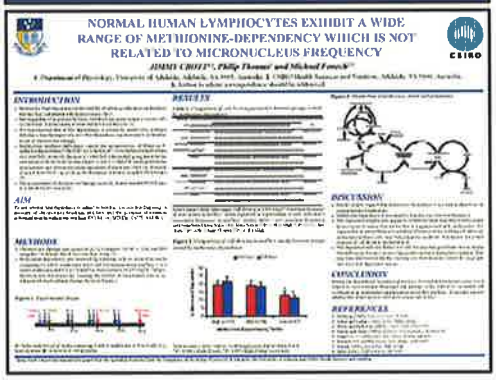
Jimmy Crott. Does impaired folate metabolism impact on Genetic stability? Oral presentation.

- Adelaide University, Dept. of Physiology seminar series. SA. AUS. 18 March 1999.



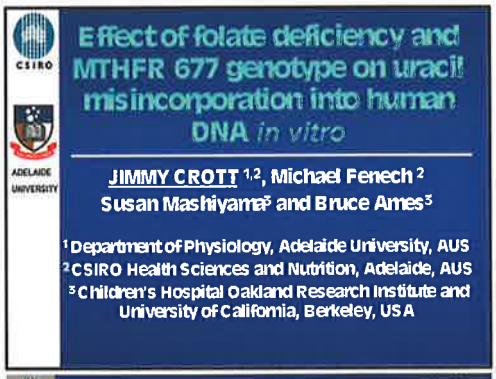
Jimmy Crott. The effect of abnormal folate metabolism on genetic stability in humans. Oral presentation.

- Adelaide University, Dept. of Physiology seminar series. SA, AUS. 21 July 2000.
- CSIRO Health Sciences and Nutrition seminar series. Adelaide. SA, AUS. 2 August 2000



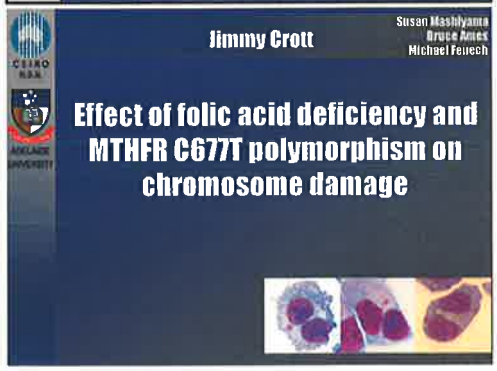
Jimmy Crott, Philip Thomas and Michael Fenech. Normal human lymphocytes exhibit a wide range of methionine-dependence which is not related to micronucleus frequency. Poster presentation.

- 7th International Conference on Mechanisms of Antimutagenesis and Anticarcinogenesis. Grand Rapids, MI, USA. 23-27 September 2000.
- Mutagenesis and Experimental Pathology Society of Australia conference. Mt Buller, VIC, AUS. 7-10 December 2000.



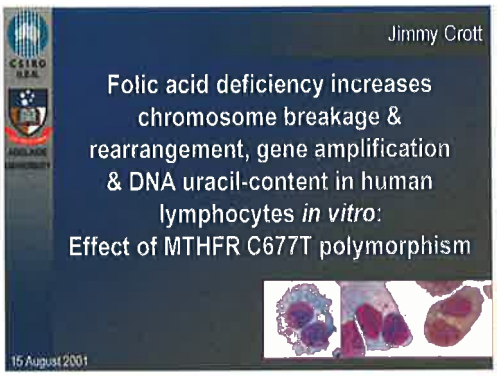
Jimmy Crott, Michael Fenech, Susan Mashiyama and Bruce Ames. Effect of folate deficiency and MTHFR 677 genotype on uracil misincorporation into human DNA *in vitro*. Oral Presentation.

- Mutagenesis and Experimental Pathology Society of Australia conference. Mt Buller, VIC, AUS. 7-10 December 2000.



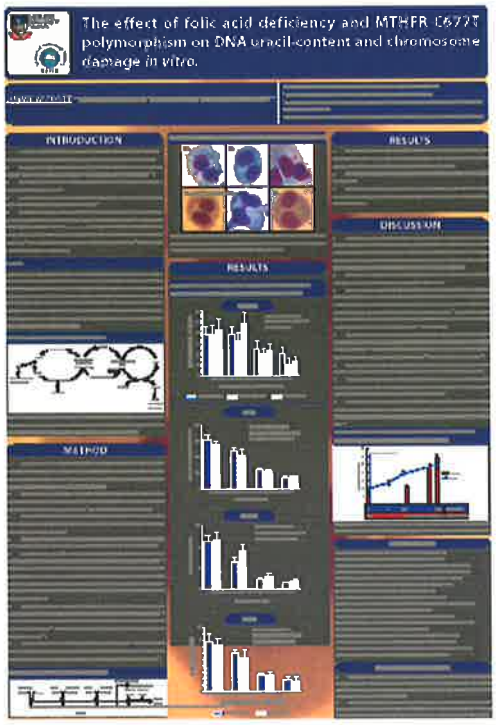
Jimmy Crott, Susan Mashiyama, Bruce Ames and Michael Fenech. Effect of folic acid deficiency and MTHFR C677T polymorphism on chromosome damage. Oral presentation.

- CSIRO Health Sciences and Nutrition seminar series. Adelaide, SA, AUS. 24 May 2001.



Jimmy Crott. Folic acid deficiency increases chromosome breakage and rearrangement, gene amplification & DNA uracil-content in human lymphocytes *in vitro*: Effect of MTHFR C667T polymorphism. Final PhD oral presentation.

- Adelaide University, Dept. of Physiology seminar series. SA. AUS. 15 August 2001.



Jimmy Crott, Susan Mashiyama, Bruce Ames and Michael Fenech. The effect of folic acid deficiency and MTHFR C677T polymorphism on DNA uracil-content and chromosome damage *in vitro*. Poster presentation.

- 8th International Conference on Environmental Mutagens. Shizuoka, Japan. 21-26 October 2001.

Chapter 2: General Method. The Cytokinesis-Block Micronucleus Assay

2.1 Introduction

In this chapter, the use of micronuclei (MNi) as a marker for chromosome damage will be discussed. The accumulation of chromosome damage over time is deleterious to health because it is thought to be involved in the aetiology of cancer and other diseases of old age such as Alzheimer's disease (Holliday, 1998; Fenech, 1998). As described in the previous chapter, folate deficiency is known to cause chromosome damage.

The experiments described in this thesis utilised a modified cytokinesis-block micronucleus (CBMN) assay to thoroughly classify the genotoxic effects of folic acid deficiency. As well as describing the method, applications and theory behind the CBMN assay, the evolution of the assay including the discovery of MNi, will be discussed in this chapter.

2.1.1 Micronuclei and micronucleus assays: history and evolution

Micronuclei (MNi) are small nuclear bodies that have been lost or extruded from the nucleus of a cell. These small bodies were first observed in the cytoplasm of erythrocytes in the late 1800s and early 1900s. First known as "fragment of nuclear material" by Howell and "corpuscules interglobularies" by Jolly, haematologists term these structures 'Howell-Jolly bodies' after these pioneering scientists who had realised that these MNi must have been of a nuclear origin (Muller and Streffer, 1994).

Early research into micronucleus (MN) induction was commonly conducted in root cells of the broad bean (*Vicia Faba*) (Thoday 1951; Evans *et al.*, 1957), with Evans and colleagues (1959) discovering that that gamma rays and neutrons were effective and dose-dependent inducers of MNi these cells. At this stage it was assumed that MNi resulted from acentric chromosome fragments, which are unable to travel to the spindle poles during mitosis and are thus excluded from the daughter nuclei when the nuclear envelope is formed (Evans, 1997).

The potential for using MNi as an assay system for genotoxic agents was realised in the nineteen seventies by Boller and Schmid, who coined the term 'micronucleus test' (1970 [in German]; cited Muller and Streffer, 1994) and Heddle (1973), both studying the induction of MNi in bone marrow erythrocytes. A major advance in the use of MNi as a measure of genetic damage was the introduction of peripheral blood lymphocytes, a cell type that can be readily obtained from volunteers, as another cellular system for MN expression (Countryman and Heddle, 1976).

Because MNi are formed when chromosome fragments or whole chromosomes lag behind at anaphase it became clear that MNi could only be expressed in dividing cell populations (Fenech, 2000b). This meant that the MN assay at that stage was a relatively imprecise measure of chromosome damage because cell division frequency may vary considerably between cultures (Fenech, 2000b). In other words, the micronucleus index of a cell culture will be very low if the frequency of cell division is low, even if MN are produced in every dividing cell. To overcome this problem of kinetics, Fenech and Morley (1985) introduced the cytokinesis-block modification to the lymphocyte MN assay whereby an actin polymerisation-inhibitor, cytochalasin-B, is added to cell cultures after anaphase in order to inhibit cytokinesis and block once-divided cells in the readily identifiable binucleated phase. The assay was now termed the 'cytokinesis-block micronucleus' (CBMN) assay. This modification revolutionised the MN assay, which is now used in the CBMN format in many laboratories around the world, over 40 of which are involved in the 'HUMN project', an international collaboration on the MN frequency in human populations (see: http://ehs.sph.berkeley.edu/holland/humn /index.html).

Because MNi may be formed due to the loss of whole chromosomes or chromosome fragments from the main nuclei, it is not possible to know the actual content of MNi using the CBMN assay in its basic format. Through the use of fluorescently labelled antibodies directed at the kinetochore proteins or fluorescently-labelled centromere-specific DNA probes, it is possible to determine whether or not MNi contain whole chromosomes (Fenech and Morley, 1989; Eastmond and Tucker, 1989). The appearance of whole chromosomes in MNi implies that one or both of the daughter nuclei are aneuploid, a situation associated with increased cancer risk (Rasnick and Duesberg, 1999). Furthermore, using these techniques it is possible to detect aneuploidy or an abnormal

distribution of chromosomes (or fluorescent signals) between the two daughter nuclei in a binucleated cell (Schuler *et al.*, 1997). In addition to targeting DNA probes to centromeric regions of the chromosomes, it is possible to direct probes at sequences that are unique to a certain chromosome. For example, using a peri-centromeric chromosome 21-specific DNA probe, Shi and colleagues (2000) showed that there was significant age-related increase in chromosome 21 nondisjunction in both males and females. Furthermore, Guttenbach and Schmid (1994) used chromosome specific probes to show that chromosomes 1, 9, 15, 16 and Y were frequently lost into MNi in response to treatment with 5-azacytidine, a chemical which causes the hypomethylation of DNA, including centromeric sequences, and under-condensation of heterochromatin.

In the 1990s, it also became apparent that the CBMN assay was relatively insensitive to genotoxic agents that predominantly induce base lesions, such UV radiation and methylating agents, because DNA lesions induced by these agents are normally repaired by base excision repair prior to replicative DNA synthesis and do not result in the formation of double-stranded DNA breaks (DSBs). In an effort to detect this type of damage, Fenech and Neville (1992) introduced the ARA modification to the CBMN assay. In this protocol, cytosine arabinoside is added to cultures to inhibit the DNA polymerisation or gap-filling step in excision repair. Consequently these excision sites are converted to single-stranded DNA breaks and then to DSBs during replication. These DSBs result in the formation of acentric chromosome fragments, which lag behind at the cell's equator during anaphase and are packaged into MNi. This protocol resulted in at least a 10-fold increase in the micronucleus frequency when applied to genotoxic agents that predominantly induce excision-repairable DNA lesions.

Despite its relative insensitivity to base lesion-inducing treatments, the standard CBMN assay remains a robust measure of the net effect of a genotoxic challenge. Current developments focus on utilising all of the information on the microscope slides to give a comprehensive picture of the genotoxic, cytotoxic and cytostatic effects of a cellular insult. For example, this laboratory has developed criteria for the scoring of apoptotic and necrotic cells on the CBMN slides (Crott and Fenech, 1999; Fenech *et al.*, 1999a). These endpoints have already been shown to be sensitive to hydrogen peroxide challenge (Crott and Fenech, 1999; Fenech *et al.*, 1999a). The incorporation of these endpoints is thought to be extremely important because the inhibition of apoptosis may allow damaged cells to

survive and proceed through replication with highly damaged or unstable DNA, a potentially carcinogenic situation. Moreover, necrosis is thought to stimulate compensative cell division and thereby may increase the risk for replication of damaged cells. It is also important to note that apoptosis and necrosis may affect the MN index because it is possible that a cell which has experienced significant DNA damage may undergo either apoptosis or necrosis instead of dividing and expressing the DNA damage as a MN (see section 2.1.3).

More recently, researchers in this laboratory have included two additional measures of chromosome damage in the CBMN assay, namely nuclear buds (see Figure 2.3.B) and nucleoplasmic bridges (see Figure 2.3.C). Nucleoplasmic bridges (NPB) are a continuous link between two nuclei in a binucleated cell and are thought to be the result of dicentric chromosomes that have been pulled to opposite poles of the cell during anaphase (Fenech, 2000a). In addition, during this project I noticed that long-term cultures with high MN frequencies (in response to folic acid deficiency) consistently tended to exhibit a large number of nuclear buds. Nuclear buds resemble MNi joined to a normal nucleus via a thin nucleoplasmic connection and may be formed when amplified DNA is extruded from the nucleus (Shimizu *et al.*, 1998; 2000). Results from this project have shown that the induction of both NPBs and nuclear buds in primary human lymphocytes is remarkably and significantly sensitive to folic acid deficiency *in vitro* (see Chapter 6).

Recent efforts have also been made to standardise the CBMN protocol across laboratories, especially in regard to slide preparation and scoring. This has been one of the main directions of the HUMN project, which also aims to find a correlation between baseline micronucleus frequencies and cancer incidence in humans (Fenech *et al.*, 1999b). It is already established that the accumulation of chromosome aberrations, such as double-stranded DNA breaks, is a risk factor for cancer (Bonassi *et al.*, 2000; Hagmar *et al.*, 1994; Hagmar *et al.*, 1998), however it is yet to be established whether the MN index correlates with cancer risk. In support of this hypothesised relationship is evidence that MNi are a good predictor of chromosomal aberrations (Norman *et al.*, 1985). The results of Duffaud and colleagues (1997), which show that the average MN frequency (micronucleated binucleates per 1000 binucleates) of cancer patients (18.7 ± 16.8) was double that of age- and sex-matched healthy subjects (9.8 ± 4.4) ($n= 54$, $P< 0.0001$) also

provide preliminary evidence in support of the hypothesis that MN frequency is related to cancer risk.

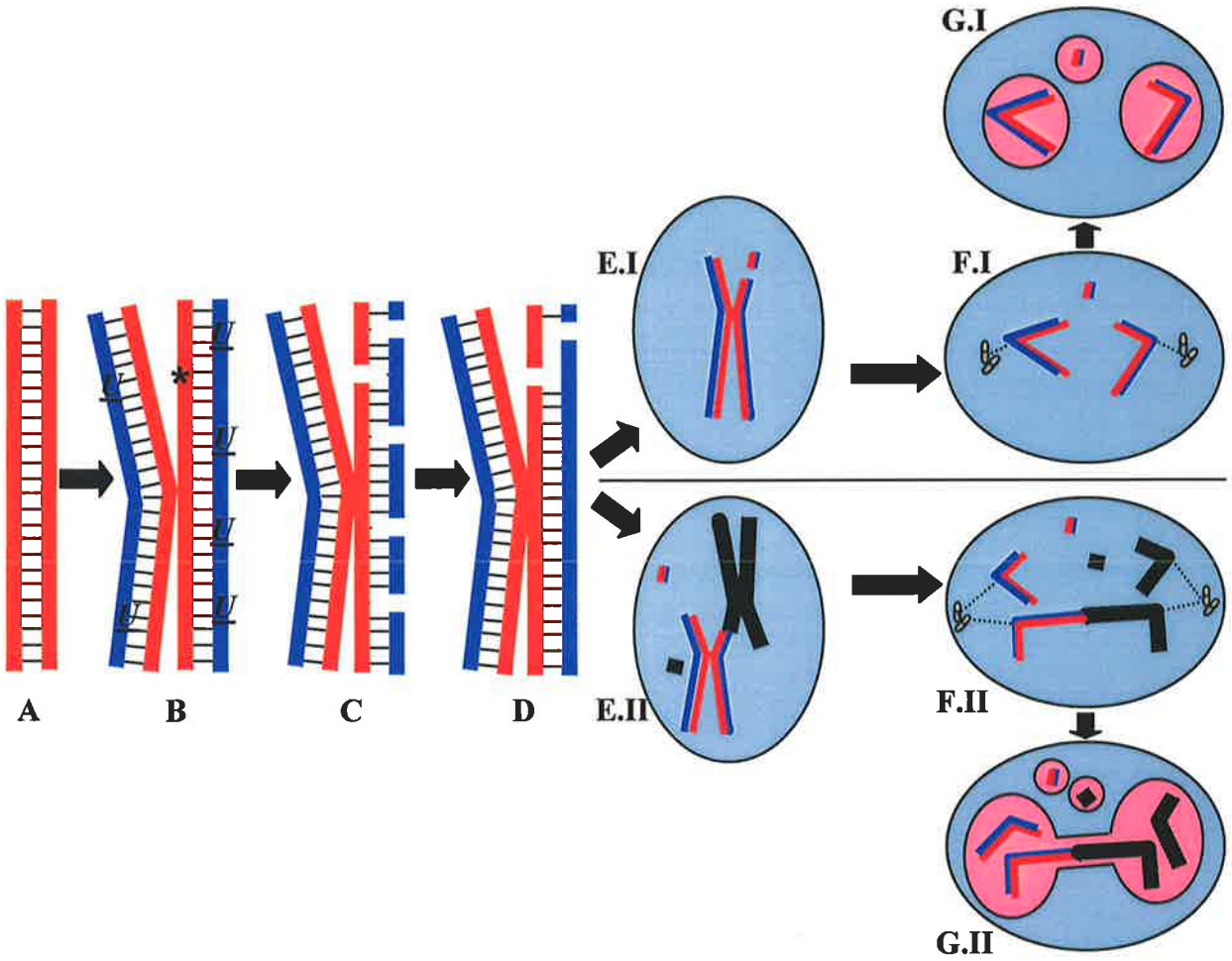
2.1.2 Mechanisms of micronucleus formation

Micronuclei form when acentric chromosome fragments or whole chromosomes with damaged centromeric regions or kinetochore proteins are not drawn to the poles of a cell during anaphase of mitosis. These fragments and/or whole chromosomes remain at the equator of the dividing cell and may be packaged into MNi during telophase when the nuclear membrane of the two main nuclei is also formed (Fenech, 2000a) (see Figure 2.1). There is also evidence that MNi are formed when damaged DNA is extruded from the nucleus in a process involving Rad51-recombination protein (Haaf *et al.*, 1999). Furthermore, gene amplification products are reportedly extruded into nuclear buds (Shimizu *et al.*, 1998; 2000), which probably break away from the main nucleus to form MNi. This event is described and discussed thoroughly in Chapter 6. In the CBMN assay, MNi are observed in binucleated cells that have been inhibited from undergoing cytokinesis (see Figure 2.3 A).

Double-stranded DNA breaks and MNi are induced by X-irradiation and as little as 5-rad of X-rays, approximately the dose received from a chest X-ray can be detected using the CBMN assay (Fenech, 1986). X-rays induce single- and double-stranded DNA breaks. Un-repaired single stranded breaks may be converted to DSBs during S-phase of mitosis when the DNA is replicated (Fenech, 2000a).

In addition to facilitating the formation of MNi, the fusion of two chromosomes that have both undergone DSB results in the formation of dicentric chromosomes. The centromeres of dicentric chromosomes may be drawn to opposite poles of the cell during anaphase, resulting in a connection spanning between the two newly forming nuclei (see Figure 2.1). When the nuclear envelope is formed the membrane also surrounds the dicentric chromosome and a nucleoplasmic bridge (NPB) is formed. These NPBs are clearly visible in cytokinesis-blocked binucleates (see Figure 2.3 C) but probably break during the process of cytokinesis. Figure 2.1 shows that the incorporation of uracil into DNA during replicative synthesis may result in the formation of DSBs and that DSBs are involved in the formation of both MNi and NPBs.

Figure 2.1 Mechanism of micronucleus and nucleoplasmic bridge formation.



When DNA is replicated (A - B) under conditions of low folate, uracil is incorporated into new DNA strands (Blount *et al.*, 1997) (B). During post-replicative DNA repair, these uracils are removed (C). At the same time, uracils from a previous division and damaged bases such as 8-hydroxy guanosine (*) are removed from the parent strand (C). If two bases, within 12 bp, are excised simultaneously from opposite strands a double-stranded DNA break may occur (Dianov *et al.*, 1991) (D). Acentric chromosome fragments (E.I) lag behind at anaphase (F.I) and are packaged into MNi (G.I). If two broken chromosomes fuse (E.II), the resulting dicentric chromatid may be drawn to opposite poles during anaphase (F.II), resulting in the formation of a nucleoplasmic bridge (E.II).

2.1.3 Cellular pathways in the CBMN system

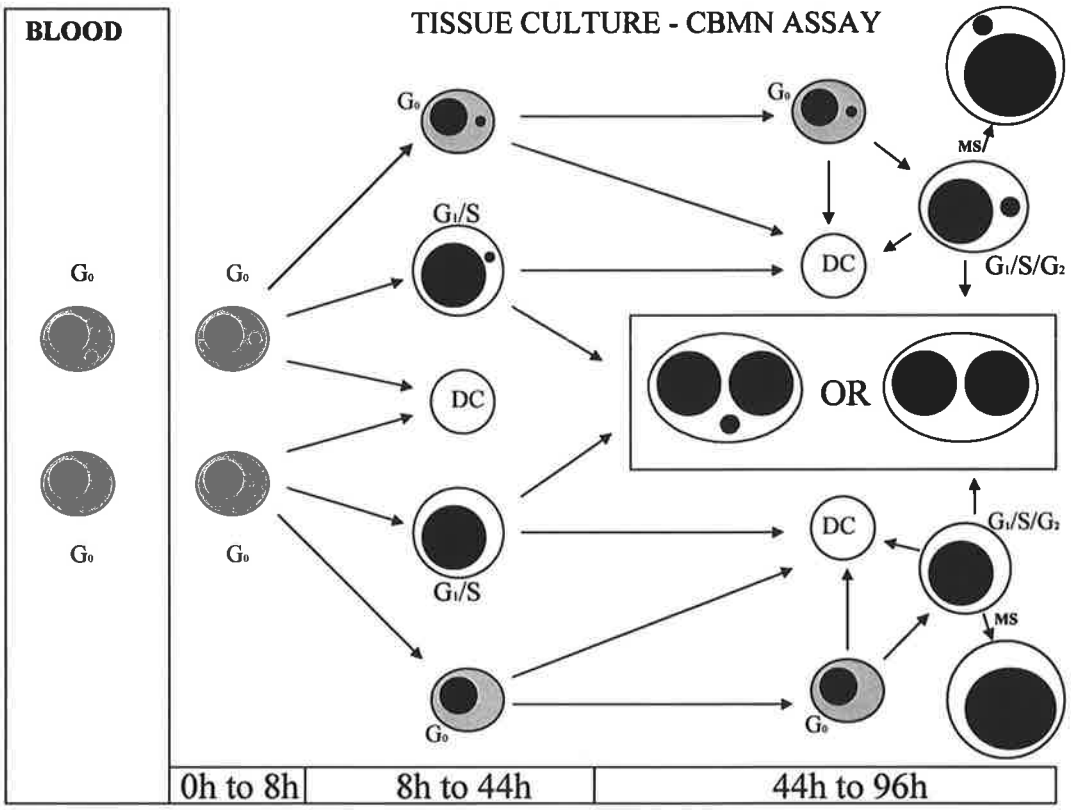
A cell can follow a number of pathways in the CBMN assay and the progression of cells along these different paths is likely to affect the observed MN frequency in binucleated cells in CBMN cultures. When lymphocytes enter the CBMN system they are predominantly in the G_0 phase of the cell cycle. These cells are stimulated to divide by the addition of a mitogen and may be exposed to certain challenges (e.g. radiation, hydrogen peroxide and nutrient deficiency). Cells may then progress through G_1 to the G_1/S cell cycle checkpoint. At this stage, there are three options available to the cell; 1) progress though to S-phase and replicate DNA, 2) undergo pre-replicative DNA repair if DNA is damaged and then enter S-phase or 3) undergo cell death via apoptosis or necrosis. Following S-phase, cells pass through another cell cycle checkpoint at the S/G_2 transition when the same three options are available to the cell (Kirsch-Volders and Fenech, 2001). It seems likely that treatments which directly induce DNA breakage or base lesions would induce pre-replicative repair in order to prevent a damaged template being copied and the damage being propagated in the daughter strands. In contrast, conditions such as folate deficiency might be expected to induce post-replicative repair because it is the actual process of DNA synthesis/replication that introduces the incorrect base (uracil) into daughter strands when the template may be free of damage. Finally, a cell that has passed through both cell cycle checkpoints will progress through anaphase into telophase and be blocked in the binucleated phase in the CBMN assay. It is the binucleated cells that are scored for the presence of MNi, NPBs and buds.

Similar to the way in which low cell division rates may have led to an underestimation of genetic damage prior to the introduction of the cytokinesis-block modification, death of damaged cells that otherwise may have expressed MNi would also contribute to a lowering of the MN frequency of cultures. In fact, the frequency of apoptosis has been shown to correlate negatively with MN frequency (Crott and Fenech, 1999).

Furthermore, cells can also replicate their DNA and exit mitosis after S-phase without undergoing normal mitotic chromatid segregation. This process, known as mitotic slippage, can be induced by exposure to spindle inhibitors (eg colchicine and taxol) and results in the formation of 4N cells. Because these cells bypass the segregation of

chromatids during anaphase and therefore the opportunity for chromosomes or chromosome fragments to lag behind, it is hypothesised that mitotic slippage may preclude the expression of MNi even if chromosome breaks are present (see Figure 2.2) (Kirsch-Volders and Fenech, 2001).

Figure 2.2 Cell fate in the CBMN system



Reproduced from Kirsch-Volders and Fenech, 2001. DC = dead cell due to necrosis or apoptosis. MS = mitotic slippage. $G_0/G_1/G_2/S$, stages of the cell cycle. Cells with a grey cytoplasm are non-dividing cells. Cells with a white cytoplasm represent cells that are actively undergoing nuclear division at the designated cell cycle stage.

2.1.4 Applications of the CBMN assay

Micronuclei are the result of chromosome aberrations and are therefore used as a method for assessing DNA damage, either baseline levels or in response to a genotoxic challenge such as radiation exposure. For example, the CBMN assay has been used to study the levels of genetic damage in children from Belarus who were exposed to radionuclides from the Chernobyl nuclear disaster (Fenech *et al.*, 1997b). It was shown that children

from Yazvenki, a highly radionuclide-contaminated town, had a MN frequency approximately four-fold higher than the relatively non-contaminated city of Minsk ($P< 0.01$).

The CBMN assay is also being applied in an effort to identify people who may be at an elevated risk for certain types of cancer. More specifically, the radiation-induced MN frequency is being studied as a tool for identifying cancer-prone individuals because radiosensitivity, in terms of chromosome damage, is though to be caused by defective DNA repair (Roberts *et al.*, 1999). Early studies on radiosensitivity were performed using chromosome aberration analysis of metaphase lymphocytes following irradiation in G_2 of the cell cycle. In these studies it was found that 40% of patients with breast cancer exhibited an abnormally high number of chromosome aberrations in response to X-irradiation (i.e. were radiosensitive) in comparison to only 6% of healthy controls (Scott *et al.*, 1994). These results were later reproduced with the CBMN assay, when it was shown that 30% of breast cancer patients were radiosensitive compared to 10% of healthy controls (Burrill *et al.*, 2000). Furthermore this radiosensitivity appeared to be heritable because 22 first-degree relatives of the cancer patients had an average MN frequency significantly higher than 68 controls (Burrill *et al.*, 2000).

In attempt to identify the genes that are involved in heritable radiosensitivity, Rothfub and colleagues (2000) showed that women who carry mutations in the breast cancer gene *BRCA1* were significantly more radiosensitive than healthy controls and family members without *BRCA1* mutations. This result suggests that the radiation-induced MN frequency may be an effective method of identifying carriers of a *BRCA1* mutation in families with familial breast and ovarian cancer. In other words, these results suggest that people who show an abnormally high radiation-induced MN frequency should be regularly screened because they may be at an elevated risk for developing breast cancer.

Apart from its applications in biological dosimetry and screening, the CBMN assay is a useful tool in determining the effect of nutritional status on levels of genetic damage. Indeed, studies have already shown that baseline MN frequencies are sensitive to blood concentrations of vitamin C (Fenech and Rinaldi, 1995), vitamin B_{12} (Fenech and Rinald, 1995; Fenech *et al.*, 1997a; Fenech *et al.*, 1998), folate (Fenech *et al.*, 1998) and homocysteine (Fenech *et al.*, 1998). In addition, *in vitro* CBMN studies such as these are

important in helping us understand how nutrient concentrations affect genetic stability and may prove to be a useful pre-clinical step in determining the optimal concentrations of nutrients needed to minimise levels of chromosome damage in cells.

2.2 Method

2.2.1 Blood Collection and lymphocyte isolation

1. Venous blood was drawn from fasted (over-night) volunteers using evacuated lithium heparin-treated 9 ml tubes (Greiner Labortechnik, Kresmunster, Austria). The tubes were inverted several times to ensure thorough mixing of the anticoagulant. Normally, 9 ml of blood taken from a healthy individual yielded approximately 5 - 10 x 10^6 lymphocytes depending on the donor and the efficiency of cell isolation.

The remainder of the lymphocyte isolation and culture set-up protocol is performed in a biohazard fume cupboard to ensure protection of the sample and the worker.

2. The blood was added to sterile 70 ml pots (Sarstedt, Adelaide, Australia) and diluted 1:1 with room-temperature sterile normal saline solution (0.89% NaCl).
3. Approximately 15 ml of Ficol-paque solution (Pharmacia Biotech, Uppsala, Sweden) was added to a sterile conical-base 50 ml tube (Becton Dickinson "Falcon", NJ, USA).
4. Approximately 30 ml of the diluted blood was then layered over the Ficol-paque taking extreme care to keep the blood in the upper layer and to not introduce blood cells to the lower layer. Red blood cells slowly penetrate the Ficol-paque layer and settle to the bottom of the tube. The tubes were then spun in a centrifuge for 25 min at 400 x g.
5. After centrifugation the solution was separated into three distinct phases. A dense dark-red suspension containing red blood cells is at the bottom. The middle layer contains the Ficol-paque solution while the upper layer contains saline and plasma. A cloudy layer of lymphocytes is located at the interface between the two upper layers. These lymphocytes were removed using a sterile glass pastuer-pipette (fitted with a rubber teat and plugged with cotton wool) into another sterile

50 ml tube. Approximately 5 ml of solution was normally removed, taking care not to disturb the lower red cell-containing layer.

6. Approximately 20 ml of Hank's balanced salt solution (HBSS). Trace Biosciences, Victoria, Australia) was then added to the lymphocyte suspension and the tube was inverted several times before being spun at 280 x g for 15 min.
7. The supernatant was then aspirated and cells are resuspended in a further 5 - 10 ml of HBSS solution and spun at 180 x g for 10 min.
8. The supernatant was again aspirated and cells were thoroughly resuspended in exactly 1 ml of HBSS.
9. Isolated lymphocytes were then counted using a Coulter Counter® (Model ZBI, Coulter Electronics, Herts, England). Fifteen µl of the cell suspension was removed into a standard 20 ml pot/vial which contained 15 ml of Isoton®II solution (Coulter Electronics, NSW, Australia) and five drops of Zapoglobin®II (Coulter Electronics, NSW, Australia) to lyse red cells. This diluted sample was counted according to manufacturers instructions.

2.2.2 Cell culture Protocol

Two scales of cell culture were used throughout the project. The small-scale CBMN assay was performed using 750 µl of culture medium in 6 ml round-bottom cell culture tubes (Becton Dickinson "Falcon", NJ, USA) while the large-scale CBMN assay was performed using 10 – 12 ml of culture medium in 25 ml culture flasks with vented lids (Sarstedt, Adelaide, Australia). The large-scale assay was used to determine the effects of longer-term nutrient deficiency on lymphocytes.

1. Lymphocyte cultures were initiated at 5 x 10^5 cells/ml. This means that either 3.75 x 10^5 cells or 5 x 10^6 lymphocytes are required, depending on whether the assay is performed in small or large-scale. Firstly, the volume of cell suspension required to deliver the chosen amount of cells was calculated. This value was subtracted from the desired culture volume in tube/flask to give the amount of medium to be added. This amount of medium was then added and warmed to 37 °C prior to the addition of the lymphocytes.
2. Mitogenesis was stimulated by the addition of phytohaemagglutinin (PHA. Murex Biotech, Kent, England) at a concentration of 22 µg/ml. Cultures were incubated at 37 °C and 5% CO_2 in a humidified incubator.

3. Cytochalasin B (cyto-B. Sigma, St. Louis, MO, USA) was added at a concentration of 4.5 μg/ml to inhibit cytokinesis in dividing cells and trap cells in a binucleated state. It is in these binucleated cells that MNi are scored. For the small-scale assay cyto-B was added 44 hr post-PHA, while in the large-scale long-term protocol eight days elapsed before a 750 μl aliquot of cell suspension was removed from the flask into a 6 ml tube and Cyto-B was added. Cells were harvested onto microscope slides approximately 24 hr later.

2.2.3 Preparation of microscope slides

1. Twenty-four to forty-eight hr after cyto-B addition, cells were harvested onto glass microscope slides. This length of time may be varied to increase the frequency of binucleated cells. For example, if cells are grown in deficient or serum-free media, they divide slower and more time is required to accumulate binucleated cells. Slide preparations with a low percentage of cells that are binucleated are difficult to score because the researcher must continuously scan across the slide, a practice which significantly increases the time required to score the slide and may induce nausea or motion sickness in the scorer. Approximately 200 μl of culture medium was removed and discarded to concentrate cells before cultures were carefully pipetted up and down to resuspend and disaggregate cells. One hundred and fifty μl of the suspension was transferred to a clean cyto-centrifuge cup, which was previously attached to a glass microscope slide using a clip, before being spun at 600 rpm in a cyto-centrifuge (Shandon Southern Products, Cheshire, England) for 5 mins.
2. After spinning, the microscope slide was removed from the assembly and allowed to air-dry for 10 mins. Cells were then fixed in methanol for approximately 10 mins. Slides were stained with Diff Quik stain (Lab Aids, NSW, Australia) without being allowed to dry. Staining involves dipping slides approximately 10 times in the red dye followed by 10 dips in the blue dye before rinsing with water. The result of this stain is similar to the Wright-Giemsa stain. Coverslips were mounted on the dry slides using DePeX mounting medium (BDH Laboratory Supplies, Poole, England).

2.2.4 Slide scoring method

CBMN slides were scored under 1000x magnification (10x objective, 100x oil immersion lens) using a conventional light microscope. Previously, laboratories have only scored CBMN slides for MNi in BNed cells and the ratio of mono- bi- and multinucleated cells. Recently this laboratory began scoring all cells on the slide to give a more comprehensive profile of the cytotoxic, cytostatic and genotoxic effects of a given treatment. Photographs of all the cell types scored for genotoxicity and cytotoxicity in this assay are provided in Figure 2.3.

All cell types on the slide including mono-, bi-, tri- and tetra-nucleated cells as well as apoptotic and necrotic cells are scored until a total of 500 cells was reached. The nuclear division index (NDI) of cell cultures was calculated using the distribution of viable cells to give an estimate of the cell division frequency. NDI is calculated according to the equation proposed by Eastmond and Tucker (1989) and is given below:

$$NDI = \{[M1 + (2 \times M2) + (3 \times M3) + (4 \times M4)]/N\},$$

where M1-M4 represents the number of cells with one to four nuclei respectively, and N is the total number of viable cells scored.

Binucleated (BNed) cells were scored until a total of 1000 is reached. BNs were scored for micronuclei, nucleoplasmic bridges and nuclear buds, which are indicative of chromosome breakage or loss, chromosome rearrangements and gene amplification respectively.

2.2.5 Cell scoring criteria

Binucleated cells

Binucleated cells (BN) were scored according to the criteria proposed by Fenech (2000a):

1. The cell membrane must be completely intact and clearly distinguishable from those of adjacent cells.
2. The two nuclei in the BNed cell must have intact nuclear membranes and be approximately equal in size, staining intensity and staining pattern.

3. Should the two nuclei be touching or overlapping, the nuclear boundaries of each nucleus must be clearly distinguishable for the cell to be classified as 'BNed'.

Micronuclei

Micronuclei (MNi) were scored according to the criteria proposed by Fenech (2000a):

1. MNi are scored in BNed cells.
2. MNi must have a diameter no larger than one-third or smaller than one-sixteenth of the diameter of the main nuclei and are stained with the same intensity.
3. MNi must not be connected to the main nuclei but may touch or overlap the main nuclei only if the boundaries of the nucleus and MN are clearly distinguishable.
4. MNi may touch but not overlap the main nuclei and the micronuclear boundary should be clearly distinguishable from the nuclear boundary.
5. MNi are non-refractile and therefore can be readily distinguished from artefact such as staining particles.

Photographs of cells containing one, eight and six MNi are shown in Figure 2.3, A I, II and III respectively. The frequency of MNi is expressed as the number of MNed BNs per 1000 BNs (MNed BNs/1000 BNs).

Nuclear Buds

Nuclear buds were scored according to the criteria developed during this project:

1. Nuclear buds are scored in BNed cells and resemble MNi that are joined to a main nucleus via a thin nucleoplasmic bridge-like structure.
2. Nuclear buds are distinct from nuclear blebs (which may simply be an earlier phase of the budding process) because the connecting bridge is clearly thinner than the diameter of the bud.
3. Although buds are attached to a nucleus, they are scored according to the same size and staining criteria described for MNi above.

Photographs of BNed cells with nuclear buds are shown in Figure 2.3, BI, II and III. The frequency of buds is expressed as the number of BNed cells with buds per 1000 BNed cells. (BNs+Bud/1000 BNs).

Nucleoplasmic bridges

Nucleoplasmic bridges (NPBs) were scored according to the following criteria proposed by Fenech (2000a):

1. NPBs are scored in BNed cells and are a continuous link between two nuclei in a cell.
2. The width of a NPB should be no greater than one-quarter of the diameter of the cell's main nuclei. NPBs commonly have a continuous narrow width but may swell in areas in response to some treatments.
3. Cells with a NPB often contain one or more MNi.

Photographs of cells with NPBs are shown in Figure 2.3, C I, II and III. Note that plate CIII shows a NPB that is swollen in two areas. It is though that this is the result of the nuclear extrusion of DNA-containing complexes, which would otherwise be removed via nuclear budding, into the NPB. The frequency of NPBs is expressed as the number of BNed cells with a NPB per 1000 BNed cells. (BNs+NPB/1000 BNs)

Apoptosis and necrosis

Apoptosis and necrosis were scored according to the criteria proposed by Crott and Fenech (1999) and Fenech *et al.* (1999a).

1. Apoptotic cells may be in one of several stages as seen in Figure 2.3, DI, II and III. These photographs represent early, intermediate and late phases of apoptosis respectively. Cells with an intact cell membrane and exhibiting chromatin condensation or intensely darker staining around the nuclear circumference (DI), pycnosis or packaging of dense nuclear material into distinct vesicles within the nuclear membrane (DII) or complete nuclear fragmentation in the cytoplasm (DIII) were classified as apoptotic.
2. Cells exhibiting a loss of well-defined nuclear boundaries, loss of cytoplasm, extensive nuclear and cytoplasmic vacuolation, complete loss of cell and/or nuclear membrane integrity without nuclear condensation and/or fragmentation were classified as necrotic. Necrotic cells are generally stained less intensely than viable and (especially) apoptotic cells. Necrotic cells are shown in Figure 2.3, EI, II and III.

Apoptosis and necrosis are expressed as a percentage of the number of cells scored for cell distribution (500) i.e. (# apoptotic or necrotic/500) x 100.

2.2.6 Age and gender adjustment of micronucleus data

It is well documented that MN frequency is positively correlated with age (Fenech and Morley, 1986) and is significantly higher in females due to the loss of an inactive X chromosome (Fenech *et al.*, 1994). The MN frequency of an individual is commonly adjusted to control for these variables.

1. The MN frequency was first corrected for gender if the mean MN frequency was significantly different between males and females. This was done by dividing the MN frequency of female volunteers by the ratio of mean female to male MN frequency.
2. If there was a significant correlation between age and MN frequency in the population studied, the MN index was adjusted to the mean volunteer age. This was done using the equation proposed by Fenech and Rinaldi (1994):

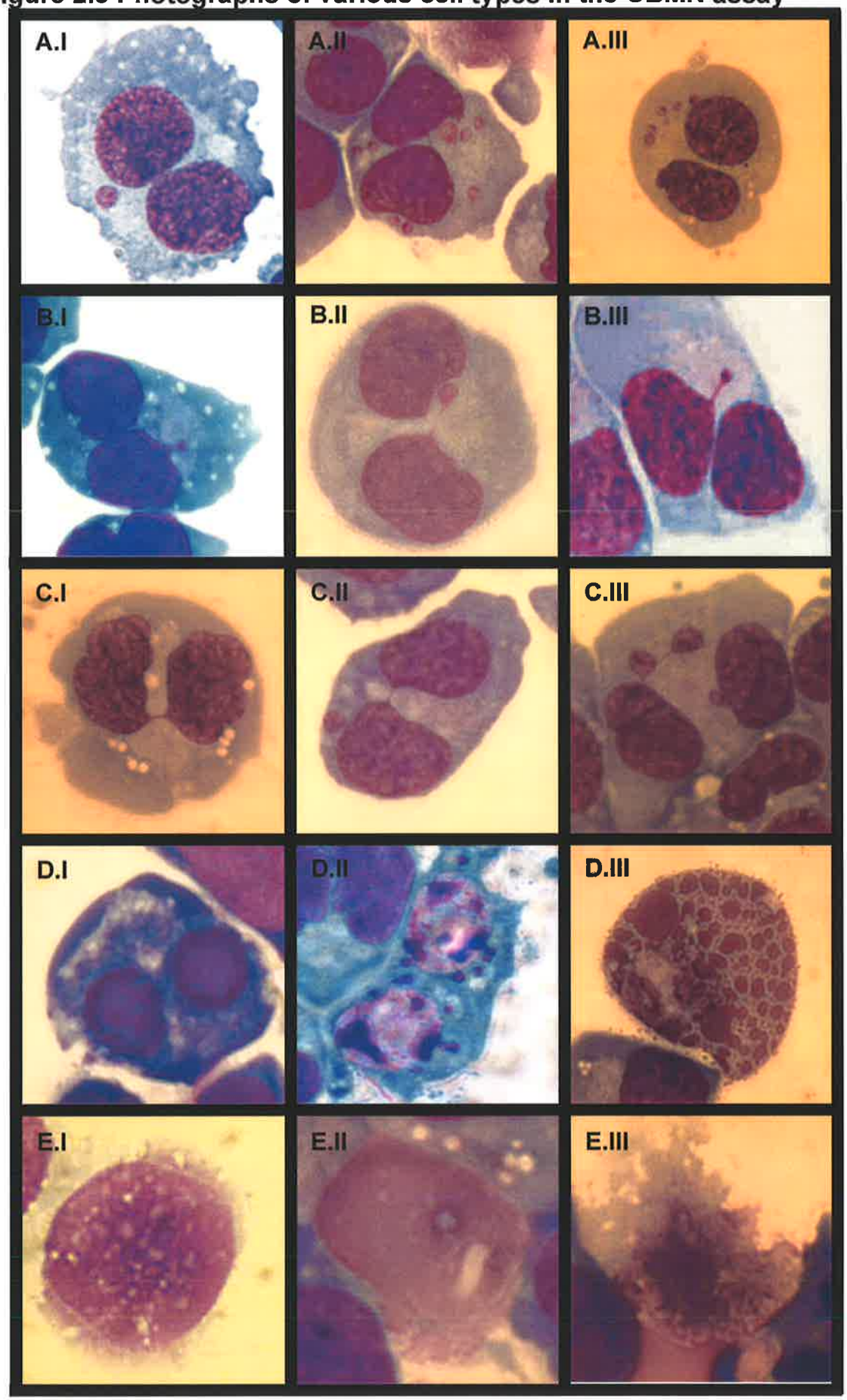
MN_{aa} = [(mean age of the group – actual age of subject) x S] + MN,

where MN_{aa} is the age-adjusted MN frequency, S is the slope of the regression line for the relationship between age and MNi and MN is the actual micronucleus frequency.

Legend to Figure 2.3 Photographs of various cell types in the CBMN assay

A, Binucleated lymphocytes with one (I), eight (II) and six (III) micronuclei; **B,** Binucleated lymphocytes with nuclear buds; **C,** Binucleated lymphocytes exhibiting a nucleoplasmic bridge (I), a nucleoplasmic bridge and a micronucleus (II) and a binucleated cell with a micronucleus and a nucleoplasmic bridge exhibiting abnormal swelling (possibly due to nuclear extrusion of DNA-containing complexes into the nucleoplasmic bridge); **D,** Lymphocytes in early (I), intermediate (II) and late stages (III) of apoptosis; **E,** Necrotic cells.

Figure 2.3 Photographs of various cell types in the CBMN assay



Chapter 3: Genotoxicity and cytotoxicity of homocysteine

AIM

To investigate the genotoxic and cytotoxic potential of high concentrations of homocysteine to primary human lymphocytes *in vitro*.

HYPOTHESIS

High concentrations of homocysteine cause cellular and genetic damage, which is expressed as an increase in the frequency of micronucleated and necrotic cells in exposed CBMN cultures.

3.1 Introduction

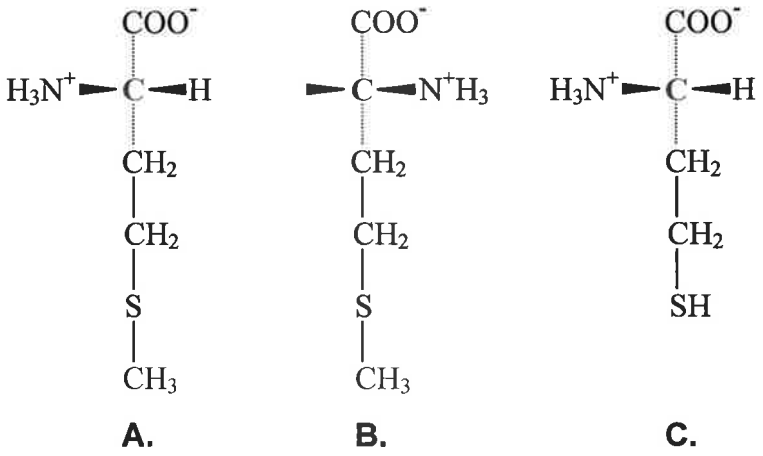
Homocysteine is the immediate precursor for the amino acid, methionine (see Figure 1.2). The structure of these two amino acids, which differ by only one methyl group, is given in Figure 3.1. In humans, plasma homocysteine concentrations may become elevated as a result of folate (Jacob *et al.*, 1998. Kang *et al.*, 1987), vitamin B_6 (Siri *et al.*, 1998) and vitamin B_{12} (Brattstrom *et al.*, 1988) deficiency. Furthermore, hyperhomocysteinemia may also be caused by mutations within the genes that encode methylene-tetrahydrofolate reductase (MTHFR) (Frosst *et al.*, 1995) and cystathione β-synthase (Mudd *et al.*, 1964).

Homocysteine is a molecule of great interest because it has been identified as a putative risk factor for arteriosclerosis (McCully, 1969), myocardial infarction (Verhoef *et al.*, 1996), peripheral arterial occlusive disease (Malinow *et al.*, 1989), subcortical vascular encephalopathy (Fassbender *et al.*, 1999), Alzheimer's disease (Clarke *et al.*, 1998) and neural tube defects (Mills *et al.*, 1995; van der Put *et al.*, 1997).

Recently the novel observation was made that homocysteine concentrations correlate positively with baseline levels of genetic damage, as measured by the cytokinesis-block micronucleus assay, in humans (Fenech *et al.*, 1997a; Fenech *et al.*, 1998). Furthermore, multiple regression analysis suggested that the effect of homocysteine was independent of

vitamin B_{12} and folate (Fenech *et al.*, 1998), both of which are known to induce chromosome damage (Reidy *et al.*, 1983; Chang *et al.*, 1996). This finding that homocysteine may induce chromosome damage at high concentrations is important because it is well established that levels of chromosome damage are significantly and positively correlated with cancer risk in humans (Hagmar *et al.*, 1994; Hagmar *et al.*, 1998; Bonassi *et al.*, 2000).

Figure 3.1 Structure of methionine and homocysteine.



A, L-methionine; **B**, D-methionine; **C**, L-homocysteine.

D- and L-isomers of amino acids (like methionine) are mirror images of each other. When the carboxyl group (COO-) is drawn above the chiral carbon (blue) and the carbon chain is drawn below the chiral carbon, L-isomers of amino acids are those where the amino group (N^+H_3) is to the left of the chiral carbon and D-isomers are those where the amino group is to the right of the chiral carbon. In these three-dimensional representations of stereoisomers, the wedge-shaped bonds project out of the plane of the page and the dashed bonds behind it. The red methyl group (CH_3) of methionine is obtained from 5-methyl tetrahydrofolate reductase in a reaction catalyzed by methionine synthase.

Studies have also shown that homocysteine promotes the expression of inducible-nitric oxide synthase (iNOS) and thereby, the production of nitric oxide (NO) in a dose-dependent fashion (Welch *et al.*, 1998; Upchurch *et al.*, 1997; Ikeda *et al.*, 1999). NO is known to inhibit methionine synthase, a key folate-metabolising enzyme (Nicolaou *et al.*,

1996). Methionine synthase inhibition causes a maldistribution of folate derivatives, which is characterized by an accumulation of 5-methyltetrahydrofolate (5-MeTHF) (Horne and Holloway, 1997). The accumulation of folate in the 5-MeTHF form ("methyl folate trapping") and subsequent lowering of 5,10-methylenetetrahydrofolate (5,10-MnTHF) promotes an increase in the ratio of dUMP to dTMP, because 5,10-MeTHF is the methyl group donor for the conversion of dUMP to dTMP, and therefore the excessive incorporation of uracil into DNA (Blount and Ames, 1995). The excision of two opposing uracils, within 12 base pairs, has been shown to cause double-stranded DNA breaks (Dianov *et al.*, 1991). Double-stranded DNA breaks or chromosome breaks result in the formation of acentric chromosome fragments, which cannot be segregated during cell division and are often packed into micronuclei. Furthermore, occupational exposure to another oxide of nitrogen, nitrous oxide (N_2O), has been reported to cause a four-fold elevation of the micronucleus frequency in lymphocytes (Chang *et al.*, 1996). N_2O is known to inactivate methionine synthase by oxidizing its cofactor, cobalamin (Drummond and Matthews, 1994).

Homocysteine has been shown to be cytotoxic to cells in culture (Wall *et al.*, 1980; Starkebaum and Harlan, 1986), which may indeed be one of the underlying causes of its postulated role in atherogenesis. However, the question remains as to whether homocysteine is genotoxic to cells or whether it is simply a biomarker of some other event that is causing DNA damage; e.g. folate deficiency which causes chromosome breakage and micronucleus (MN) formation (Titenko-Holland *et al.*, 1998). Various reports show that supraphysiological concentrations of homocysteine (100 μM to 10 mM) facilitate the generation of hydrogen peroxide (H_2O_2) *in vitro* (Wall *et al.*, 1980; Starkebaum and Harlan, 1986; Stamler *et al.*, 1993). The plasma concentration range for homocysteine in humans approximately 5 – 18 μM, depending on plasma folate concentrations (Kang *et al.*, 1987). It is thought that H_2O_2 is a product of the oxygen-dependent oxidation of homocysteine (Starkebaum and Harlan, 1986). H_2O_2 is known to induce necrosis and MN in human lymphocytes (Crott and Fenech, 1999).

In an effort to clarify whether homocysteine is a cause of chromosome damage or simply a biomarker of another chromosome-damaging event, experiments were designed to determine whether high concentrations of homocysteine are genotoxic to human

lymphocytes in folate and vitamin B_{12} replete conditions *in vitro* by using the Cytokinesis-Block Micronucleus assay. The relative importance of necrosis, a passive cell death process, was also assessed. Cultures challenged with homocysteine were compared with control cultures challenged with similar concentrations of methionine. Homocysteine (and methionine) concentrations of between 50 and 400 μM were chosen in order to maximize the chance of detecting an effect on MN induction. Plasma homocysteine concentrations generally range from 5 – 18 μM depending on blood folate concentrations but may reach up to 69 μM in some cases (Kang *et al.*, 1987).

3.2 Materials and methods

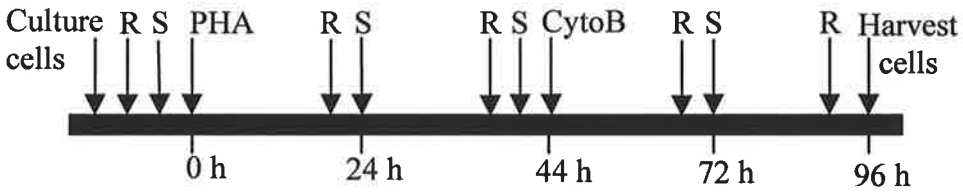
Blood samples (36 ml in lithium-heparinized vacuettes) were collected from four fasted male volunteers (22-23 yrs) and lymphocytes were isolated as previously described (Section 2.2.1)

3.2.1 Culture protocol for homocysteine-supplemented medium

Eighteen cultures ($1x10^6$ cells/ml) were prepared for each volunteer in 1 ml of RPMI 1640 medium (Trace Biosciences, Australia) without methionine, foetal calf serum (FCS) or interleukin-2 (IL-2) in 6 ml culture tubes (Becton Dickinson, USA). Serum free conditions were used to have an optimal control on methionine and homocysteine concentrations. Preliminary experiments in this laboratory showed that it is feasible to obtain sufficient binucleated cell frequencies in the CBMN assay with serum and IL-2 free medium, however, it is necessary to extend culture time by harvesting cells at 96 hr, i.e. 52 hr after the addition of cytochalasin-B rather than 28 hr. Tubes were then gently spun and 100 μl of supernatant was removed and stored at –20°C for the measurement of D,L-homocysteine concentrations. One hundred μl of medium containing either 0, 1, 2, 4 or 8 mM D,L-homocysteine (Hcy) or 0.5, 1, 2 or 4 mM L-methionine (Met) (for comparison) was then added to the cultures to achieve concentrations ranging from 0-800 μM in the cell culture. Because homocysteine is only commercially available as a 50/50 mixture of the D- and L-isomers and only the L-isomer is metabolically active, double the amount of the D,L-homocysteine to L-methionine is added to ensure that equal quantities of the metabolically active isomer were present in the cultures.

Cell cultures were prepared in duplicate. After sampling and spiking cultures with Hcy or Met, phytohaemagglutinin (PHA, 22 μg/ml) (Murex Biotech, England) was added to stimulate mitogenesis. Cultures were then placed in a humidified incubator set at 37°C with 5% CO_2. The process of sampling and spiking cultures was repeated at 24, 44 and 72 hr after the addition of PHA. At 44 hr post-PHA, cytochalasin B (Cyto-B, 4.5 μg/ml)(Sigma, USA) was added (immediately after spiking) to inhibit cytokinesis. At 96 hr post-PHA, cultures were sampled for the final time before cells were transferred to microscope slides as described in Section 2.2.3. The precise sequence of the above events is illustrated in Figure 3.2. Slides were scored for micronuclei and necrotic cells as described in Section 2.2.4.

Figure 3.2 Experimental design



R= Removal of 100 μl media which was stored frozen. S= Spiking with 100 μl of media containing 0 – 4 mM L-methionine or 0 – 8 mM D,L-homocysteine.

3.2.2 HPLC determination of homocysteine concentrations

The concentration of homocysteine in cell culture medium was measured by high performance liquid chromatography (HPLC) with fluorescent detection using the method of Vester and Rasmusen (1991). Twenty μl of an internal standard (0.1 M mercaptoproprionyl glycine. Sigma, St. Louis, MO, USA) was added to each sample (60 μl) in a 0.6 ml eppendorf-type tube. Eight μl of reducing solution (10% v/v tri-n-butyl phosphine in dimethylformamide) was then added and samples were chilled at 4°C for 30 mins before the addition of 50 μl perchloric acid (5% v/v in water with 37.2 mg Na-EDTA per 100 ml). After 10 mins standing at room temperature, samples were spun at 10,000 x g and 40 μl of supernatant was removed into a new tube. Eighty μl of 0.5 M

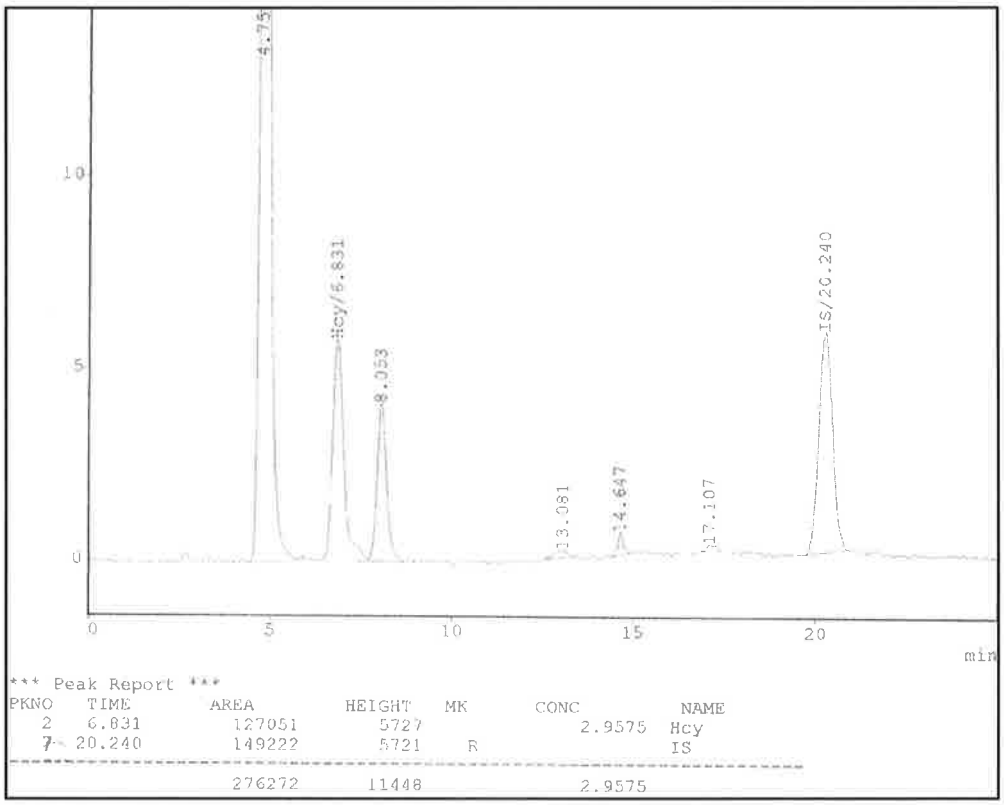
potassium tetraborate/ 5mM Na-EDTA (pH 10.5) was added and then homocysteine was derivatised by the addition of 40 µL 1 mg/ml 7-fluorobenzofurazine-4-sulphonic acid (Fluka, Buchs, Switzerland) in 0.1 potassium tetraborate/ 2 mM EDTA. Tubes were allowed to stand for 10 mins at room temperature before being incubated at 60°C for 60 mins. Tubes were then chilled at 4°C in a Shimadzu SIL-10A auto-injector unit fitted with a cooling rack until analysis.

Twenty µl of sample was injected into the system and separation was achieved on a C18 reverse phase microsorb column (25 cm x 4.6 mm, 5µm particle size. Varian, Walnut Creek, CA, USA) protected by an ODS2 guard column (SGE, Victoria, Australia). Flow rate was 1 ml/min. The presence of homocysteine and internal standard was detected using a Shimadzu RF-10A spectrofluorometric detector (excitation wavelength= 385 nm, emission wavelength= 515 nm). Two mobile phases were used, consisting of 19.95 ml glacial acetic acid, 3.5 L water, approximately 900 ml of 0.1M sodium acetate to adjust pH to 4.0 and methanol to either 4% (mobile phase A) or 15% (mobile phase B). A linear gradient was run from 100% mobile phase A to 100% mobile phase B over the first seven mins, followed by 10 mins of mobile phase B only, a two min linear gradient back to 100% phase A and four mins of A only before the next sample. The total run time was 23 mins per sample. The homocysteine concentration in samples was automatically calculated by the Shimadzu class LC10 version 1.0 software by comparing the ratio of the homocysteine peak area to the internal standard peak area to a standard curve. A typical chromatogram is shown in Figure 3.3.

3.2.3 Statistical analyses

Because L-homocysteine is the metabolically active form, the relevant homocysteine concentration is half that of D,L-homocysteine and comparisons with methionine treated cultures were made on that basis. Comparisons between different doses were made using repeated-measure ANOVA (with Tukeys' post-test) and Kruskal-Wallis (with Dunn's post-test) analyses. Comparisons between treatments (along with doses) were made using a two-way ANOVA followed by individual paired t-tests. Statistical calculations were performed using GraphPad Prism® Version 2.01 (GraphPad Inc., USA). Significance was accepted at $P \leq 0.05$.

Figure 3.3 Chromatogram from the homocysteine assay



A typical HPLC-fluorescence chromatogram showing a homocysteine peak at approximately 6.8 mins and the internal standard peak at 20.2 mins.

3.3 Results

As shown in Figure 3.4, Hcy accumulated over the culture period in both Hcy and Met treated cultures. At 96 hr the mean concentration of Hcy ranged from 19.45 ± 2.34 to 149.02 ± 28.16 μM and 0.91 ± 0.17 to 2.15 ± 0.9 μM in the Hcy and Met-spiked cultures respectively depending on dose. These concentrations and those shown in Figure 3.4 are those immediately before spiking and are therefore a measure of the amount of Hcy remaining in the culture medium approximately 24 hr after spiking with Hcy or Met.

Lymphocytes from two of the four volunteers exhibited a marked Met-dependency and hence the mitotic index was very low in Hcy-supplemented media (without Met). Methionine-dependency is the inability of cells to proliferate in media where methionine has been replaced by its immediate precursor, homocysteine. This made it impossible to score micronuclei at any Hcy concentration for one volunteer and for the two lowest Hcy

doses for another. No cells were able to proliferate in the simultaneous absence of Hcy and Met and therefore micronucleus data in once-divided cells could not be obtained.

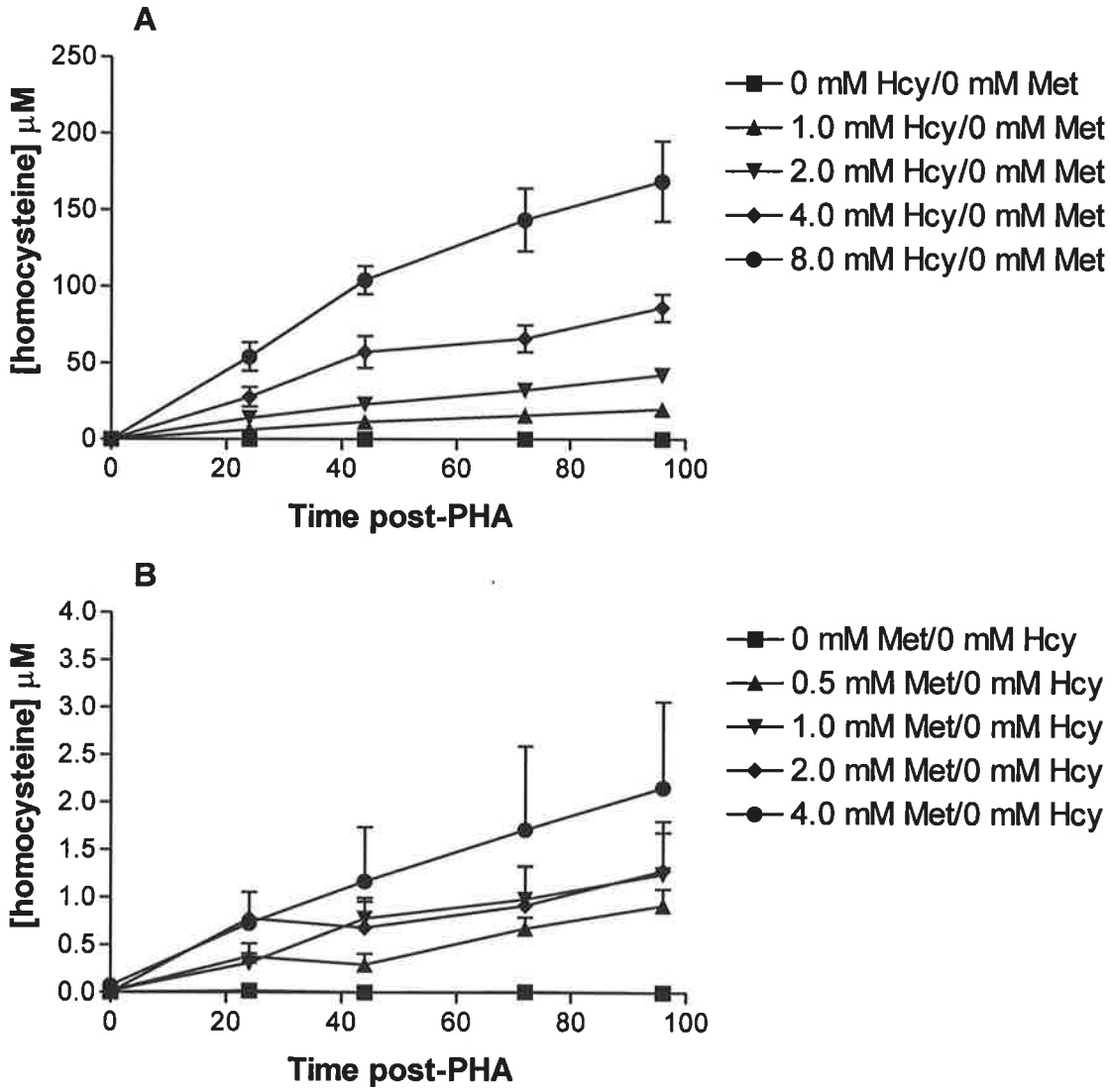
Treatments are described in terms of the spiking solution concentration (in the mM range) and not the final concentration after spiking (in the μM range).

There was no significant dose-related change in the micronucleus frequency of cultures spiked with Met (P= 0.93) or Hcy (P= 0.24) (Table 3.7). Two-way ANOVA performed on this data yielded a P value of 0.0688 for the comparison between treatments and a P value of 0.7447 for the dose effect (in accordance with the one-way ANOVA results). Because there was no clear dose-response effect of Hcy or Met on micronucleus frequency, the data from all Hcy and Met cultures were pooled for comparison. The P value of the one-tailed paired t-test was 0.04. These data suggest that Hcy treatment causes a marginal but significant increase in micronucleus frequency compared to Met treated cultures (Figure 3.5).

The addition of Hcy and Met significantly increased %BNs compared to 0 Hcy/Met. In fact, Hcy concentrations (added) were positively correlated with %BNs (P= 0.0083, r^2= 0.17). When the 0 mM Met/Hcy point was omitted from the analysis, a negative correlation between Met concentration and %BNs approached significance (P= 0.072, r^2= 0.11).

Spiking cultures with 0.5- 2 mM Met significantly increased necrosis compared to 0 mM Hcy/Met (Table 3.1). The addition of 4 mM L-hcy significantly increased necrosis compared to 0 and 0.5 mM L-Hcy. Furthermore, Hcy concentrations were significantly and positively correlated with necrosis (P< 0.0005, r^2= 0.276).

Figure 3.4 Concentration profile of D,L-homocysteine in cell cultures spiked with D,L-homocysteine (A) or L-methionine (B).



Concentrations shown are those before spiking at each time point. Data as mean ± SEM

Table 3.1 Effect of L-homocysteine and L-methionine concentration on micronuclei, binucleate frequency and necrosis.

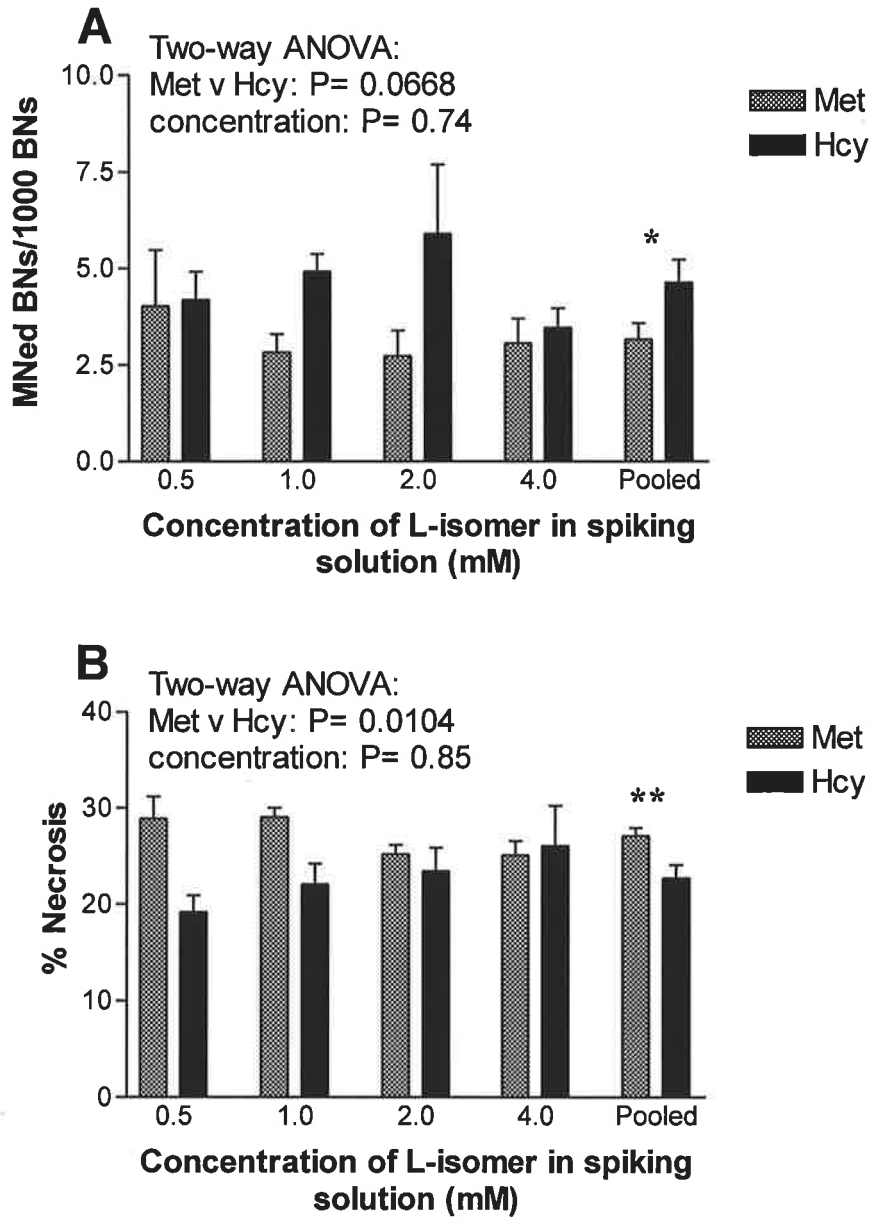
Spike	MNed BNs/1000 BNs		% BNs (of viable)		% Necrosis	
mM	Met	Hcy	Met	Hcy	Met	Hcy
0	NA		2.2 ± 1.3		20.3 ± 1.2	
0.5	3.9 ± 1.3	4.2 ± 0.6 [2]	26.0 ± 3.9 [a]	9.4 ± 3.6	29.3 ± 3.1 [a]	19.8 ± 1.6
1.0	2.8 ± 0.4	4.9 ± 0.4 [2]	22.7 ± 2.3 [a]	10.8 ± 3.9 [a]	30.2 ± 1.7 [a]	23.4 ± 2.2
2.0	2.7 ± 0.5	5.9 ± 1.3 [3]	19.3 ± 2.0 [a]	12.6 ± 3.1 [a]	28.8 ± 2.6 [a]	24.9 ± 1.8
4.0	3.1 ± 0.5	3.5 ± 0.5 [3]	18.4 ± 2.8 [a]	15.9 ± 3.4 [a]	26.1 ± 1.4	30.2 ± 3.5 [a b]
P value	P= 0.93	P= 0.24	P< 0.01	P< 0.01	P= 0.01	P= 0.01

Data are reported as mean ± SEM (n=4). NA: insufficient binucleated cells to determine MNed BN frequency. P value is for dose effect. Kruskal-Wallis test used for MNi data and repeated measures ANOVA used for %BNs and %Necrosis. [a] P< 0.05 v 0 mM Met/Hcy, [b] P< 0.05 v 0.5 mM Hcy, [2] n=2, [3] n=3.

At the two lower spiking concentrations there is a larger amount of necrosis occurring in cultures treated with Met than with Hcy. Two-way ANOVA performed on the necrosis data yielded a P value of 0.01 for the comparison between treatments and a P value of 0.85 for the dose effect. P values for the (paired t-test) comparison between Met and Hcy were 0.07, 0.09, 0.46 and 0.82 at 0.5, 1.0, 2.0 and 4.0 mM (L-isomer) respectively. Necrosis data for all Hcy and Met cultures were pooled into treatments and compared using a paired t-test. This analysis revealed that necrosis was significantly higher in cultures spiked with Met than with Hcy (P= 0.019) (Figure 3.5 B).

Cell necrosis was significantly and positively correlated with %BNs (P< 0.0001, r^2= 0.302).

Figure 3.5 Comparison of the frequency of micronucleated (A) and necrotic (B) cells between cultures spiked with L-methionine and D,L-homocysteine.



Data are reported as mean ± SEM. N=4, except for MNi values for Hcy treated cultures where n= 2,2,3 and 3 for 0.5, 1, 2 and 4 mM Hcy respectively. * paired t-test P= 0.04. ** paired t-test P= 0.02.

3.4 Discussion

The results from this experiment provide only limited support to the hypothesis that high concentrations of homocysteine elevate the frequency of micronucleated lymphocytes compared to similar molar concentrations of methionine *in vitro*. There was a trend for a higher frequency of micronucleated cells in cultures exposed to L-homocysteine compared to cells grown in the presence of L-methionine. These data can only be considered preliminary due to the methionine-dependency of two of the four volunteers, which prevented the scoring of micronuclei in some homocysteine-spiked cultures. More experiments are required to verify these observations in lymphocytes from a larger number of subjects whose lymphocytes are methionine-independent as well as in those who are methionine-dependent.

Methionine-dependency is the inability of cells to proliferate in media where methionine has been replaced by homocysteine (Hoffman, 1982). Interestingly, methionine-dependency is normally associated with cancerous cells and even as a trait acquired during transformation (Hoffman, 1982; Stern *et al.*, 1984). However, it has been reported that some people with methionine-dependent tumours also exhibit this trait in non-cancerous cells (Mikol and Lipkin, 1984). Further research on the prevalence of methionine dependency and the relationship between this phenotype and the micronucleus index in a larger study group is presented in Chapter 4.

Previously it has been reported that plasma homocysteine concentrations correlate positively with micronucleus frequency in humans (Fenech *et al.*, 1997a; Fenech *et al.*, 1998). Because the results presented here do not support a strong role of homocysteine in micronucleus formation it seems likely that the high plasma homocysteine concentrations seen in people with high micronucleus counts (Fenech *et al.*, 1997a; Fenech *et al.*, 1998) are a biomarker for conditions, such as folate or vitamin B_{12} deficiency, that promote chromosome damage rather than being a cause of the damage. Indeed, negative correlations between plasma vitamin B_{12} and micronucleus frequency were also evident in the study groups that exhibited a positive correlation between homocysteine concentration and micronucleus frequency (Fenech *et al.*, 1998). It must be noted however that this experiment utilized a regime of acute exposure to homocysteine *in vitro*. To obtain a definitive answer as to whether or not homocysteine is genotoxic *in vivo*, further

experiments testing the effects of artificially and chronically elevated plasma homocysteine concentrations in laboratory animals under conditions of B vitamin repletion need to be conducted.

A possible confounding factor in these studies is that elevated homocysteine concentrations could have increased S-adenosyl methionine levels considerably by increasing the rate of methionine synthesis. Elevated S-adenosyl methionine concentrations would inhibit MTHFR (Kutzbach and Stokstad, 1971) and thereby divert folate towards the methylation of dUMP to form dTMP which may, in turn, reduce micronucleus frequency and partially explain the absence of a clear dose-response effect for homocysteine.

High concentrations of D,L-homocysteine caused a significant elevation in the frequency of cells that were necrotic. Furthermore, homocysteine concentrations positively correlated with the percentage of cells that were necrotic. This is in agreement with several reports of dose-dependent cytotoxicity to various cell types by homocysteine *in vitro* (Wall *et al.*, 1980; Hultberg *et al.*, 1997; Starkebaum and Harlan, 1986). A protective effect of catalase in these experiments suggests a role for hydrogen peroxide (H_2O_2) in the cellular damage caused by homocysteine (Wall *et al.*, 1980; Starkebaum and Harlan, 1986). Although H_2O_2 is an effective inducer of micronucleus formation, it is clear that the major event in H_2O_2 is necrosis (Crott and Fenech, 1999). The significantly higher necrosis in the methionine treated cultures, which had much lower homocysteine concentrations, does not support the hypothesis that homocysteine is the cause of necrosis in lymphocyte cultures. In fact, the observed changes in necrosis may simply be a side effect of mitotic activity with which necrosis was significantly and positively correlated ($r^2= 0.11$, $P= 0.0005$).

Most of the pathologies that are associated with elevated plasma homocysteine levels involve the cardiovascular system. Recently it was discovered that the tissue of the cardiovascular system (endothelial and smooth muscle) has a limited ability to process homocysteine due to a low expression of cystathione β-synthase (Chen *et al.*, 1999), one of the two enzymes that processes homocysteine in most tissue cells. (The liver also possesses betaine:homocysteine methyl-transferase, which converts homocysteine and

betaine to methionine and dimethyl glycine). Since an important avenue of homocysteine removal is absent in the cells of the cardiovascular system, these cells may be more susceptible to hyperhomocysteinemia than lymphocytes. For this reason, it seems necessary to investigate whether high concentrations of homocysteine result in damage to the DNA in these cardiovascular cells and whether such DNA damage is one of the underlying causes of atherosclerosis.

It must also be noted that the great majority (99%) of homocysteine in plasma occurs in its various oxidized forms, which are indistinguishable by most homocysteine assays (Jacobsen, 1998). It is therefore plausible that the positive correlation observed between homocysteine and micronucleus frequency *in vivo* (Fenech *et al.*, 1997a; Fenech *et al.*, 1998) is due to alternate potentially genotoxic forms of homocysteine and not due to homocysteine itself.

In summary, this experiment shows that homocysteine does not strongly induce micronucleus formation in human lymphocytes *in vitro*. Further research is needed to verify these preliminary results and to determine the effects of chronic hyperhomocysteinemia, during B vitamin repletion, not only on lymphocytes, but also on a diverse range of cell types, especially those of the human cardiovascular and nervous systems.

3.5 Publication resulting from this chapter

Crott,J. and Fenech,M. (2001) Preliminary study on the genotoxic potential of homocysteine in human lymphocytes *in vitro*. *Mutagenesis*, **16**(3), 213-217.

Chapter 4: Methionine-dependency and genetic stability

AIM

To investigate whether methionine-dependence is related to genetic stability and also to study the frequency and cause of methionine-dependence in human lymphocytes.

HYPOTHESIS

Methionine-dependence is a common phenotype that is related to elevated micronucleus frequencies in human lymphocytes.

4.1 Introduction

Methionine-dependence is defined as the inability of cells to proliferate when methionine is replaced by homocysteine, one of its immediate precursors. This phenotype has been reported in numerous cancer cell lines (Hoffman, 1982; Stern *et al.*, 1984) and human tumours (Hoshiya *et al.*, 1995) and has long been considered a property unique to cancer cells (Hoffman, 1985). For this reason, treatments involving methionine-restriction have been proposed as a potential method for controlling the growth of methionine-dependent cancer cells (Guo *et al.*, 1993; Breillout *et al.*, 1990; Hoshiya *et al.*, 1995).

The rationale for the present experiment is that methionine-dependency may be a phenotypic marker for methionine synthase deficiency and as such may impact on various markers of chromosome stability. Methionine-dependency in human lymphocytes had previously been observed in two of four volunteers participating in an experiment where the potential genotoxic and cytotoxic effects of homocysteine were being studied, suggesting that methionine-dependence can be observed in cells from people without cancer (see section 3.3).

It is becoming evident that methionine-dependency is not solely a property of cancer cells (Kano *et al.*, 1982; Mikol and Lipkin, 1984; Christa *et al.*, 1986; Judde and Frost, 1988). For example, Mikol and Lipkin (1984) reported that non-cancerous skin fibroblasts taken from patients with hereditary colon cancer are relatively methionine-dependent compared

with those of controls with no familial history of colon cancer. Because methionine-dependency can exist in both normal and cancer cells in the same individual (Mikol and Lipkin, 1984) it seems possible that methionine-dependent cancer cells simply develop in individuals who were originally methionine-dependent. In this scenario, methionine-dependency is a pre-existing phenotype that is not acquired during transformation. Whether methionine-dependence is a risk factor for cancer is not yet clear.

The culturing of methionine-dependent cells in methionine deficient, homocysteine supplemented (met^-hcy^+) media results in a reversible late cell cycle arrest (Hoffman and Jacobsen, 1980; Guo *et al.*, 1993). Elevated levels of transmethylation, and hence a higher demand for methyl groups, is one of the proposed mechanisms for methionine-dependent cell cycle progression (Hoffman, 1985; Judde *et al.*, 1989).

Methionine-dependent cells are reported to have lower S-adenosyl methionine (SAM) concentrations than methionine-independent cells (Stern *et al.*, 1984), a finding consistent with elevated rates of transmethylation. Because SAM is an inhibitor of methylenetetrahydrofolate reductase (MTHFR) (Kutzbach and Stokstad, 1971), a decline in SAM levels may accelerate the conversion of 5,10-Methylene tetrahydrofolate (5,10-MnTHF) to 5-methyl tetrahydrofolate (5-MeTHF) by MTHFR, resulting in a diversion of folate away from thymidine synthesis. These conditions may elevate intracellular dUMP: dTMP ratios and subsequently increase the rate of uracil misincorporation into DNA (Blount *et al.*, 1997). The simultaneous removal of uracil residues within 12 base pairs of each other and on opposite strands during excision repair may result in the formation of double-stranded DNA breaks (DSB) (Dianov *et al.*, 1991). The introduction of DSBs leads to chromosome breakage and rearrangement, events that significantly correlate with the risk for cancer (Hagmar *et al.*, 1994; Hagmar *et al.*, 1998; Bonassi *et al.*, 2000).

The recycling of homocysteine to methionine is catalysed by vitamin B_{12}-dependent methionine synthase in a reaction where 5-MeTHF is the methyl group donor (see Figure 1.2). Theoretically, the reduced activity of methionine synthesis is an attractive mechanism for the aetiology of methionine-dependence. This mechanism is supported by the finding that a methionine-independent revertant cell line had a 31% greater methionine synthase activity than the methionine-dependent cell line from which it was

isolated (Judde *et al.*, 1989). Furthermore, of the other cancer cell lines tested methionine synthase activity was at least 20% greater in methionine-independent cells than in methionine-dependent cells (Judde *et al.*, 1989). It has also been reported that methionine-dependent glioma cells (P60) remethylate homocysteine to methionine substantially slower than methionine-independent glioma cells (P60H), possibly due to a reduced capacity of P60 cells to furnish methylated cobalamin (vitamin B_{12}) to methionine synthase (Fiskerstrand *et al.*, 1997).

Occupational exposure to nitrous oxide, an inhibitor of methionine synthase, is reported to cause a significant elevation in micronucleus frequency (Chang *et al.*, 1996). Methionine synthase inhibition causes folate trapping (Horne and Holloway, 1997) and a reduction in the amount of folate available for thymidine synthesis as described above. Furthermore, low vitamin B_{12} (the essential cofactor for methionine synthase) concentrations, are associated with an elevated micronucleus index *in vivo* (Fenech *et al.*, 1997a; Fenech *et al.*, 1998). It follows that if reduced methionine synthase activity is a cause of methionine dependency, a cell's methionine requirements may be related to markers of genetic damage (e.g. micronucleus frequency).

Although it is not known whether the common A2756G mutation (Leclerc *et al.*, 1996) in the gene encoding methionine synthase causes a reduction in enzyme activity, it is possible that such a mutation may affect the (exogenous) methionine requirements of normal human lymphocytes in culture.

In contrast to the above results, which support a role of methionine synthase in methionine-dependency, some earlier studies show that methionine-dependent cells have similar methionine synthase activities to methionine-independent cells (Hoffman and Erbe, 1976; Hoffman *et al.*, 1978; Christa *et al.*, 1986; Stern *et al.*, 1984).

It is also conceivable that mutations in the gene encoding MTHFR could affect a cell's methionine requirements. MTHFR catalyses the conversion of 5,10-MnTHF to 5-MeTHF, the methyl donor required for the conversion of homocysteine to methionine (see Figure 1.2). Two common mutations in the MTHFR gene, C677T (Frosst *et al.*, 1995) and A1298C (van der Put *et al.*, 1998), are reported to reduce MTHFR activity by 70% and

39% respectively, in homozygous mutants. This may affect a cell's requirement for exogenous methionine by limiting the remethylation of endogenous homocysteine.

There is a clear need to clarify the cause and effects of methionine-dependence, not only in cancer cells but also in normal human cells. Indeed the mechanisms behind this phenotype may even vary between cell types. This study was designed to test the hypothesis that methionine-dependence is related to baseline levels of genetic damage, as measured by the CBMN assay, in normal human lymphocytes. Furthermore, to determine whether mutations in two key folate-metabolising enzymes (MTHFR and methionine synthase) affect lymphocyte methionine requirements *in vitro*.

4.2 Materials and methods

4.2.1 Volunteers

Fifty-two healthy asymptomatic people (23 male, 29 female) aged between 29-65 years who were not receiving anti-folate therapy or cancer treatment and participating in an on-going study at CSIRO were recruited for this study. Each gave their informed consent and ethics approval was obtained from the CSIRO Health Sciences and Nutrition and Adelaide University human ethics committees.

4.2.2 Visit 1- Baseline measures

On the first visit, volunteers donated a blood sample (18 ml in lithium-heparinized vacuettes) after an overnight fast. Lymphocytes were isolated and baseline levels of genetic damage were measured using the standard CBMN assay as described in Chapter 2. The micronucleus frequency of female volunteers was adjusted by dividing by a factor of 1.87 (the ratio of the average female micronucleus frequency to male micronucleus frequency in this study). The data were then age adjusted to an age of 49.5 years (average age of volunteers) as described in Section 2.2.5. Research assistants in this laboratory previously performed these micronucleus assays.

Plasma homocysteine concentrations in these samples were measured by HLPC with fluorescent detection using the internal standard method of Vester and Rasmussen (1991) as described in Section 3.2.2. A research assistant from this laboratory had previously performed these homocysteine measures.

4.2.3 Detection of MTHFR C677T polymorphism

The presence of the C to T transition at position 677 in the gene encoding MTHFR was determined using the restriction fragment length polymorphism (RFLP) technique described by Frosst *et al.* (1995). Briefly, a 198 bp region of the MTHFR gene containing position 677 is amplified from genomic DNA using the polymerase chain reaction (PCR).

The reaction mix contained 100 ng of genomic DNA, 3 µl 25 mM $MgCl_2$ (Boehringer Mannheim, Germany), 5 µl 10x PCR buffer (Boehringer Mannheim, Germany), 1 µl 10 mM (of each) nucleotide mix (Boehringer Mannheim, Germany), 5 µl of each primer (2 µM; Geneworks, Adelaide, Australia), 1 µl Taq DNA polymerase (Boehringer Mannheim, Germany) and distilled water to make a total volume of 50 µl. The primer sequences were as follows:

5'- TGA AGG AGA AGG TGT CTG CGG GA- 3' (exonic)

5'- AGG ACG GTG CGG TGA GTG- 3' (intronic)

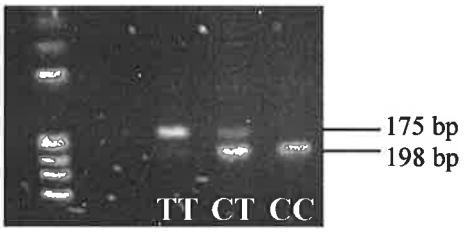
The program for the thermal cycler consisted of an initial 5 min denaturation at 96°C followed by 35 cycles of denaturation at 93°C for 50 secs, annealing at 55°C for 50 secs and extension at 72°C for 30 secs. A final extension of 7 mins at 72°C was also used.

The 198 bp PCR product was then digested with HinfI restriction endonuclease. The C677T polymorphism results in the formation of a HinfI site which, when cut, produces a 175 bp and a 23 bp fragment. The digestion reaction contained 20 µl of PCR product, 2 µl 10x digestion buffer (supplied with enzyme), 2 µl bovine serum albumin (BSA; supplied with enzyme) and 1 µl 10 U/µl HinfI enzyme (Geneworks, Adelaide, Australia) and was incubated at 37°C for 2 hrs. After 2 hrs, the digestion was stopped with a drop of stop buffer (50% glycerol, 50 mM EDTA, 0.05% bromophenol blue). Four µl of digest was then mixed with 2 µl standard loading buffer and 2 µl water and loaded into a 3% agarose

gel. One µl of molecular weight marker (pBR323 HaeIII digest; Sigma, St. Louis, MO, USA) was mixed with water and loading buffer and loaded onto the gel in a similar fashion. The gel contained 2 µl (10 mg/ml) ethidium bromide/ 40 ml gel and was run at 67 V for approximately 90 mins in 0.5x TAE solution (50x = 242 g Tris base, 33.62 g Na_2EDTA and 57.1 ml glacial acetic acid per litre, pH 8.0). The gel was visualised under UV light when complete (approximately 45 mins).

As shown in Figure 4.1, wildtypes (CC) exhibit one band at 198 bp, heterozygotes (CT) exhibit a band at 198 bp and one at 175 bp with a 23 bp fragment that has run-off of the gel and homozygous mutants (TT) exhibit one band at 175 bp. A research assistant in this laboratory performed this genotyping.

Figure 4.1 Photograph of agarose gel used for MTHFR 677 genotyping



4.2.4 Detection of MTHFR A1298C polymorphism

The MTHFR A1298C polymorphism was detected using the RFLP method of van der Put *et al.* (1998). This mutation destroys a MboII restriction enzyme site. The mutation is detected by amplifying a 163 bp DNA fragment. Homozygous mutants (1298CC) exhibit four fragments following digestion with MboIII, namely 84, 31, 30 and 18 bp. In wildtypes (1298AA) the 84 bp fragment is cut and results in the formation of five fragments, namely 56, 31, 30, 28 and 18 bp.

The PCR mix contained 200 ng genomic DNA, 6 µl 25 mM $MgCl_2$ (Boehringer Mannheim, Germany), 5 µl 10x PCR buffer (Boehringer Mannheim, Germany), 1 µl 10 mM nucleotide mix (Boehringer Mannheim, Germany), 5 µl of each primer (2 µM; Geneworks, Adelaide, Australia), 1 µl Taq DNA polymerase (Boehringer Mannheim,

Germany) and water to make up a volume of 50 µl. The primer sequences were as follows:

5'-CTT TGG GGA GCT GAA GGA CTA CTA C - 3' (forward)

5'- CAC TTT GTG ACC ATT CCG GTT TG- 3' (reverse)

The thermal cycler program consisted of an initial 92°C denaturation for 2 mins followed by 35 cycles of denaturation at 92°C for 60 secs, annealing at 60°C for 90 secs and extension at 72°C for 30 secs. A final extension of 7 mins at 72°C was also used.

Twenty µl of PCR product was digested with 1 µl of MboII enzyme (Geneworks, Adelaide, Australia) in the presence of 2 µl BSA (supplied with enzyme) and 2 µl 10x digestion buffer (supplied with enzyme) for 2 hrs at 37°C. The reaction was stoped and the product was run on an agarose gel as described above (section 4.2.2.) except a 3.5% Metaphase gel was used and run at 100 V for 75 mins. A research assistant in this laboratory performed this genotyping.

4.2.5 Detection of methionine synthase A2756G polymorphism

The MS A2756G polymorphism was detected using the RFLP method of Leclerc *et al.* (1996). This polymorphism forms a HaeIII restriction enzyme site, which is detected by the amplification of a 189 bp fragment, followed by its digestion with HaeIII. Wildtypes (AA), heterozygotes (AC) and homozygous mutants (CC) show a single 189 bp fragment, a 189 bp and a 159 bp fragment and a single 159 bp fragment respectively. In ACs and CCs the smaller 30 bp fragment is run-off of the gel.

The PCR mix contained 150 ng genomic DNA, 3 µl 25mM $MgCl_2$ (Boehringer Mannheim, Germany), 2.5 µl 10x PCR buffer (Boehringer Mannheim, Germany), 1 µl nucleotides (Boehringer Mannheim, Germany), 3 µl of each primer (2 µM; Geneworks, Adelaide, Australia), 1.5 µl (units) Taq polymerase and water to make up to a volume of 50 µl. The primer sequences were as follows:

5'- GAA CTA GAA GAC AGA AGA AAT TCT CTA- 3' (intronic)

5'- CAT GGA AGA ATA TGA AGA TAT TAG AC- 3' (exonic)

The thermal cycler program consisted of an initial 96°C denaturation for 4 mins followed by 35 cycles of denaturation at 95°C for 60 secs, annealing at 60°C for 90 secs and extension at 72°C for 60 secs. A final extension of 4 mins at 72°C was also used.

Twenty µl of PCR product was digested with 1 µl of HaeIII enzyme (Geneworks, Adelaide, Australia) in the presence of 2 µl BSA (supplied with enzyme) and 2 µl 10x digestion buffer (supplied with enzyme) for 2 hrs at 37°C. The reaction was stopped and the product was run on an agarose gel as described above (section 4.2.2).

4.2.6 Visit 2- Methionine-dependency screening

On the second visit, fasted volunteers donated another blood sample (9 ml, in lithium-heparinized vacuettes) and lymphocytes were isolated. Methionine-dependency was assessed by preparing three lymphocyte cultures per person. Each culture contained $1x10^6$ cells in 900 µl of RPMI 1640 medium (Trace Biosciences, Australia) without foetal calf serum or methionine. Cultures were then spiked with 100 µl of RPMI 1640 medium containing either 1 mM L-methionine, 2 mM D,L-homocysteine or 4 mM D,L-homocysteine (Sigma, St. Louis, MO, USA) in order to achieve concentrations of 0.1 mM methionine (met^+hcy^-), 0.2 mM homocysteine (0.2-hcy^+) and 0.4 mM homocysteine (0.4-hcy^+) respectively.

The culture protocol is exactly as described for the homocysteine dose-response experiment (see section 3.2.1)

Cultures were repeatedly spiked (daily) because preliminary experiments in this laboratory showed that a single addition of homocysteine does not support cell division. Because FCS was not used, cell culture time was increased from 72 to 96 hrs in order to increase the yield of binucleated cells. A minimum total of 500 cells were scored per slide. Cell division in met^+hcy^- medium is represented as a percentage of viable cells that are binucleated. Tri- and tetranucleated cells, although infrequent, were regarded as 1.5 and 2 binucleated cells respectively. For each individual, cell division in met^-hcy^+ medium is expressed as a percentage of cell division in met^+hcy^- medium. Nuclear

division index was calculated according to the equation of Eastmond and Tucker (1989). The experimental design is illustrated in Figure 3.2.

4.2.7 Statistical analyses

Data were tested for a normal (Guassian) distribution in order to determine whether parametric or non-parametric analyses should be employed. Relationships between data were investigated using Pearson correlation tests and multiple regression analyses. Comparisons between groups were made using a one-way ANOVA (with Tukeys' post-test). Analyses were performed using Prism® version 2.01 and InStat® version 3.0, both from GraphPad Software Inc., USA. Significance was accepted at $P \leq 0.05$.

4.3 Results

Data for rates of cell division in $met^{+}hcy^{-}$ and $met^{-}hcy^{+}$ media exhibited normal distributions. Volunteer age was negatively correlated with division in $met^{+}hcy^{-}$ media ($P < 0.001$, $r^2 = 0.19$). There was a positive correlation between cell division in $0.2\text{-}hcy^{+}$ media and $0.4\text{-}hcy^{+}$ media ($P < 0.0001$, $r^2 = 0.46$).

Volunteers were sorted into tertiles of methionine-dependence according to the division rate of their cells in $met^{-}hcy^{+}$ media (relative to division in $met^{+}hcy^{-}$ media). Those in the lowest tertile of growth in $met^{-}hcy^{+}$ media were classified in the highest tertile of methionine-dependence and vice versa. Two methods of sorting were used, one based on cell division rate in $0.2\text{-}hcy^{+}$ media and the other on cell division rate in $0.4\text{-}hcy^{+}$ media. There is no age difference between tertiles for both $0.2\text{-}hcy^{+}$ and $0.4\text{-}hcy^{+}$ (Table 4.1).

There was no association between age/gender-adjusted micronucleus frequency (Table 4.1) or plasma homocysteine concentrations (Table 4.3) and methionine-dependency phenotype.

Table 4.1 Comparison of age and micronucleus frequency between groups sorted into tertiles methionine-dependence.

Sorted by		Methionine-dependence Tertile			ANOVA
		High (n=17)	Mid (n=18)	Low (n=17)	P
Cell division in 0.2-hcy^+	Volunteer age	45.1 ± 2.4 (29 – 58)	51.9 ± 2.7 (33 – 63)	51.6 ± 2.2 (34 – 65)	P= 0.09
	MNi frequency	5.41 ± 0.61 (1.90 – 12.92)	6.31 ± 0.87 (1.03 – 14.91)	5.67 ± 0.62 (1.59 – 13.30)	P= 0.65
Cell division in 0.4-hcy^+	Volunteer age	48 ± 2.5 (29 – 62)	49.8 ± 2.8 (30 – 64)	50.8 ± 2.3 (33- 65)	P= 0.7
	MNi frequency	5.56 ± 0.58 (2.67 – 12.92)	6.37 ± 0.91 (1.03 – 14.91)	5.46 ± 0.58 (1.90 – 12.27)	P= 0.61

Data presented as mean ± SEM, range in parentheses. MNi = Age and gender-adjusted micronucleus frequency from methionine and FCS-supplemented cultures (expressed as the number of micronucleated binucleates per 1000 binucleates).

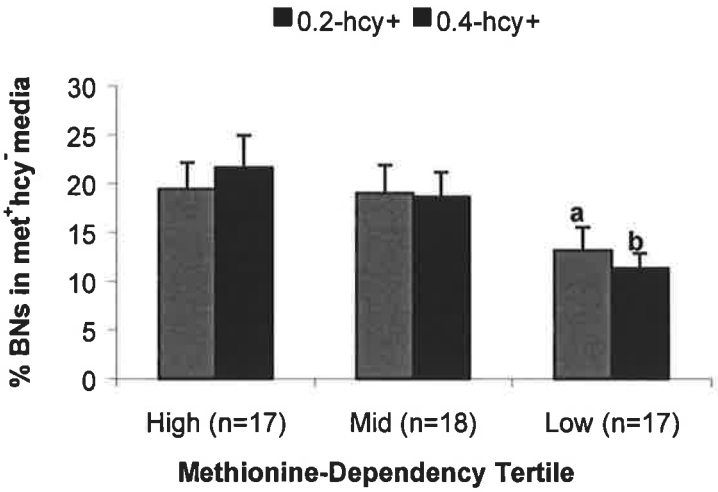
Cell division rate in met^-hcy^+ media was correlated negatively with %BNs in met^+hcy^- media (P= 0.05 r^2= 0.07 and P= 0.007, r^2= 0.14 for 0.2-hcy^+ and 0.4-hcy^+ respectively). Lymphocytes with the highest methionine-dependence were found to have higher rates of cell division in met^+hcy^- media than lymphocytes in the lowest tertile of methionine-dependence (Table 4.2, Figure 4.2). In other words, increased methionine-dependence was associated with increased cell division in methionine-supplemented, serum-free media compared to methionine-independent lymphocytes. However, the methionine-dependence phenotype was not associated with increased cell proliferation when cells were grown in medium supplemented with 10% FCS, (see Table 4.3).

Table 4.2 Comparison of cell division parameters in serum-free medium between tertiles of methionine-dependence.

Sorted by		Methionine-dependence Tertile			ANOVA P
		High (n=17)	Mid (n=18)	Low (n=17)	
Cell division in 0.2-hcy$^+$	Cell division in 0.2-hcy$^+$	5.03 ± 0.8 (0 – 11.08)	25.78 ± 2.08 (13.36 – 41.79)	69.74 ± 7.48 (43.99 – 157.05)	
	%BNs in met$^+$hcy$^-$	19.46 ± 2.7 (4.41 – 44.85)	19.03 ± 2.86 (2.95 – 48.05)	13.15 ± 2.37 [a] (2.52 – 34.05)	P= 0.19
	NDI in met$^+$hcy$^-$	1.16 ± 0.02 (1.04 – 1.32)	1.16 ± 0.012 (1.04 – 1.34)	1.12 ± 0.02 (1.03 – 1.27)	P= 0.24
Cell division in 0.4-hcy$^+$	Cell division in 0.4-hcy$^+$	28.96 ± 3.37 (7.3 – 51.19)	69.53 ± 3.5 (52.90 – 99.63)	142.44 ± 9.28 (100.05 – 223.89)	
	%BNs in met$^+$ hcy$^-$	21.66 ± 3.28 (4.18 – 48.05)	18.64 ± 2.51 (4.41 – 42.01)	11.35 ± 1.5 [b] (2.51 – 27.07)	P= 0.01
	NDI in met$^+$hcy$^-$	1.18 ± 0.02 (1.04 – 1.34)	1.16 ± 0.02 (1.05 – 1.27)	1.11 ± 0.01 [c] (1.03 – 1.23)	P= 0.03

Data presented as mean ± SEM, range in parentheses. Cell division in 0.2/0.4-hcy$^+$= binucleate frequency of cells grown in met$^-$hcy$^+$ media expressed as a percentage of each individual's binucleate frequency in met$^+$hcy$^-$ media. NDI= nuclear division index. [a] P= 0.089 v High (T-test), [b] P< 0.05 v High (Tukeys' post-test), [c] P< 0.05 v High (Tukeys' post-test).

Figure 4.2 Comparison of cell proliferation in met$^+$hcy$^-$ media between groups sorted by methionine-dependence phenotype.



Data presented as mean ± SEM. [a] P= 0.089 v High (T-test) (ANOVA P= 0.19). [b] P< 0.05 v High (Tukey's post-test) (ANOVA P= 0.01)

Table 4.3 Comparison of % binucleates in FCS-supplemented, methionine-replete media and plasma homocysteine concentrations between tertiles of methionine-dependence.

Sorted by		Methionine-dependence Tertile			ANOVA P
		High (n=17)	Mid (n=18)	Low (n=17)	
Cell division in 0.2-hcy$^+$	%BNs in FCS-media	26.74 ± 2.15 (15.5 - 44.5)	26.22 ± 2.35 (8.5 – 53.5)	27.76 ± 3.0 (12.5 – 49)	P= 0.91
	plasma [hcy]	8.57 ± 0.55 (5.13 - 15.76)	8.81 ± 0.38 (5.79 – 11.62)	9.96 ± 0.98 (6.68 – 23.95)	P= 0.31
Cell division in 0.4-hcy$^+$	%BNs in FCS-media	23.68 ± 1.71 (17 – 41.5)	27.81 ± 2.87 (8.5 – 53.5)	29.15 ± 2.59 (12.5 – 49)	P=0.28
	plasma [hcy]	8.64 ± 0.56 (5.13 – 15.76)	9.7 ± 0.92 (5.79 – 23.95)	8.95 ± 0.44 (6.68 – 14.83)	P= 0.53

Data presented as mean ± SEM, range in parentheses. FCS = Foetal calf serum,

The growth of lymphocytes in $met^{+}hcy^{-}$ and $met^{-}hcy^{+}$ media was not significantly different between homozygous wildtypes, heterozygotes or homozygous mutants for all three polymorphisms studied (Table 4.4). Furthermore, when the values 0, 1 and 2 were assigned to represent the number of mutant alleles it was found that the frequency of mutant alleles was similar between the tertiles of division in $met^{+}hcy^{-}$ and $met^{-}hcy^{+}$ media ($P > 0.05$).

Table 4.4 Comparison of cell division between MTHFR and methionine synthase genotypes.

Locus		Genotype			
		CC	**CT**	**TT**	**P value**
MTHFR	n	22	20	10	-
677	%BNs in $met^{+}hcy^{-}$	16.1 ± 2.1	18.3 ± 2.7	17.8 ± 14.1	0.81
	Cell divn. in 0.2-hcy^{+}	27.8 ± 5.3	37.3 ± 8.1	37.7 ± 8.1	0.58
	Cell divn. in 0.4-hcy^{+}	79.3 ± 9.3	83.7 ± 12.9	74.7 ± 20.7	0.91
		AA	**AC**	**CC**	**P value**
MTHFR	n	23	23	6	-
1298	%BNs in $met^{+}hcy^{-}$	19.3 ± 2.6	16.7 ± 2.2	11.8 ± 3	0.34
	Cell divn. in 0.2-hcy^{+}	32.8 ± 5.1	36.6 ± 8.8	23.2 ± 10.6	0.67
	Cell divn. in 0.4-hcy^{+}	65.5 ± 7.7	98.6 ± 13.1	65.4 ± 21.5	0.08
		AA	**AG**	**GG**	**P value**
MS	n	37	14	0	-
2756	%BNs in $met^{+}hcy^{-}$	17.1 ± 1.8	16.9 ± 3.48	-	0.95
	Cell divn. in 0.2-hcy^{+}	36.2 ± 5.9	25.6 ± 6.2	-	0.31
	Cell divn. in 0.4-hcy^{+}	83.6 ± 8.9	51.3 ± 13.7	-	0.37

Data presented as mean ± SEM. Cell divn. (division) in 0.2/0.4-hcy^{+}= binucleate frequency of cells grown in $met^{-}hcy^{+}$ media expressed as a percentage of each individual's binucleate frequency in $met^{+}hcy^{-}$ media.

For the MTHFR 677 and 1298 and methionine synthase 2756 loci the frequency of mutant alleles was 38.5% (T), 33.65% (C) and 13.7% (G) respectively. The population

studied was found to be in Hardy-Weinberg equilibrium with respect to all three genotypes.

4.4 Discussion

The results of this experiment clearly suggest that methionine-dependence is a common phenotype in lymphocytes taken from normal healthy human beings. At least one-third of the volunteers tested had cell growth rates in $met^{-}hcy^{+}$ media that were half the rate of cell division in $met^{+}hcy^{-}$ media (Table 4.2). Mikol and Lipkin (1984) have previously reported that non-cancerous skin fibroblasts taken from patients with hereditary colon cancer are relatively methionine-dependent compared with those of controls with no familial history of colon cancer. However, to the best of our knowledge this is the first report of primary lymphocytes, taken from volunteers with no history of cancer, exhibiting varied degrees of methionine dependency.

Kano and co-workers (1982) have previously reported that peripheral blood lymphocytes taken from normal volunteers fail to proliferate in $met^{-}hcy^{+}$ media containing 30 mg/L (220 μM) homocysteine. Experiments described in this thesis have shown that when homocysteine is added to PHA-stimulated lymphocytes in cell culture at a concentration of 200 μM, the amount of homocysteine remaining after 24 hr is only 12.77 ± 3.39 μM (see Figure 3.3). Furthermore, in preliminary experiments it was observed that a single addition of homocysteine at 200 and 400 μM cannot support proliferation in lymphocytes, however as shown in these experiments lymphocytes are able to proliferate when the homocysteine is added to the cell culture every 24 hr. In light of these results, we suggest that the observations made by Kano and co-workers (1982) cannot be interpreted as methionine-dependency of lymphocytes because it is likely that the amount of homocysteine present in their system could not support the growth of methionine-independent cells, nor methionine-dependent cells.

The most reasonable explanation for methionine-dependency is a failure to convert homocysteine to methionine by methionine synthase, a model supported by some experimental evidence (Judde *et al.*, 1989. Fiskerstrand *et al.*, 1997). We hypothesised that those with a methionine-dependent phenotype may have a higher micronucleus

frequency than those with a methionine-independent phenotype because they may be expected to have a lower intracellular SAM concentration, which would facilitate folate trapping and increase uracil misincorporation into DNA. This hypothesis is not supported by the results of this experiment, which show that there is no difference in the age and gender-adjusted micronucleus frequency of people in the highest (methionine-independent) and lowest (methionine-dependent) tertiles of cell division in $met^{-}hcy^{+}$ media. If methionine synthase deficiency explains the methionine-dependency phenotype then a possible explanation for the null effect of methionine-dependence on MNi frequency is that methionine synthase deficiency (or inhibition) may result in a build up of homocysteine, S-adenosyl homocysteine (SAH) and S-adenosyl methionine (SAM) when methionine is supplied in the culture medium or diet (see Figure 1.2). The known inhibitory effect of SAM on MTHFR (Kutzbach and Stokstad, 1971) may serve to protect against DNA damage by allowing sufficient availability of 5,10-MnTHF for the efficient conversion of dUMP to dTMP. Therefore, methionine-dependence would only favour a low SAM concentration, elevated dUMP and MNi from chromosome breaks during conditions of methionine deficiency which would also coincide with induction of MNi from chromosome loss events caused by hypomethylation of heterochromatin. It is also possible that differences in micronucleus frequency between methionine-dependence phenotypes may only be detectable under conditions of folate deficiency, because a high folate concentration may facilitate the provision of sufficient 5,10-MnTHF for thymidine synthesis despite a methionine synthase deficiency-induced folate trapping. Future studies should be directed towards comparing the genetic stability of methionine-dependent and methionine-independent cells under conditions of methionine and/or folate deficiency conditions that allow cell division to proceed. It may also be important to use 5-MeTHF as the folate source in these cultures, because this folate species requires a methionine synthase-mediated conversion to THF (which is then converted to 5,10-MnTHF) before it can participate in thymidine synthesis. A lack of effect in this system may be due to the fact that folic acid was used as the folate source, a folate species that does not require any methionine synthase-mediated conversions before it can participate in thymidine synthesis.

The theory that methionine-dependency may be influenced by mutations in key folate-metabolising enzymes was not supported by the results presented here (Table 4.4). It is

not known to what extent the A2756G mutation affects methionine synthase activity, however, there is evidence indicating that people without the mutation are at a modestly higher risk for hyperhomocysteinemia than hetero- or homozygous mutants (Harmon *et al.*, 1999; Tsai *et al.*, 2000). It is evident that the A2756G mutation or lack of it in methionine synthase cannot account for the methionine-dependent phenotype. Furthermore, because the C677T and A1298C MTHFR polymorphisms are known to reduce enzyme activity by 70% (Frosst *et al.*, 1995) and 39% (van der Put *et al.*, 1998) in homozygous mutants respectively, it seems likely that MTHFR activity does not affect methionine-dependency in this system.

The results of this experiment suggest that relatively methionine-dependent lymphocytes proliferate faster than methionine-independent cells in $met^{+}hcy^{-}$ serum-free media. This finding is in agreement with that of Fiskerstrand *et al.* (1997) who reported that a methionine-dependent glioma cell line (P60) proliferated at a faster rate than the methionine-independent variant (P60H) in $met^{+}hcy^{-}$ media. One plausible explanation for an elevated division rate of methionine-dependent cells in $met^{+}hcy^{-}$ media is that homocysteine levels are elevated due to an inefficient recycling to methionine (which we speculate to be the underlying cause of methionine-dependency). This, in turn, may cause a build up of SAM and a subsequent increase in polyamine synthesis in methionine-independent cells. Polyamines are inducers of cell proliferation (Heby, 1981). However, the data on plasma homocysteine concentrations do not appear to support the view that methionine-dependence phenotype is a cause of hyperhomocysteinemia.

Because methionine-dependency can exist in both normal and cancer cells of the same individual (Mikol and Lipkin, 1984) it seems likely that methionine-dependent cancer cells may simply develop in individuals who are originally methionine-dependent. These observations do not exclude the possibility that methionine-dependence may be a risk factor for cancer. Although our studies have shown no relationship between methionine-dependency and DNA damage, as measured by the cytokinesis-block micronucleus assay, results in serum-free medium suggested an increased rate of cell proliferation which may be a risk factor for cancer by increasing the probability of uncontrolled cell division in the early and late stages of cancer. It is possible that the consumption of methionine-rich foods (such as meat, fish and dairy products) may increase the risk for cancer or increase

cancer growth by a (polyamine-mediated?) stimulation of cell division. Methionine-restriction has been proposed as a method for controlling the growth of methionine-dependent tumours (or tumours in individuals with the methionine-dependent phenotype) (Guo *et al.*, 1993. Breillout *et al.*, 1990. Hoshiya *et al.*, 1995).

In conclusion we have shown that normal human lymphocytes exhibit a wide variation of methionine-dependency or abilities to proliferate in $met^{-}hcy^{+}$ media. Furthermore methionine-dependency is not related to baseline micronucleus frequency and is not determined by common mutations in MTHFR and methionine synthase. We found that methionine-dependency is related to the ability of cells to proliferate in serum-free $met^{+}hcy^{-}$ media and speculate that this may be due to a higher production of growth-stimulating polyamines in methionine-dependent cells.

4.5 Publication resulting from this chapter

Crott,J., Thomas,P. and Fenech,M. (2001) Normal human lymphocytes exhibit a wide range of methionine-dependency which is related to altered cell division but not micronucleus frequency. *Mutagenesis*, **16**(4), 317-322.

Chapter 5: Developing a long-term CBMN culture protocol for studying B-vitamin deficiency

The conventional CBMN assay involves culturing human lymphocytes for three days. Preliminary experiments in this laboratory suggested that three days, the time taken for most primary lymphocytes to undergo only one or two rounds of cell division, is insufficient time to observe the effect of low folic acid concentrations on micronucleus (MN) induction. This chapter describes the development of a nine-day culture protocol, which enables the effects of folic acid deficiency on various markers of chromosome damage to be observed.

5.1 Cell culture medium

The experiments described in this chapter involved testing the dose-related effects of folic acid, vitamin B_{12} and vitamin B_6 concentrations in culture medium on cell growth and MN frequencies. Because there are no commercial suppliers of RPMI-1640 medium (the medium generally used for lymphocyte and CBMN cultures) without these three vitamins, it was necessary for me to custom make the medium for these experiments in this laboratory.

Except for folic acid, vitamin B_{12} and vitamin B_6, the RPMI-1640 medium was made to the specifications of Moore and Woods (1976). All chemicals were of tissue culture grade and were obtained from Sigma (St. Louis, MO, USA).

5.1.1 Concentrates

Because all of the chemicals in cell culture medium are present in such small amounts, concentrated solutions had to be prepared to ensure that the correct amount of each chemical was added. The use of concentrates also minimised variation in medium composition from batch to batch. Concentrates were made for the vitamin mix, salt mix, sodium phosphate and calcium nitrate. The concentration of these mixes was determined by the solubility of the constituents in water and/or the minimum amount that could be weighed accurately (0.01 g in this case).

5.1.1.a Vitamin mix

The vitamin mix was prepared as a 50x concentrate. All vitamins were weighed in small disposable plastic weighing trays and tipped into a clean 200 ml beaker. Weighing trays were rinsed several times with milli-Q water. The lights were turned down in the laboratory and the beaker was chilled on ice kept covered with aluminium foil because some vitamins are heat and light sensitive. The amount of each vitamin required to prepare one litre of a 50x concentrate are given in Table 5.1. After all vitamins were added to the beaker, the solution was poured into a 1L volumetric flask and rinsed several times with milli-Q water. The volume in the flask was then made up to 1L and the solution was sterilised using a 0.22 μm filter before the mixture was accurately aliquoted into 20 ml volumes aliquots in labelled 70 ml pots and stored at -20 °C.

Table 5.1 Vitamin concentrate

Vitamin	Cat. #	MWt	g/L in RPMI	moles/L	50x (g)
P-amino benzoic acid	A9580	137.1	0.001	7.29 e-6	0.05
D-biotin	B4639	244.3	0.0002	8.19 e-7	0.01
D-panothenic acid	P5155	238.3	0.00025	1.05 e-6	0.01
Choline chloride	C7527	139.6	0.003	2.15 e-5	0.15
Myo-inositol	I7508	180.2	0.035	1.94 e-4	1.75
Niacinamide	N0636	122.1	0.001	8.19 e-6	0.05
Riboflavin	R9504	376.4	0.0002	5.31 e-7	0.01
Thiamine HCL	T1270	337.3	0.001	2.96 e-6	0.05
Glutathione	G6013	307.3	0.001	3.25 e-6	0.05

Cat. #, Sigma catalogue number; MWt, molecular weight

5.1.1.b Salt concentrates

Three salt concentrates were prepared as described in Table 5.2. Solutions were made in 1L volume using a volumetric flask and stored at room temperature. All of the salts could not be added together in the same concentrate because they precipitate out of solution.

Table 5.2 Salt concentrates

Compound	Cat. #	MWt	g/L in RPMI	moles/L	g
10x Salt concentrate					10x (g)
Potassium chloride	P5405	74.55	0.4	5.36 e-3	4
Magnesium sulphate	M2643	120.4	0.0488	4.05 e-4	0.5
Phenol red	P5530	376.4	0.005	1.33 e-5	0.05
10x Sodium phosphate concentrate					10 x (g)
Sodium phosphate	S5136	142	0.8	5.6 e-3	8
50x Calcium nitrate concentrate					50x (g)
Calcium nitrate	C7652	236.2	0.1	4.23 e-4	5

5.1.2 Preparation of medium

1. Add 100 ml of the 10 x salt concentrate to a 2L beaker. The solution was stirred using a magnetic flea.
2. Add approximately 600 ml of Milli-Q water.
3. Add 100 ml of 10 x sodium phosphate concentrate.
4. Add 5.6 ml of 1M HCl.
5. Slowly add 20 ml 50x calcium nitrate.
6. Add 20 ml of 50x RPMI 1640 amino acid concentrate (R7131).
7. Add 6 g sodium chloride (S5886), 2 g sodium bicarbonate (S5761) and 2 g D-glucose (G7021).
8. Add 0.06 g streptomycin and 0.1 g penicillin G.
9. Add 10 ml of 10 mM L-glutamine (G7513).
10. Add 20 ml 50x vitamin concentrate (turn lights off in lab).
11. Add sterile folic acid/vitamin B_{12}/vitamin B_6 as required.
12. Adjust pH to 7.2 using 1M NaOH or 1M HCL.
13. Transfer the media to a clean 1L volumetric flask and make the volume up to 1L with milli-Q water.
14. Sterilise the medium using a (pre-sterilised) 1L filter unit fitter with a 0.22 μm filter.
15. * Pipette 56.2 ml of dialysed foetal calf serum (1000 MWt cut-off)(FCS)(Trace Biosciences, Victoria, Australia) and 1 ml of interleukin-2 (IL-2; 10,000 U/ml) (Roche diagnostics, Basel, Switzerland) into the medium in the lower part of the filter unit and then thoroughly mix the medium by gently stirring with the [sterile 10 ml] pipette. This is equivalent to 5 % FCS (v/v) and 10 U/ml IL-2.

* Experiments 1 and 2 involved testing how much FCS and IL-2 was required for the long-term protocol. Therefore step 15 is not applicable to the medium prepared for these experiments.

5.2 Experiment 1

5.2.1 Aim

The aim of this experiment was to determine whether 1) lymphocytes could grow for up to 9 days in culture, 2) how much FCS is required to support this growth and 3) whether multiple additions of the mitogen is required to maintain proliferation.

5.2.2 Method

Folic acid, vitamin B_6 and vitamin B_{12} concentrations in cell culture medium

One of the general aims of these experiments was to study the effect of folic acid, vitamin B_6 and vitamin B_{12} deficiency on MN induction at concentrations that are in a physiological range. The first step in this process involved selecting concentrations which a) were sufficient to maintain low MN frequencies and b) caused a significant increase in MN frequencies. Concentrations for preliminary experimentation were chosen following an extensive review of the literature. A summary of both plasma and *in vitro* vitamin concentrations used in previous research and the corresponding effect of these concentrations is given in Table 5.3.

From the literature it seems that the average plasma range for folic acid is 10 – 30 nM while *in vitro* experiments have generally used concentrations that are outside the normal physiological range. Most *in vitro* experiments have compared the cellular response to zero folic acid and millimolar folic acid concentrations, both of which are outside the normal physiological range. Duthie and Hawdon (1998) do however show that cells can be supported in culture at concentrations within the physiological range. At this early stage of medium refinement, the folic acid levels chosen for medium were as follows:

Replete medium: 60 nM

Low folic acid medium: 24 nM (40% replete)

Deficient folic acid medium: 12 nM (20% replete)

Plasma vitamin B_{12} concentrations range from 160 – 400 pM and although very little research has been done to determine the effect of vitamin B_{12} concentration on cells *in vitro*, the concentration in normal RPMI medium is 370 pM which is within this range. The medium vitamin B_{12} concentrations selected were as follows:

Replete medium: 350 pM

Low vitamin B_{12} medium: 140 pM (40% replete)

Deficient vitamin B_{12} medium: 70 pM (20% replete)

Vitamin B_6 is present in plasma in a number of different forms or vitaminers, which include pyridoxine, pyridoxal 5'-phosphate, pyridoxine and pyridoxic acid. On average the sum of all vitaminers in plasma ranges from 100 – 200 nM. In culture, it has been shown that medium containing 300 nM of pyridoxine-HCl can support cell growth effectively (Matthews *et al.*, 1994). The medium vitamin B_6 (in the form of pyridoxine-HCl) concentrations selected were as follows:

Replete medium: 100 nM

Low vitamin B_6 medium: 40 nM (40% replete)

Deficient vitamin B_6 medium: 20 pM (20% replete)

Table 5.3 Cited folic acid and vitamin B_6 and B_{12} concentrations

Reference	Status	[folate] nM	Comment
PLASMA FOLATE CONCENTRATIONS (*in vivo* studies)			
Blount *et al.*, 97	BL low folate BL normal folate	9.7 26	8x ↑ uracil than normal
Fenech & Rinaldi, 94	BL	27 - 32	
Fenech & Rinaldi, 95	BL, vego F (41-60 y) BL, non-vego M (20 – 40 y)	44 21	
Fenech *et al.*, 97a	Control, M (51-74 y) 700 μg FA/d (8 wk), M (50-70 y)	13 44	No change in MN
Jacob *et al.*, 98	Depletion- 56 μg FA/d (5 wk)	19.5 to 9.3	↓ DNA-Me, ↑uracil, ↑ hcy
Titenko-H. *et al.*, 98	Samples from Jacob *et al.*, 98	19.5 to 9.3	30%↑ MN
MEDIA FOLIC ACID CONCENTRATIONS (*in vitro* studies)			
Duthie & Hawdon, 98	Dose-response	0, 2.3, 22, 226 nM	↑ DNA with ↓ FA
James *et al.*, 94	Medium +/- FA	0 or 2.9 μM	↑ DNA-uracil, ↓ division w/o folic acid
Moore and Woods, 76	Normal RPMI-1640	2.26 μM	
Reidy *et al.*, 83	Medium +/- FA	0 or 2.26 μM	↑ DNA gaps + breaks w/o FA
Reidy, 87	Medium +/- FA	0 or 2.26 μM	↑ DNA breaks w/o FA
Summary:	***in vivo* 10 – 30 nM, *in vitro* 0 – 2.26 μM**		
Reference	**Status**	**[B_{12}] pM**	**Comment**
PLASMA VITAMIN B_{12} CONCENTRATIONS (*in vivo* studies)			
Fenech & Rinaldi, 94	BL	260 - 270	
Fenech & Rinaldi, 95	BL, vego M (20 – 40 y) BL, non-vego M (20 – 40 y)	160 352	
Fenech *et al.*, 98	Control (25 y) 7-20 μg B_{12}/d (24 wk) (25 y)	344 397	↓ MN in high MN people (also on FA supplement)
MEDIA VITAMIN B_{12} CONCENTRATIONS (*in vitro* studies)			
Moore and Woods, 76	Normal RPMI-1640	370	
Summary:	***in vivo* 160 – 400 pM, *in vitro* 370 pM**		
Reference	**Status**	**[B_6] nM ***	**Comment**
PLASMA VITAMIN B_6 CONCENTRATIONS (*in vivo* studies)			
Brussard *et al.*, 97	BL, M (20 – 49 y) BL, M (50 – 79 y) BL, F (20 – 49 y) BL, F (50 – 79 y)	48 – 53, 13 - 17 32 – 49, 10 - 17 33 – 48, 10 – 15 36 – 55, 11 - 16	PLP, PL PLP, PL PLP, PL PLP, PL
Kang-Y. & Kirsey, 92	BL, F (20 – 30 y) 27 mg/d PN (7days), F (20 – 30 y)	57, 21, 16, 13 (93) 424, 108, 77, 11 (521)	PLP, 4-PA, PL, PN (total all vitaminers) PLP, 4-PA, PL, PN (total all vitaminers)
Vermaak *et al.*, 90	BL, non-smokers BL, smokers	15, 7 19, 8	PLP, PL PLP, PL
MEDIA VITAMIN B_6 CONCENTRATIONS (*in vitro* studies)			
Matthews *et al.*, 1994	CBFI low B-vitamin medium	300 nM	PN- hydrochloride
Moore and Woods, 76	Normal RPMI-1640	4.86 μM	PN-hydrochloride
Summary: (all vitaminers)	***in vivo* 100 – 200 nM, *in vitro* 300 nM or 4.86 μM**		

BL, baseline; F, female; FA, folic acid; hcy, homocysteine; M, male; 4-PA, pyridoxic acid; PL, pyridoxal; PLP, pyridoxal 5' phosphate; PN, pyridoxine; vego, vegetarian; w, weeks; y, years of age. * vitamin B_6 is present in a number of different forms (vitaminers) in plasma. ↑, increase; ↓, decrease.

Cell culture protocol

Approximately 45 ml blood was drawn from myself by venipuncture using lithium-heparinized evacuated containers. Lymphocytes were isolated as described in section 2.2.1 and cultured at a concentration of 5 x 10^5 cells/ml in 4 ml of culture medium in 25 ml culture flasks with vented lids (Sartstedt, Adelaide, Australia). Three sets of cell culture flasks, in duplicate, were set up for this experiment (a total of 18 flasks). Each set contained replete medium (without IL-2, see above) with either 0, 5 or 10% dialysed FCS. Cell division was initiated by the addition of PHA (22 μg/ml) and flasks were incubated at 37°C (5% CO_2) laying flat in humidified incubator.

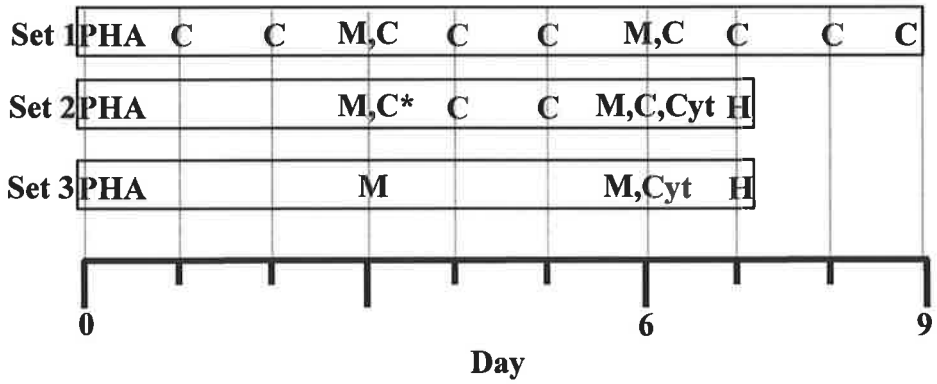
Culture set 1 was counted every 24 hr to monitor cell growth between the media with different FCS contents. Culture set 2 involved the addition of PHA after the media change at day three. The purpose of set 2 was 1) to compare the cell counts with set 1 at days four, five and six to determine whether a second addition of PHA is needed to maintain cell growth in terms of cell number and 2) to compare binucleated cell frequencies (on microscope slides prepared on day 7) with set 3 to determine whether a second PHA addition affected the frequency of binucleated cells, the cells in which MNi are scored.

Set 1: Cell number and viability were determined using a coulter counter and a haemocytometer with trypan blue staining every 24 hr in these cells. Culture medium was changed at days three and six. At these times the cell suspension was removed into a 10 ml conical base tube, which was then gently spun (100 x g) to pellet the cells before the supernatant was removed by aspiration. Approximately 500 μl of supernatant was left, to which 5 ml of fresh medium was added before the cells were resuspended and returned to the culture flasks.

Set 2: Cultures were set up as described above, however PHA (22 μg/ml) was added for a second time after the medium was changed on day three. Cells from this set were only counted on days three, four, five and six because they were identical to set 1 prior to this time. After the medium change on day six, cytochalasin-B was added (4.5 μg/ml) to block cells in the binucleated stage. Cells were harvested onto microscope slides 24 hr later (on day seven) in order to count binucleated cell frequency as described in section 2.2.3 and 2.2.4.

Set 3: This set was similar to set 2, however PHA was only added to cultures once. The experimental design is illustrate in Figure 5.1 below.

Figure 5.1 Experimental design



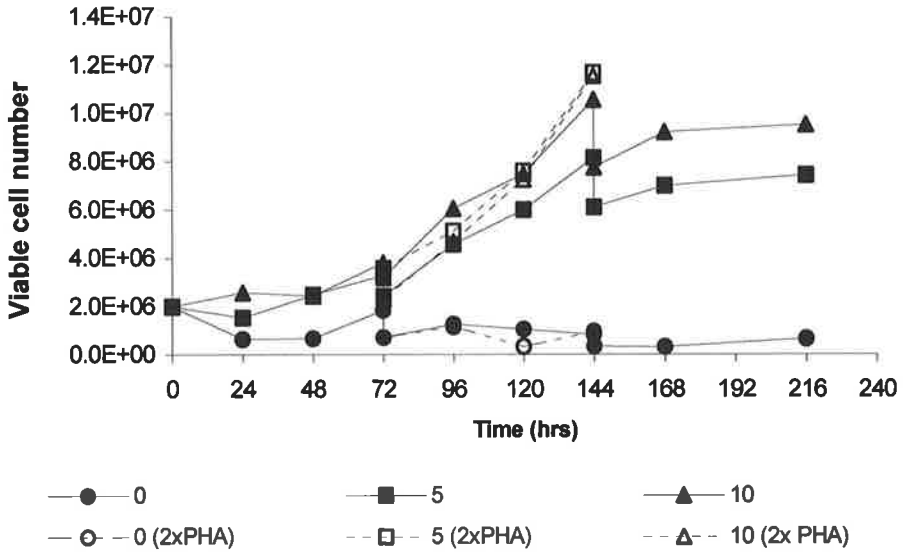
C, count cells; Cyt, addition of cytochalasin-B to block cells in a binucleate phase; H, harvest cells onto microscope slides; M, change medium; PHA, phytohaemagglutinin addition; * second PHA addition.

5.2.3 Results

The cell proliferation data for set 1 and 2 are shown in Figure 5.2. It is clear that proliferation was fastest in medium containing 10% FCS, while 0% FCS did not support cell proliferation. Cell numbers in medium with 5 and 10% FCS were very similar up to day 3, at which time cell proliferation in 10% FCS continued at a faster rate than in 5% FCS. It is also clear from the graph that a second addition of PHA was not necessary to maintain cell proliferation, however it appeared to improve cell proliferation slightly in medium with 5% FCS.

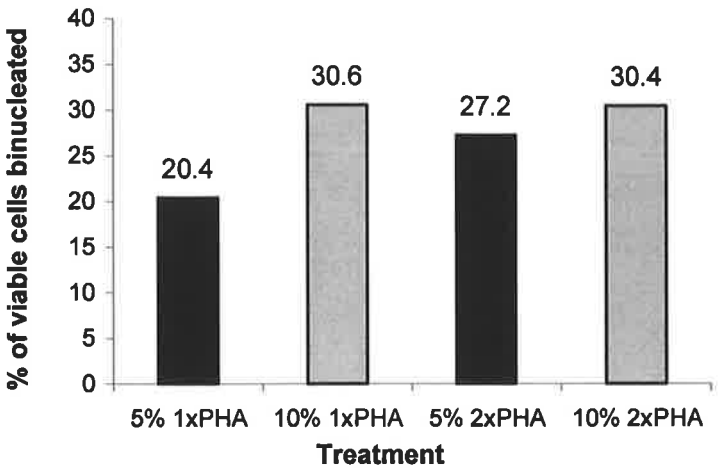
Binucleated cell (BN) frequencies for the cytokinesis-blocked cultures (set 2 and 3) are shown in Figure 5.3. BN data is not presented for cells grown in 0% FCS because these cultures contained no viable cells. Furthermore, as seen in Figure 5.2, the cell numbers for these cultures did not increase from the initial number.

Figure 5.2 Effect of FCS and second PHA addition on lymphocyte proliferation



Medium changed at days 3 and 6. Cultures with a second PHA addition (dashed lines and open symbols) are identical to the other set up to day 3.

Figure 5.3 Effect of FCS and second PHA addition on binucleate frequencies in cytokinesis-blocked lymphocyte cultures



1 x PHA, single PHA addition at start of culture; 2 x PHA, PHA added at start and after medium change on day 3; 5/10 %, percentage of FCS in the medium. Binucleates counted on microscope slides under 1000x magnification.

5.2.4 Conclusions

The data from this experiment clearly indicate that lymphocytes can be successfully cultured for 9 days in the medium prepared in this laboratory. Furthermore, the results show that while a there is some increase in cell division with 10% FCS, 5% FCS can effectively support cell growth in this system. Medium containing 5% FCS was chosen for all later experiments because this would introduce smaller quantities of additional B vitamins to the system than the higher amount of FCS. When comparing the frequency of binucleated cells in the cytokinesis-blocked cultures it can be seen that cultures with 10% FCS have approximately 10% more binucleated (BNed) cells than cultures with 5% FCS. Although the higher BN frequency makes scoring CBMN slides easier it was anticipated that the BN frequency could be enhanced by other means. For example, the use of growth factors such as interleukin-2 as suggested by O'Donovan *et al.* (1995) was expected to improve cell division. This was tested in experiment 2.

In summary, the results suggest that a single addition of the mitogen is sufficient to maintain proliferation in these cells. While a second addition of PHA slightly improves cell number and BN frequency in cultures with 5% FCS, it was hoped that proliferation can be significantly improved with the use of IL-2. Furthermore, the second PHA addition also complicates the culture procedure unnecessarily and was not continued in further experiments.

5.3 Experiment 2

5.3.1 Aim

To determine 1) whether the addition of interleukin-2 (IL-2) to culture medium increases the rate of cell division and 2) to test cell growth in medium deficient in folic acid, vitamin B_{12} and vitamin B_6.

5.3.2 Method

Eighteen ml of blood was obtained from two asymptomatic male volunteers (both 24 yr old) and lymphocytes were isolated as previously described (section 2.2.1). Lymphocytes from each volunteer were cultured in four types of RPMI-1640 media, each containing 5% dialysed FCS. The concentrations of folic acid, vitamin B_6 and vitamin B_{12} in each of the media are given in Table 5.4.

Table 5.4. Media folic acid, vitamin B_{12} and vitamin B_6 concentrations

Medium	[folic acid] nM	[vitamin B_{12}] pM	[Vitamin B_6] nM
Replete	60	350	100
Folic acid deficient	12	350	100
Vitamin B_{12} deficient	60	70	100
Vitamin B_6 deficient	60	350	20

Cells were cultured at 7.5 x 10^5 cells/ml in three ml of medium (note that not enough cells were available to increase the culture volume) in 25 ml flasks and PHA (22 μg/ml) was added prior to incubation. The culture duration was nine days; with media being changed at day three and six, at which time cell number and viability was also determined. At day three, cultures were split into two flasks and IL-2 (10 U/ml) was added to one of the flasks. Interleukin-2 was added to these cultures a second time after the media was changed on day nine.

5.3.3 Results

As shown in Figure 5.4, cell number steadily increased with time in most cultures. Cell growth was very similar between the media types up to day three, at which time an effect of vitamin concentrations and IL-2 became apparent.

Cell number at day nine was higher in every type of medium and in both volunteers when IL-2 was added (open symbols) compared to when IL-2 was omitted (closed symbols). Furthermore, the cultures with the highest cell number at day nine were grown in replete media with IL-2 while the cultures with the lowest cell number were grown in low folic acid media without IL-2. For cultures grown with IL-2, the omission of vitamin B_6 and B_{12} from the media had only a very minor effect on cell number at day 9 but without IL-2 there was a greater effect (range 2 and 3 x 10^6 v 8 and 3 x 10^6 cells for volunteers 1 and 2, with and without IL-2 respectively).

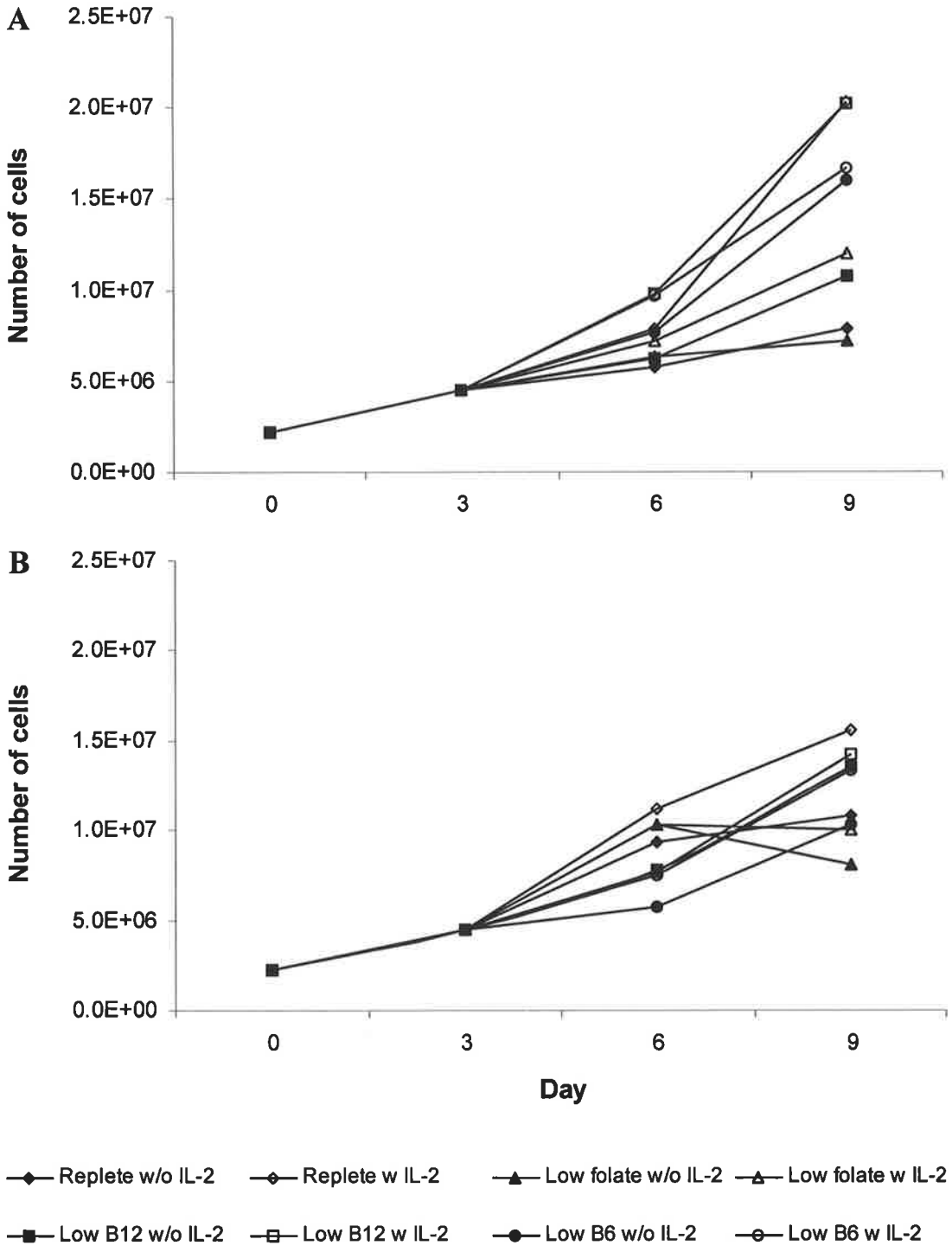
Interestingly, in the cultures without IL-2, it was not the replete media that has the highest cell number at day nine but the low B_6 and low B_{12} media for volunteer 1 and 2 respectively.

5.3.4 Conclusions

The results of this experiment clearly indicate that the in-house medium can effectively support cell growth and that the concentration of folic acid, vitamin B_6 and vitamin B_{12} does have some effect on cell growth. In contrast to the majority of published experiments, which use sub- and supra-physiological concentrations, these experiments use concentrations of folic acid vitamin B_{12} and vitamin B_6 that are in the physiological range.

The results also clearly indicate that IL-2 increases cell division in all of the media types tested. When the media is changed, the growth factors that have been produced by the cells over the past three days are removed. It is likely that the exogenous IL-2 acts by replacing some of the growth stimulus provided by the cell's natural growth factors that are secreted during culture period but are removed when the medium is refreshed. All further experiments were conducted using medium containing IL-2. The protocol of this experiment involved adding the IL-2 only after a media change but further experiments used media containing IL-2 from the start of culture in an effort to further enhance cell division. Furthermore, adding IL-2 directly to the medium during its preparation minimises variation in the actual amount of IL-2 added to each culture flask.

Figure 5.4 Effect of IL-2 and deficient medium on lymphocyte proliferation



A, volunteer 1; **B**, volunteer 2; w, with; w/o, without; IL-2, interleukin-2. Because cultures were split at day three into two sets (one with and one without IL-2) the cell number is halved at this time. The graph shows the cell number calculated assuming no cells were removed from the culture by using the percentage increase in cell number and applying it to the pre-split number.

Although this experiment established that the vitamin concentration, folic acid in particular, affects the rate of cell division in this system, it is not known whether levels of genetic damage (MN frequency) are also modulated. The next experiments involved testing the effect of these same media on MN induction in lymphocytes.

5.4 Experiment 3

5.4.1 Aim

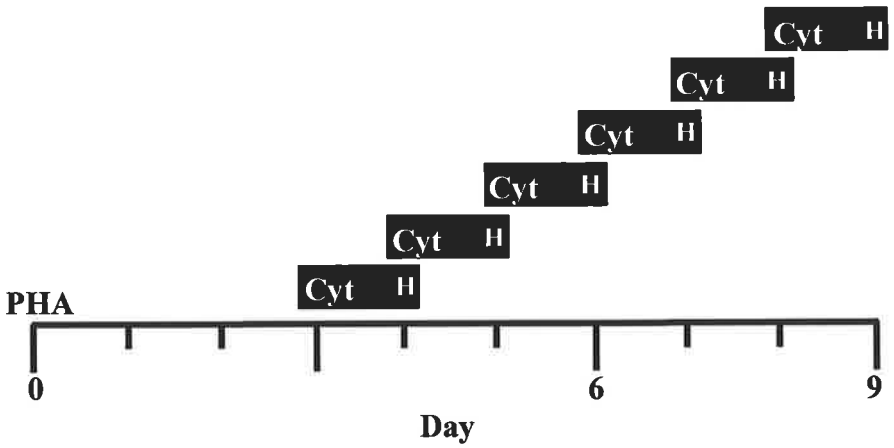
To determine 1) the effect of low folic acid, vitamin B_{12} and vitamin B_6 concentrations on MN induction and 2) the optimal time to block cytokinesis and harvest cells in terms of MN induction.

5.4.2 Method

Thirty-six ml of blood was collected from two asymptomatic male volunteers and lymphocytes were isolated as described (section 2.2.1). Volunteer #1 was 24 yr old while volunteer #2 was 44 yr old. Four lymphocytes cultures were initiated for each volunteer at a concentration of 5 x 10^5 cells/ml in eight ml of media and in 25 ml culture flasks. The four types of media are those used in experiment 2, namely replete, folic acid deficient, vitamin B_{12} deficient and vitamin B_6 deficient medium. Each contained 5% FCS and 10 U/ml IL-2. The culture volume was increased from the previous two experiments and the flasks were placed in the incubator in the upright position rather than laying flat. It was suggested that lymphocytes might proliferate faster when their concentration is increased at the bottom of the flask to increase cell-to-cell contact (Michael Fenech, personal communication). Furthermore, there was a need to increase the cell yield because this system was being developed to grow cells in which DNA uracil-content was to be measured, a procedure that requires a relatively large amount of DNA.

After placing cells in culture, PHA (22 μg/ml) was added and cultures were incubated as described previously. Culture medium was refreshed at day three and six. On day three, four, five, six, seven and eight a 750 μl aliquot of cell suspension was removed from each flask into six ml culture tubes and cytokinesis was inhibited with cyto-B (4.5 μg/ml). This was done to determine which was the best time, in terms of MN expression, for blocking cytokinesis. Twenty-four hr after the addition of cyto-B, cells were transferred to microscope slides, stained and scored for MNi as previously described (section 2.2.3 and 4).

Figure 5.5 Experimental design



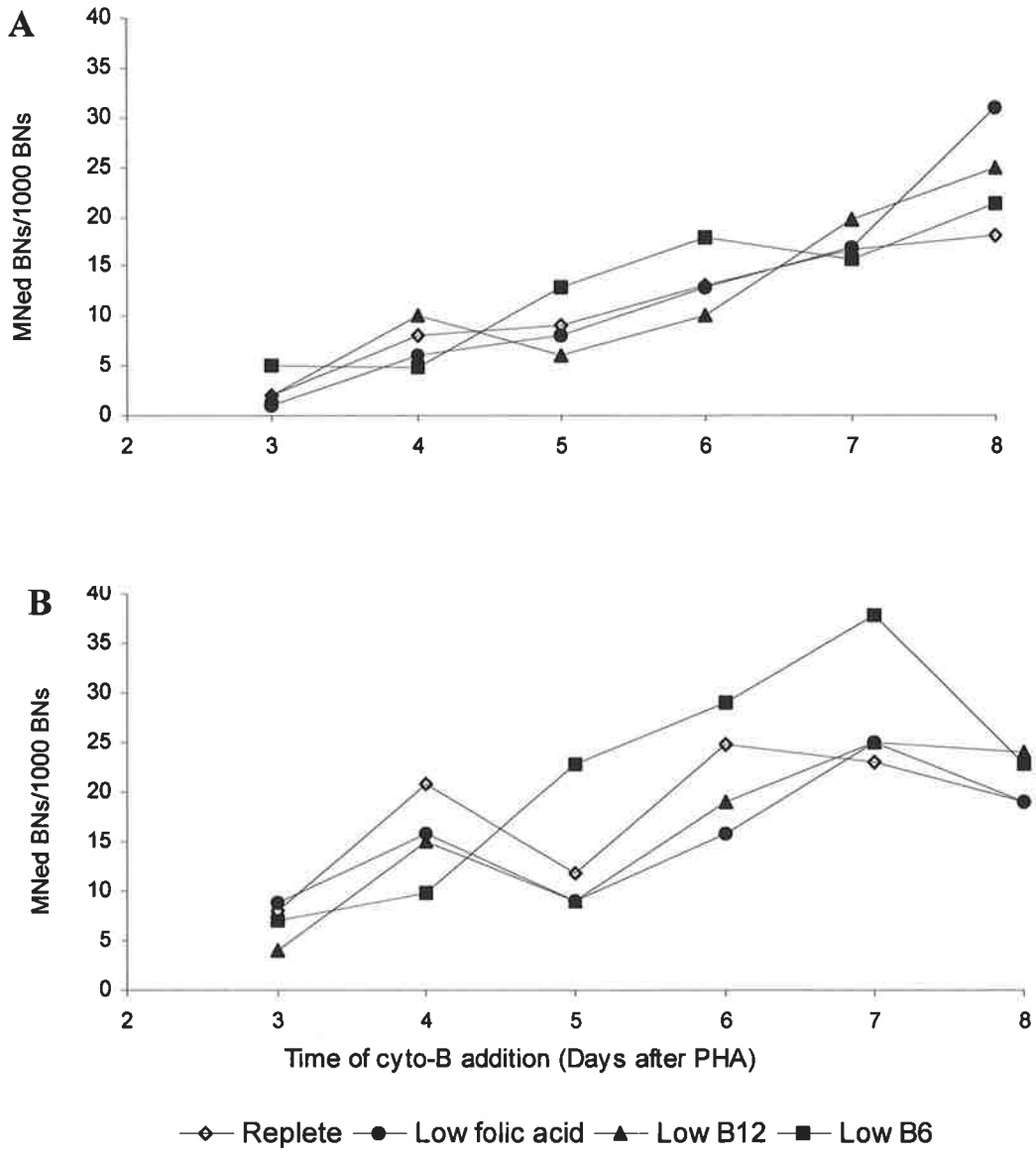
Cyt, addition of cytochalasin-B; H, harvest cells onto microscope slide; PHA, addition of phytohaemagglutinin. Cells were grown in culture flasks in four types of medium. On day 3, 4, 5, 6, 7 and 8, an aliquot of cells was removed from the flasks into culture tubes, cytokinesis was inhibited and cells were harvested 24 hr later.

5.4.3 Results

As shown in Figure 5.6, there is a steady increase in the MN frequency for all medium types and for both volunteers with culture time. This result suggests that the nine-day protocol, where cyto-B is added at day eight, may be the optimal schedule in terms of maximising the chance of observing differences in MN frequency between cultures.

There is no clear effect of medium vitamin-content on MN induction, however cultures grown in 'replete' medium do seem to have the lowest or equal lowest MN frequencies. Furthermore, as culture time increases the difference between the lowest and highest MN frequency tends to increase.

Figure 5.6 Effect of harvest time and B-vitamin concentration on micronuclei



A, results for volunteer 1; **B**, results for volunteer 2; cyto-B, cytochalasin B; MNed BNs/1000 BNs, micronucleated binucleates per 1000 binucleates; PHA, phytohaemagglutinin.

Replete medium = 60 nM folic acid, 350 pM vitamin B_{12}, 100 nM vitamin B_6; Low folic acid medium= 12 nM folic acid (replete B_6 and B_{12}); Low B_{12} medium= 70 pM vitamin B_{12} (replete folic acid and B_6); Low B_6 medium= 20 nM vitamin B_6 (replete folic acid and B_{12}).

Cells were transferred to microscope slides 24 hr after the addition of cytochalasin-B.

5.4.4 Conclusions

Although there was no clear effect of vitamin concentration on MN induction seen for this experiment, the results suggest that the nine-day protocol in which cytokinesis is inhibited after eight days may be an effective tool for testing the genotoxic effects of various culture conditions.

It is likely that the vitamin concentrations used here were of too narrow a range to observe differences in MN induction. In the case of folic acid especially, it is likely that 60 nM may already be a 'deficiency' dose because it is insufficient to minimise or prevent a rise in MN frequency during a nine day culture period. The results of these experiments suggest that the concentration of folate required to maximise cell proliferation in lymphocytes is less than that required to minimise MN induction. For example Figure 5.4 clearly shows that cell numbers after nine days are much lower in cultures with 12 compared to 60 nM folic acid (both with and without IL-2 present). In contrast, the results shown in Figure 5.6 show that MN frequencies are quite similar between these two concentrations. The next section describes a dose-response experiment that was designed to determine the effect of folic acid concentration, over a broader dose range on MN induction. The 'replete' folic acid concentration was revised to 120 nM, with the next lowest concentration being ten-fold lower, or 12 nM in an effort to maximise the chance of observing a difference in MN induction.

In conclusion, the information obtained from this experiment facilitated further refinement of the cell culture protocol and better selection of vitamin concentrations that cause changes in MN induction.

5.5 Experiment 4

5.5.1 Aim

To perform dose response experiments for folic acid, vitamin B_6 and vitamin B_{12} in order to select concentrations that cause a dose-related increase in MN induction.

5.5.2 Method

Approximately 40 ml of blood was collected from three asymptomatic male volunteers (all 24 yr of age) and lymphocytes were isolated as described previously (section 2.2.1). Lymphocytes were cultured at a concentration of 5 x 10^5 cells/ml in seven ml of RPMI-1640 medium (5% dialysed FCS and 10 U/ml IL-2) and in 25 ml culture flasks. The medium was divided into three groups, each with five different concentrations of either folic acid, vitamin B_6 or vitamin B_{12}. The vitamin concentrations of the media were as follows:

Group 1- Folic acid dose response: 0, 3, 6, 12 and 120 nM folic acid (each with 700 pM vitamin B_{12} and 200 nM vitamin B_6).
Group 2- Vitamin B_{12} dose response: 0, 17.5, 35, 70 and 700 pM vitamin B_{12} (each with 120 nM folic acid and 200 nM vitamin B_6).
Group 3- Vitamin B_6 dose response: 0, 5, 10, 20 and 200 nM vitamin B_6 (each with 120 nM folic acid and 700 pM vitamin B_{12}).

Upon placing cells in the pre-warmed culture medium, cell division was stimulated by the addition of PHA (22 μg/ml) and culture flasks were incubated in the upright position. Medium was refreshed on day three and six after cell number and viability had been determined. Cells were returned to the flasks in 500 μl of spent medium in an effort to return some of the endogenously produced growth factors to the system. Approximately 5 x 10^6 cells were returned to the flask in this volume before a further 9.5 ml of fresh medium (pre-warmed) was added to the flask. On day eight, a 750 μl aliquot of cell suspension was removed from each flask into six ml culture tubes and cyto-B was added (4.5 μg/ml). These cells were harvested onto glass microscope slides, stained and scored

for MNi as described in section 2.2.3 and 4. When cell number was determined for the culture flasks at day nine, values were corrected for the removal of the 750 µl aliquot.

Cell division data is a projection of cell proliferation because only 5 x 10^6 cells were returned to the cultures at days three and six. The curves were generated by calculating the percentage increase in cell number in the last two three-day periods and applying it to the number of cells present at the end of the previous three day period (see below).

Projected cell # = end cell # + (end cell # x (%change in cell # /100)

where % change in cell# = (end cell # - begin cell #)/begin cell #) x 100

At the end of the culture period, cells from the folic acid dose response experiment were stored frozen for the future analysis of DNA-uracil content as described in section 6.2.4. These samples were analysed at the same time as those from chapter six, approximately one year after the MN data was collected.

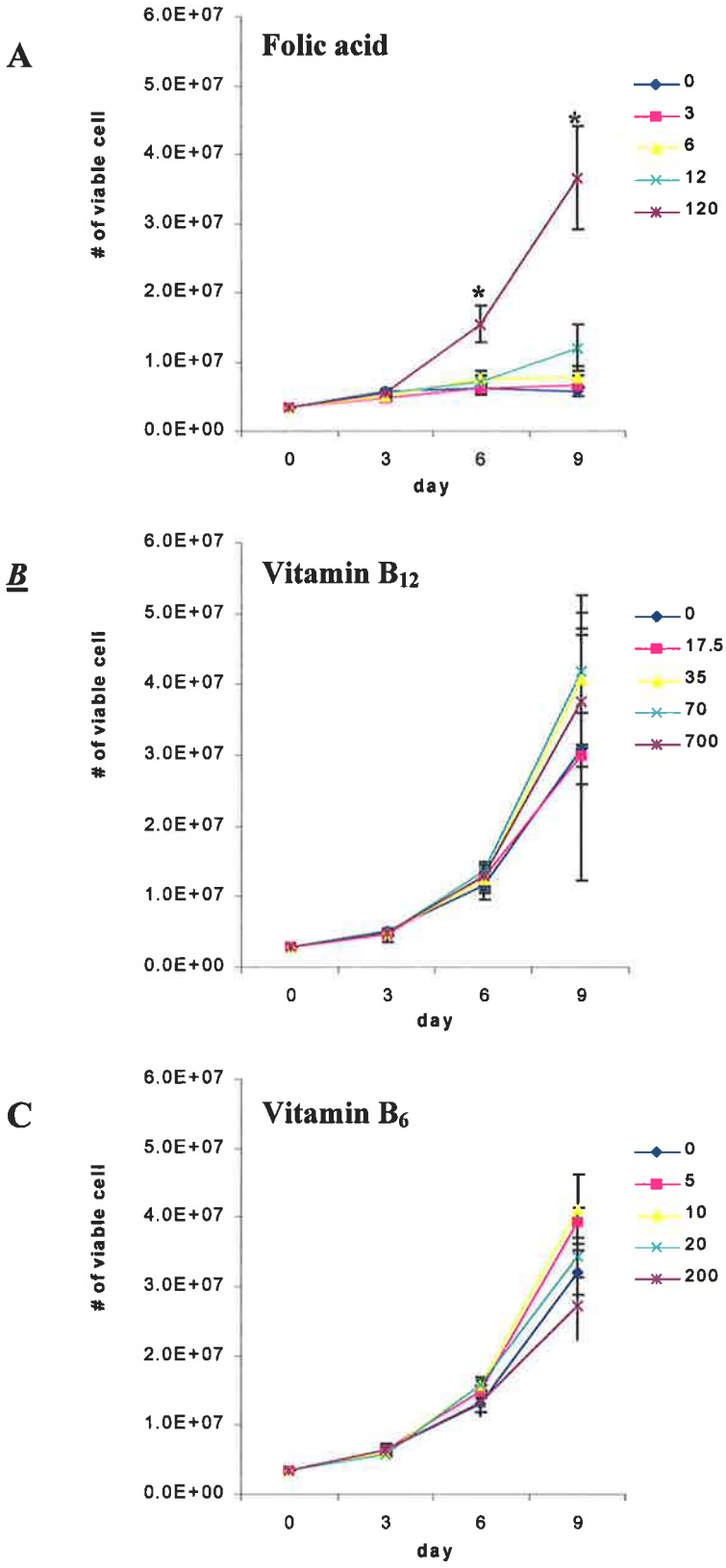
5.5.3 Results and Discussion

5.5.3.a Folic acid dose response

The cell proliferation data are presented in Figure 5.7 A. Two way ANOVA revealed that both folic acid concentration in the medium and culture time are significant determinants of cell number ([folic acid $P< 0.0001$; day $P< 0.0001$). Folic acid concentrations of below 12 nM supported only a very minor increase in cell number over the nine-day culture period. Both 12 and 120 nM supported a steady increase in cell number. Maximal cell division was seen in media with 120 nM with a projected cell number at nine days of approximately three-fold higher than for cultures grown in medium with 12 nM folic acid (Tukey's $P< 0.01$).

As well as supporting better cell growth, 120 nM folic acid minimised MN induction (Figure 5.8 A). The MN frequency of cultures grown with 120 nM folic acid was 8.1 ± 1.2 MNed BNs/ 1000 BNs, approximately 2.5-fold lower than the 20 ± 0.7 MNed cells observed with 12 nM. Furthermore, the MN frequency at 120 nM was significantly lower than at all other concentrations ($P< 0.05$). The highest MN frequency was observed with

Figure 5.7 Effect of B-vitamin concentration on cell proliferation (Expt. 4)



A, dose response to folic acid (nM); **B**, dose response to vitamin B_{12} (pM); **C**, dose response to vitamin B_6 (nM). N= 3 for each data point. Media is replete for vitamins not being tested. * P< 0.01 v all other cultures (Tukey's post test).

0 nM added folic acid (25.3 ± 1.7) although this concentration barely supported a maintenance of the initial cell number (Figure 5.7 A).

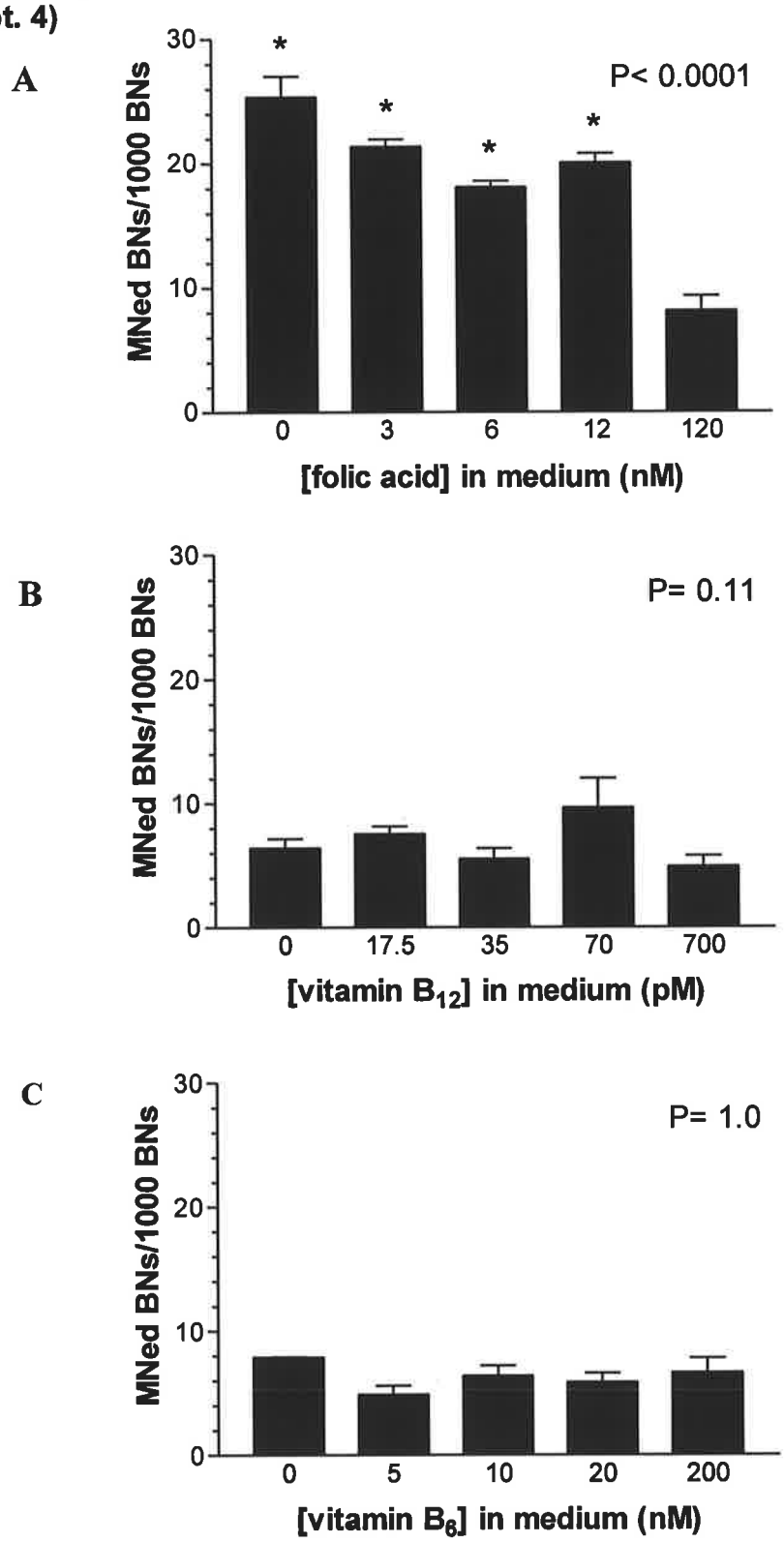
There was a significant negative correlation between MN frequency and folic acid concentrations (r^2= 0.26, P= 0.0013).

Whilst scoring the slides for this experiment, it became apparent that the slides that had a high frequency of MNed cells also seemed to have a high frequency of cells exhibiting nuclear budding. Nuclear buds are similar in appearance to MNi but are connected to a nucleus via a thin nucleoplasmic connection. The significance of these buds is introduced in section 2.2.4 and discussed thoroughly in Chapter 6. It is thought that nuclear budding is a method for the extrusion of amplified DNA from the nucleus of a cell. Photographs of cells exhibiting nuclear budding are shown in Figure 2.3 B.

As shown in Figure 5.9, folic acid concentration is negatively correlated with DNA uracil-content. Together with the fact that DNA uracil-content positively correlates with MN frequency (Figure 5.10), these results support the hypothesis that folic acid deficiency causes an accumulation of uracil in the DNA, which can result in the formation of double-stranded DNA breaks following excision-repair (Dianov *et al.*, 1991).

These results clearly indicate that this system is effective in studying the effects of folic acid deficiency on cell division and chromosome damage as measured by the CBMN assay.

Figure 5.8 Effect of B-vitamin concentration on micronucleus induction (Expt. 4)



A, folic acid dose response; **B**, vitamin B_{12} dose response; **C**, vitamin B_6 dose response. MNed BNs/1000 BNs, micronucleated binucleates per 1000 binucleates. * P< 0.001 v 120 nM folic acid.

5.5.3.b Vitamin B_{12} dose response

As shown in Figure 5.7 B, there was no apparent dose response effect of vitamin B_{12} concentration on cell number (two way ANOVA, [vitamin B_{12}] P= 0.9, day P < 0.0001). On day nine there was a 1 x 10^7 difference in cell number between the lowest and highest projected cell number, however this difference was not significant (P= 0.91). The two lowest cell numbers (3.1 x 10^7 and 3.0 x 10^7) came from cultures grown with 0 and 17.5 pM of added vitamin B_{12}. Somewhere between 17.5 and 35 pM, there seemed to be a threshold effect of vitamin B_{12} concentration on cell number as the three highest concentrations yielded similar cell numbers at day nine (3.8 – 4.2 x 10^7 cells).

There was no significant effect of vitamin B_{12} concentration on MN frequency (P= 0.11)(Figure 5.8 B). The MN frequency was quite consistent across treatments with all values being between 4.9 and 9.7 MNed BNs/1000 BNs. It was expected that the omission of vitamin B_{12} from medium would result in a trapping of folate as 5-methyl tetrahydrofolate (see section 1.6.1). There are three possibilities as to why the absence of vitamin B_{12} from the medium had no effect on MN induction when it has been repeatedly shown that *in vivo* vitamin B_{12} deficiency or inactivation causes chromosome breakage and MN induction.

1) Although there was no vitamin B_{12} added to the medium there was vitamin B_{12} in the FCS. Since the molecular weight of vitamin B_{12} (1355.4) is larger than the molecular weight cut-off of the filter used in dialysis (1000) of the FCS, this could be a significant source of vitamin B_{12}. Analysis of the FCS indicates that the vitamin B_{12} concentration is 363 pM which equates to 18.1 pM in the complete medium when present at 5% v/v. Although 18.1 pM is far lower than the normal plasma concentration range (160 – 400 pM), it is possible that the high folic acid concentration masked the effects of the vitamin B_{12} deficiency. Indeed, it is well documented that the symptoms of vitamin B_{12}-induced anaemia can be ameliorated by folic acid supplementation (see section 1.6.1). The following experiment (section 5.6) aimed to test the effect of the vitamin B_{12} deficiency under low folic acid conditions.

2) Folic acid was used for the experiment rather than 5-methyltetahydrofolic acid (5-MeTHF), which is the predominant folate species found in plasma (see Figure 1.1). Because folic acid is up-stream from 5,10-methylene tetrahydrofolate (5,10-MnTHF) in

the folic acid cycle, the folic acid added to this system can participate in the synthesis of thymidine without requiring any methionine synthase/vitamin B_{12}-mediated interconversion. In contrast, it is possible that if 5-MeTHF was used as the folate source, different results may have been observed. The reason for this is that 5-MeTHF requires the methionine synthase/vitamin B_{12}–mediated conversion to THF before it could participate in the synthesis of thymidine. In this situation, low vitamin B_{12} concentrations may cause a depletion of 5,10-MnTHF, the methyl donor for thymidine synthesis, and facilitate an intracellular increase in uracil concentrations which is know to lead to double-stranded DNA breakage (see section 1.4.2). It is clear that future experiments should test whether the type of folate used in culture medium affects the cellular response to vitamin B_{12} or vitamin B_6 deficiency.

3) High concentrations of methionine in the medium (15 mg/ml) may have prevented the trapping of folate in the 5-MeTHF form, which is caused by a lack of inhibition of MTHFR by S-adenosyl methionine (SAM). It is expected that high methionine concentrations would result in high SAM concentrations. S-adenosyl methionine is known to inhibit MTHFR (Kutzbach and Stokstad, 1971) and may have resulted in a diversion of the available folate towards thymidine synthesis.

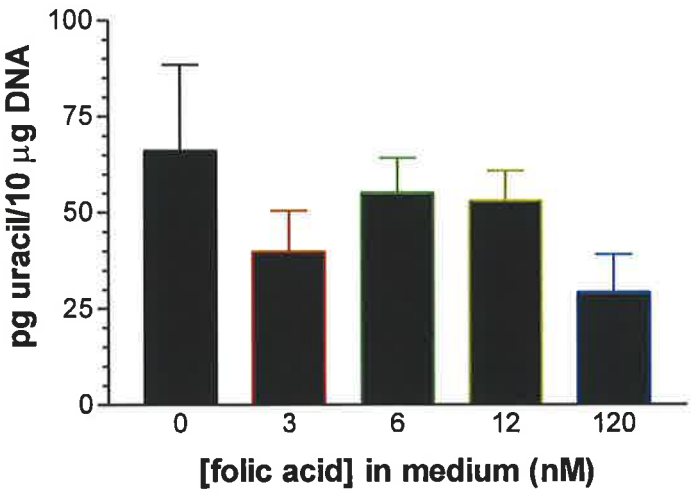
5.5.3.c Vitamin B_6 dose response

The results for the vitamin B_6 dose response are very similar to those of the vitamin B_{12} experiment. There was a definite variation in cell number between cultures at day nine, which was in the order of 1.4×10^7 cells (Figure 5.7 C). This range is very similar to that observed for the vitamin B_{12} data, however there are no dose-related trends in cell number ($P > 0.05$). In fact, the cell number of cultures seems completely random with cell number increasing for the following concentrations in ascending order; 200, 0, 20, 5, 10 nM vitamin B_6. Because vitamin B_6 is bound to albumin in serum (Bender, 1999), it is likely that very little of the vitamin is removed from the FCS during dialysis. Although the vitamin B_6 concentration in the FCS was not measured it is possible that the FCS contributed sufficient vitamin B_6 to the medium to maintain cell growth.

Similarly, vitamin B_6 concentration had no effect on MN induction ($P = 1.0$) (Figure 5.8 C). Because vitamin B_6 is the cofactor for the serine hydroxy methyltransferase-mediated conversion of THF to 5,10-MnTHF and folic acid (in medium) is upstream of THF in the

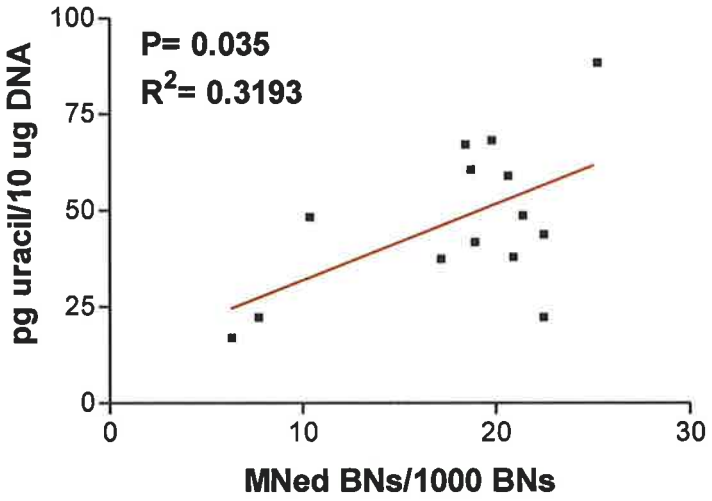
cycle, it was hypothesised that vitamin B_6 depletion would induce a depletion of 5,10-MnTHF. A depletion of 5,10-MnTHF, by any cause, would be expected induce the build up and subsequent incorporation of uracil into DNA (see section 1.4.2). Although there are no published studies that investigate the effect of vitamin B_6 deficiency on DNA damage, the theoretical link is clear. Furthermore, it has been reported that people who have low serum B_6 levels are at higher risk for lung (Hartman *et al.*, 2001) and prostate cancer (Key *et al.*, 1997). The role of vitamin B_6 in genomic stability requires further in depth research, which is outside the scope of this project.

Figure 5.9 Effect of folic acid concentration on DNA uracil-content



N= 3 (in duplicate) for each data point. ANOVA P> 0.05. Pearson correlation P= 0.013, r^2= 0.26

Figure 5.10 Relationship between micronuclei and DNA-uracil content



N=14. Pooled data for five different folic acid concentrations.

5.5.4 Conclusions

The results of these three dose response experiments suggest that proliferation and MN induction in lymphocytes are both sensitive to medium folic acid but not vitamin B_6 and vitamin B_{12} concentrations when the other vitamins are in abundance. Furthermore, it is clear that, with respect to folic acid, these changing cellular responses can be observed over a physiological concentration range. The response with folic acid seems to be quite reproducible judging from the small magnitude of the SEM in this dose-response study.

In an effort to clarify whether lower folic acid status affects the cellular response to vitamin B_{12} and vitamin B_6 deficiency a dose response to these vitamins was performed (in the next experiment) using medium that contained only 24 nM folic acid.

5.6 Experiment 5

5.6.1 Aim

To determine whether the effects of vitamin B_6 and vitamin B_{12} deficiency on MN induction become apparent at low folic acid concentrations in this *in vitro* system.

5.6.2 Method

Approximately 80 ml of blood was collected from three male volunteers (see experiment 4) and lymphocytes were isolated as previously described (section 2.2.1). Lymphocytes were cultured in eight types of RPMI-1640 medium (5% FCS, 10 U/ml IL-2) containing differing amounts of folic acid, vitamin B_6 and vitamin B_{12} as described in Table 5.5.

Table 5.5 Experiment 5 medium B-vitamin concentrations

Medium	[FA] nM	[B_{12}] pM	[B_6] nM	
1	120	700	200	Replete control
2	24	700	200	Low FA control
3	24	70	200	Low B_{12}
4	24	0	200	Absent B_{12}
5	24	700	20	Low B_6
6	24	700	0	Absent B_6
7	24	70	20	Low B_{12} and B_6
8	24	0	0	Absent B_{12} and B_6

B_6, vitamin B_6; B_{12}, vitamin B_{12}; FA, folic acid.

Twenty-four nM folic acid was chosen because it was expected to ameliorate the low growth rate observed with lower concentrations of folic acid, which may enhance the possibility of discerning an independent effect of the vitamin B_6 and B_{12} deficiency on MN induction.

After placing cells in culture, PHA addition, medium changing and harvesting were performed as described in the previous experiment (section 5.5.2).

5.6.3 Results and Discussion

As seen in Figure 5.11, the major determinant of projected cell number at day nine was the concentration of folic acid in the medium. One way ANOVA showed that, at days six

and nine, cell number was significantly higher in medium 1 than all other media ($P< 0.05$) and that there was no significant differences in cell number for media 2 – 8, which all contained 24 nM folic acid ($P> 0.05$). The largest number of cells at day nine was observed in cultures grown in replete medium that contained 120 nM folic acid (6.5×10^7 cells). In comparison, the medium replete for vitamins B_6 and B_{12} but with low folic acid (24 nM) contained 2.4×10^7 cells, very similar to all other media ($P> 0.05$). The range in cell number at day nine of all of the media containing 24 nM was relatively small (0.5×10^7 cells). This result indicates that even the complete omission of vitamin B_6 and vitamin B_{12} had little or no effect on cell proliferation and that all of the observed effect on cell number was due to reduced folic acid concentration.

Similarly, folic acid concentration significantly affected MN induction (medium 1 v 2, $P< 0.01$) but vitamin B_6 and vitamin B_{12} did not (Figure 5.12). There was no significant difference in MN frequency between any of the seven cultures grown in medium containing 24 nM folic acid but with different concentrations of vitamin B_6 and vitamin B_{12}. The MN frequency for the replete medium was 9.3 ± 3.3 MNed BNs/1000 BNs. This was similar to the MN frequency observed for the same volunteers in replete medium (containing 120 nM folic acid, 700 pM vitamin B_{12} and 200 nM vitamin B_6) in experiment 4 (8.1 ± 1.2, 4.9 ± 0.8 and 6.6 ± 1.2 MNed BNs/1000 BNs) and highlights the reproducibility of this assay system.

When the media containing 24 nM folic acid, 200 nM vitamin B_6 and either 0, 70 or 700 pM vitamin B_{12} are compared there was no difference between concentrations when tested by ANOVA ($P= 0.53$) and no correlation between vitamin B_{12} concentration and MN frequency ($P= 0.78$). Similarly, there was no difference in MN frequency between the three concentrations of vitamin B_6 (with 120 nM folic acid and 700 pM vitamin B_{12}) when tested by ANOVA ($P= 0.9$) and no correlation between vitamin B_6 concentration and MN frequency ($P= 0.86$).

It was hypothesised that folate deficiency was required to observe the effects of vitamin B_6 and vitamin B_{12} deficiency in this system. As discussed earlier, the presence of these two vitamins in the FCS may contribute to the lack of effect on proliferation and MN induction. When studying vitamin B_{12}, the type of folate used, in this case folic acid, may

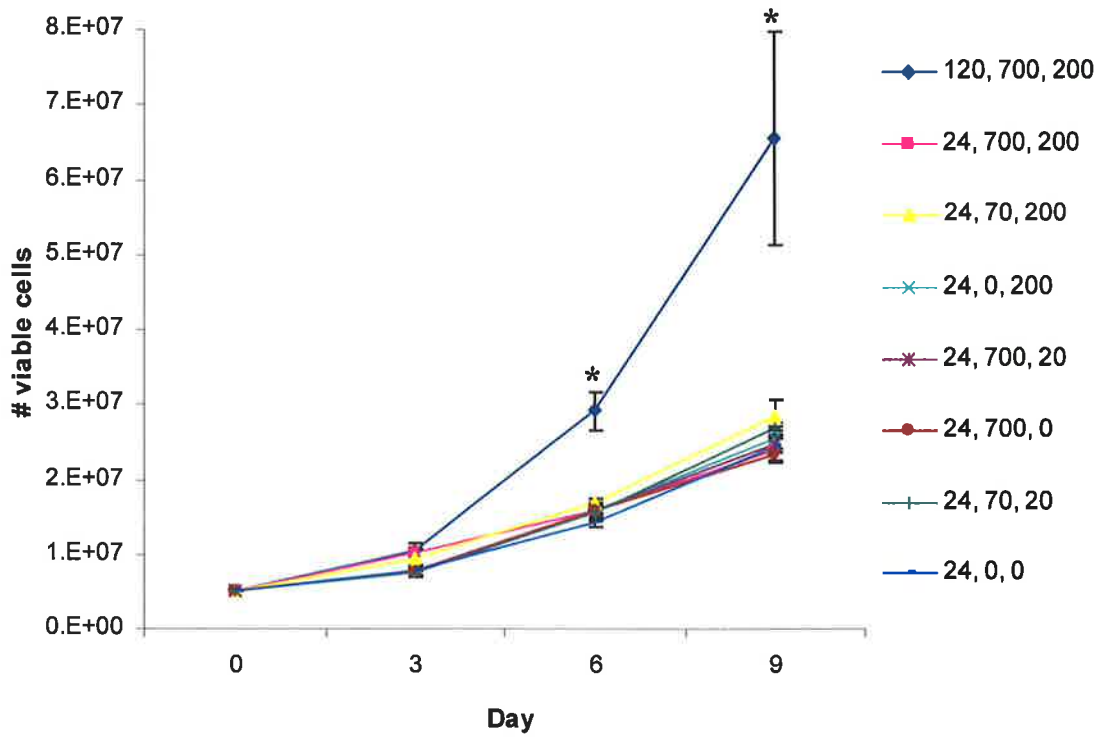
be important in determining whether an effect can be seen due to vitamin B_{12} deficiency. As discussed in section 5.5.3, it may be that 5-MeTHF may facilitate an effect for vitamin B_{12} deficiency on MN because this folate species requires a vitamin B_{12}-dependent conversion before it can participate in thymidine synthesis, whereas folic acid does not.

Because the culture system described here is effective for the study of folic acid deficiency (the main focus of this work) but is unable to detect any effect of vitamin medium B_{12} deficiency, it was decided that testing of vitamin B_{12} deficiency be discontinued. An alternative system incorporating the changes suggested above as well as longer culture duration may be required make further progress in this area.

In the case of vitamin B_6 deficiency, a lack of effect on MNi and cell division may be due to the presence of vitamin B_6-independent pathways for the conversion of THF to 5,10-MnTHF. In fact, as mentioned in section 1.3.1 A, there are three other reactions by which THF can be converted to 5,10-MnTHF that occur exclusively in the mitochondria (Wagner, 1995). It is possible that these pathways produce sufficient 5,10-MnTHF for thymidine synthesis so that only severe long-term vitamin B_6 deficiency, where the serine hydroxy methyltransferase mediated reaction is completely disabled, can impact on 5,10-MnTHF pools. To date, the only indirect evidence available that vitamin B_6 deficiency may cause genetic instability is that people who have low plasma vitamin B_6 levels have a slightly elevated risk for lung (Hartman *et al.*, 2001) and prostate (Key *et al.*, 1997) cancer. It remains unclear how vitamin B_6 is related to genetic stability. Further study involving culture durations of longer than nine days may be required to test any association. In addition, the use of serum free media or FCS completely stripped of vitamin B_6 is required. The use of vitamin B_6 antagonists such as L-canaline (Rosenthal, 1997) may also shed further light onto this topic.

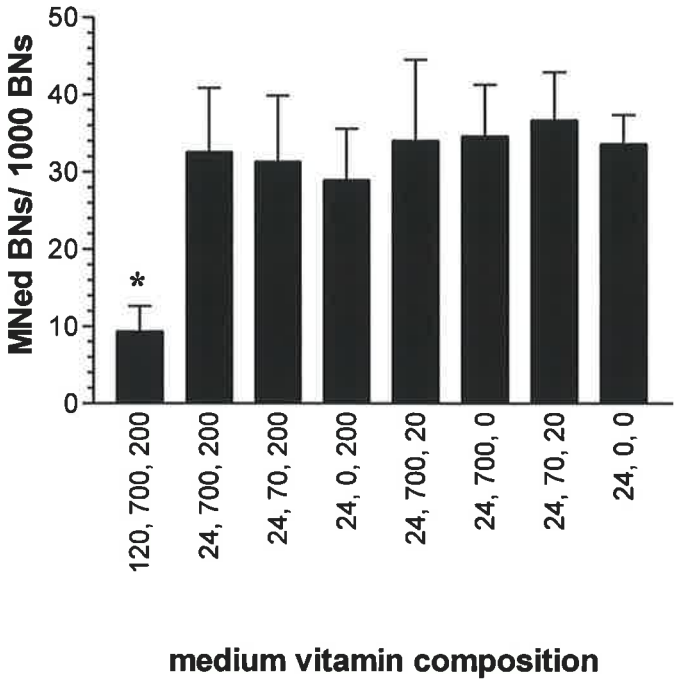
Because of the lack of effect of vitamin B_6 deficiency on MN frequency in this *in vitro* system, it was decided that experimentation using this vitamin be discontinued until a better protocol design is established. It is apparent that further study, which is outside the scope of this project, is required to determine the role, if any, of vitamin B_6 in maintaining genetic stability.

Figure 5.11 Effect of B-vitamin concentration on cell proliferation (Expt. 5)



Values in key refer to concentrations of folic acid (nM), vitamin B_{12} (pM) and vitamin B_6 (nM) respectively. N=3 for each data point. * P< 0.05 v all other cultures.

Figure 5.12 Effect of B-vitamin concentration on micronucleus induction (Expt.5)



Values in axis label refer to concentrations of folic acid (nM), vitamin B_{12} (pM) and vitamin B_6 (nM) respectively. N=3 for each data point. * P< 0.01 v all other media.

5.7 General Conclusion

This chapter has described the development of a culture protocol that can reproducibly detect changes in chromosome damage, as measured by MN induction, in response to folic acid deficiency over a physiological concentration range. Furthermore, it has been shown that this system is appropriate in determining the DNA uracil-content of cells in parallel to these MN measures. The results also show that MNi and DNA uracil-content exhibit a significant positive correlation that supports the hypothesis that folate deficiency-induced chromosome breaks are caused by the presence of uracil.

Although this system is sensitive to fluctuations in folic acid concentration, no effects of vitamin B_6 or B_{12} deficiency were detected. This may be due to the type of folate used, the presence of these vitamins in the FCS or insufficient culture duration. It is clear that further research is required on this topic, however this is beyond the scope of this thesis. Further experiments focussed on characterising, in greater detail, the effects of folic acid deficiency on markers of cell and chromosome damage.

Chapter 6: The effect of folic acid deficiency and MTHFR C677T polymorphism on genetic stability

AIM

1. To thoroughly characterise the effect of folic acid deficiency on chromosome damage using the new comprehensive long-term CBMN assay.
2. To determine the optimal folic acid concentrations for genetic stability in human lymphocytes *in vitro*.
3. To determine whether lymphocytes from people who are homozygous for the MTHFR C677T polymorphism are protected against uracil incorporation into DNA and chromosome damage compared to wildtypes over a physiological concentration range *in vitro*.

HYPOTHESES

1. Low concentrations of folic acid in culture medium induce chromosome damage in primary lymphocytes, which is expressed at MNi, NPBs and nuclear buds and these markers of chromosome damage are minimised *in vitro* at folic acid concentrations above those that are considered to be in the normal range for plasma.
2. Lymphocytes from TTs incorporate less uracil into DNA compared to CCs over a physiological concentration range of folic acid *in vitro*.

6.1 Introduction

Cancer is one of several diseases that have been associated with low blood concentrations of folate (Mason, 1995). Folate provides methyl groups for DNA methylation and the conversion of uracil (dUMP) to thymidine (dTMP). Therefore, folate deficiency can cause DNA hypomethylation (Jacob *et al.*, 1998) and induce excessive incorporation of uracil into DNA (Blount *et al.*, 1997). Simultaneous removal of two uracil bases within 12 base pairs and on opposite strands may result in the formation of double-stranded DNA breaks (DSBs) (Dianov *et al.*, 1991) (see Figure 6.1 A-C). The promotion of uracil incorporation into DNA and subsequent chromosome breakage caused by DSBs is important because it

is now established that the accumulation of chromosome aberrations such as DSBs is a risk factor for cancer (Hagmar *et al.*, 1998; Bonassi *et al.*, 2000) and may be one of the underlying mechanisms of aging (Fenech, 1998). The mutagenic potential of uracil is highlighted by the fact that, of eight known human glycosylases involved in DNA repair, four remove uracil from the code (Lindahl and Wood, 1999). Studies have shown that global DNA hypomethylation, a marker of folic acid depletion, may induce chromosome loss, probably due to the under condensation of peri-centromeric heterochromatin (Guttenbach and Schmid, 1994). Chromosome loss leads to micronucleus formation and aneuploidy, the latter now recognised as an important risk factor for cancer (Rasnick and Deusburg, 1999).

Perhaps one of the most intriguing facets of the relationship between folate status and cancer risk is how polymorphisms in key folate-metabolising enzymes can modulate disease risk. As described in section 1.5, a number of polymorphisms have been identified in the gene encoding methylenetetrahydrofolate reductase (MTHFR). Data from this laboratory show that the frequency of one such polymorphism, a C to T transition at position 677 of the gene, is present in the homozygous state in approximately 10% of South Australians. This frequency is similar to that of other predominantly Caucasian populations (Botto and Yang, 2000). Individuals who are heterozygous (CT) or homozygous (TT) for this polymorphism have an *in vitro* enzyme activity of 65% and 30% of wildtypes (CC) respectively (Frosst *et al.*, 1995). It is now well established that TTs have elevated plasma homocysteine concentrations (Frosst *et al.*, 1995; Zittoun *et al.*, 1998; Kluijtmans *et al.*, 1997), which is thought to be due to the inefficient recycling of homocysteine to methionine. The TT genotype is associated with an elevated risk for cardiovascular disease (Kluijtmans *et al.*, 1997; Gallagher *et al.*, 1996), which may be related to homocysteine. In addition, TTs are reported to have a 2.8-fold higher risk for endometrial cancer (Esteller *et al.*, 1997), a 2.7- and 2.8-fold higher risk for Crohn's disease and ulcerative colitis respectively (Mahmud *et al.*, 1999) and a 2.6- (Hobbs *et al.*, 2000) to 3.2- (James *et al.*, 1999) fold higher risk for Down syndrome in offspring. Various reports also show that TT babies have a 1.6- (Sheilds *et al.*, 1999), 1.8- (van der Put *et al.*, 1997) or 2.2- (Christensen *et al.*, 1999) fold higher risk for neural tube defects.

In contrast, various reports document that TTs have a 1.2- (Slattery *et al.*, 1999), 1.7- (Chen *et al.*, 1996) and 3.0- (Ma *et al.*, 1997) fold reduced risk for colorectal carcinoma.

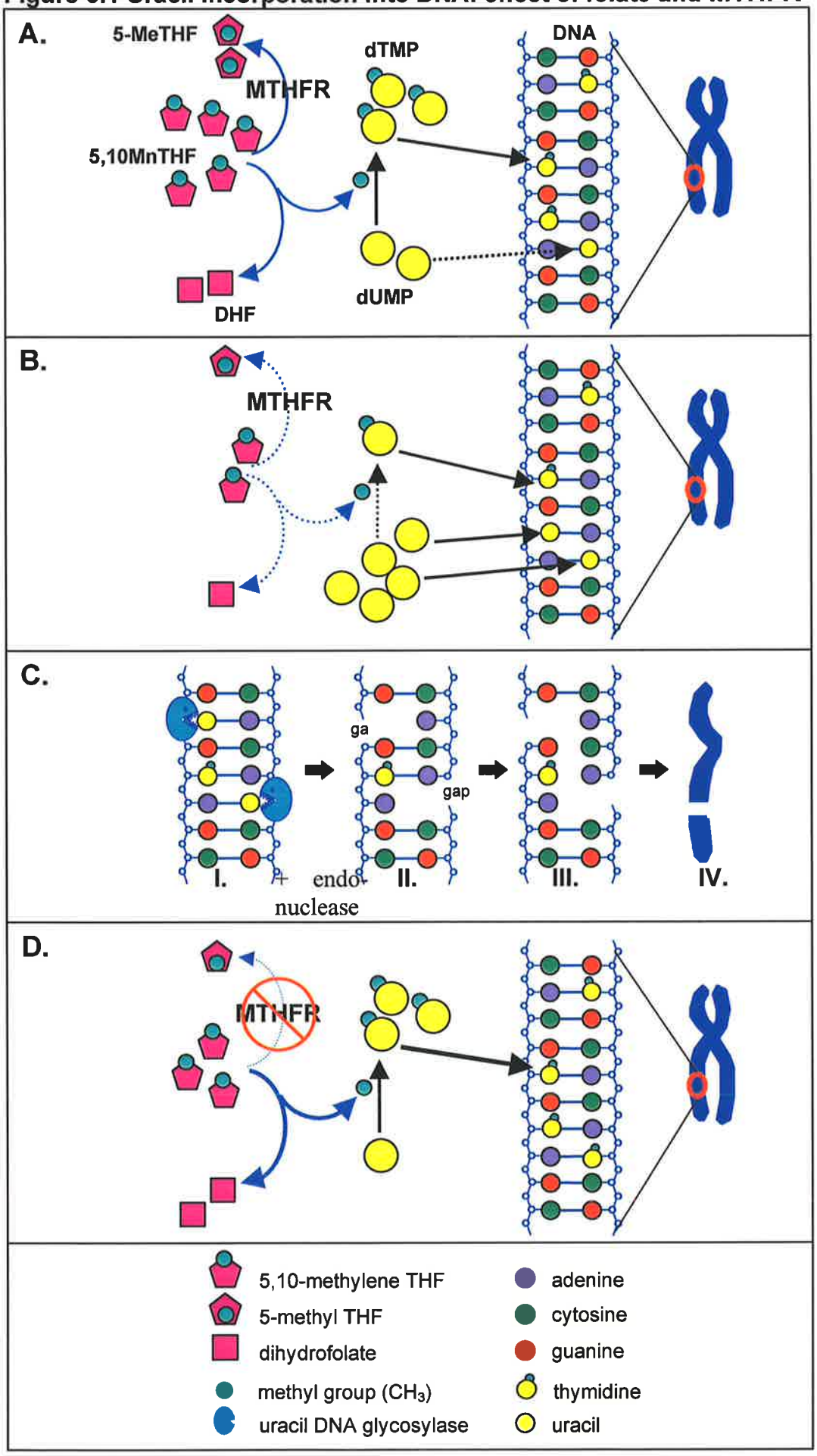
Despite this evidence relating to carcinomas, the TT genotype does not seem to afford any protection against the formation of colorectal adenomas (Chen *et al.*, 1998; Marugame *et al.*, 2000) and may even increase risk for adenoma if dietary intakes of folic acid, vitamin B_{12}, vitamin B_6 and methionine are low (Ulrich *et al.*, 1999). Recently, the TT genotype has also been associated with a 4.3-fold reduction in the risk for developing acute lymphocytic leukaemia (Skibola *et al.*, 1999). It has been hypothesised that TTs are protected against some cancers because reduced MTHFR activity causes a diversion of 5,10-methylenetetrahydrofolate (5,10-MnTHF) towards thymidine synthesis (Blount *et al.*, 1997) (Figure 6.1 D). This altered folate distribution is thought to minimise intracellular dUMP:dTMP ratios, therefore minimising the chance of uracil being incorporated into DNA and thus the chance for DNA breakage.

This chapter presents a comparison of DNA uracil-content and markers of chromosome damage in lymphocytes from CCs and TTs during folate depletion and repletion. Two novel biomarkers for *in vitro* folic acid deficiency are also described and validated, these being 1) nucleoplasmic bridges, which indicate chromosome rearrangement and 2) nuclear buds, which are caused by the nuclear exclusion of amplified or damaged DNA.

Legend to Figure 6.1 Uracil incorporation into DNA: effect of folate and MTHFR

A) Under folate replete conditions, there is sufficient 5,10-methylene tetrahydrofolate (5,10-MnTHF) present to support thymidine (dTMP) production and maintain a low uracil (dUMP): thymidine ratio, however a small amount of uracil may still be incorporated into DNA. **B)** When folate intake is low, 5,10-MnTHF concentrations are reduced, the synthesis of dTMP is compromised and the ratio of dUTP: dTTP increases. This results in the excessive incorporation of uracil into DNA. **C)** During post-replicative DNA repair, glycoslyase enzymes remove uracil from the DNA (I), which results in gap formation after the action of an endonuclease (II). The simultaneous removal of two uracils on opposite strands and within 12 base pairs may result in the formation of a double-stranded DNA break (III) which is effectively a chromosome break (IV). **D)** In people who are homozygous for the MTHFR C677T mutation (TTs) the reduced MTHFR enzyme activity results in a diversion of folate away from 5-methyl THF (5-MeTHF) synthesis and towards dTTP synthesis. This situation is thought to minimise the intracellular ratio of uracil: thymidine and thereby reduce the basal level of uracil incorporation into DNA. The subsequent lowered rate of double-stranded DNA breakage is thought to be the underlying mechanism for the reduced leukaemia and colorectal cancer risk seen in TTs.

Figure 6.1 Uracil incorporation into DNA: effect of folate and MTHFR



6.2 Materials and Methods

Approval for this study was obtained from CSIRO Health Sciences and Nutrition and Adelaide University human ethics committees. Informed consent was obtained from all participating volunteers.

6.2.1 Volunteers

Ten volunteers homozygous for the C677T polymorphism in MTHFR (TT) and twenty age- and gender-matched controls (14 wildtypes (CC) and 6 heterozygotes (CT)) were selected from a database of previous study participants. The presence of this polymorphism was determined previously using the method of Frosst *et al.* (1995). Volunteers were also previously tested for the presence of the A1298C MTHFR and A2756G methionine synthase polymorphisms using the methods of van der Put *et al.* (1998) and Leclerc *et al.* (1996) respectively as described in sections 4.2.3-5. Controls were divided into two groups of ten. The first control group (Ctrl 1) consisted entirely of CCs who were optimally matched for age, gender and the MTHFR A1298C and MS A2756G polymorphisms. The second control group (Ctrl 2) consisted of a mixture of CCs and CTs matched for age and gender but not as well for the other MTHFR and MS polymorphisms. Volunteer characteristics are listed in Table 6.1.

	TT	Ctrl 1	Ctrl 2
n	10	10	10
Male/Female	4/6	4/6	4/6
Ages in years* (range)	52.5 ± 2.9 (36 – 64)	52.4 ± 3.2 (34 – 65)	50.6 ± 3.3 (33 - 65)
n MTHFR 677 CC/CT/TT	0/0/10	10/0/0	4/6/0
n MTHFR 1298 AA/AC/CC	7/3/0	5/5/0	7/3/0
n MS 2756 AA/AG/GG	8/2/0	8/2/0	6/4/0

Table 6.1 Volunteer details

* ANOVA P= 0.86.

6.2.2 Nine-day lymphocyte culture protocol

Volunteers donated approximately 90 ml of blood (lithium-heparin) after an overnight fast on one occasion. Lymphocytes were isolated using Ficol-paque gradients (Pharmacia Biotech, Uppsala, Sweden) and cultured (5 x 10^5 cells/ml) in 10 ml of medium in eight 25

ml culture flasks with vented lids (Sarstedt, Adelaide, Australia) as described previously (Section 2.2.1 and 2). Duplicate cultures were established in four different types of media (described below). A diagrammatic representation of the culture protocol is shown in Figure 6.2. The development and optimisation of this nine-day culture protocol is described in Chapter 5.

Cell culture media was custom made (in house) RPMI-1640 which contained 5% dialysed foetal calf serum (FCS)(Trace Biosciences, Victoria, Australia), 10 units/ml interleukin-2 (Roche Diagnostics, Basel, Switzerland) and either 120, 60, 24 or 12 nM folic acid. The amount of vitamin B_{12} added to media was 700 pM and all other constituents were standard for RPMI-1640 as described by Moore *et al.* (1976) and were all purchased from Sigma (St Louis, MO, USA). The dialysed FCS contained 356 pM vitamin B_{12} and 9 nM folic acid which equates to a contribution of 17.8 pM B_{12} and 0.45 nM folic acid in the complete media containing 5% FCS. The method for preparing RPMI 1640 is given in section 5.1.

After three days, cell number and viability were determined. Cells were resuspended and disaggregated by gently pipetting up and down the cell suspension. Fifteen µl of cell suspension was diluted 1000x and cells were counted using a Coulter counter as described in section 2.2.1. A further 15 µl of cells was removed to determine cell viability by Trypan blue exclusion. This 15 µl of cells was mixed with 15 µl of 0.4% (w/v) Trypan blue solution in an eppendorf tube (0.6 ml, generic brand) and allowed to stand for approximately 5 - 10 mins. Approximately 10 µl of the solution was then transferred to the chamber of a standard hemocytometer and allowed to settle for a 1 - 2 mins. Cells from five squares of the hemocytometer were counted. Dead cells are unable to exclude the dye and therefore appear blue while viable cells appear clear.

The remaining cell suspension was then transferred to a sterile 10 ml conical base tube (generic brand) and spun at 100 x g. The supernatant was removed and cells were resuspended in 500 to 1500 µl of this spent medium at a concentration of 10 $x10^6$ viable cells/ml. This volume was calculated so that 5 x 10^6 viable cells could be returned to the flask in 500 µl of spent medium. This was done to return some of the growth factors from the spent medium to the system. 9.5 ml of fresh medium was added to each flask and

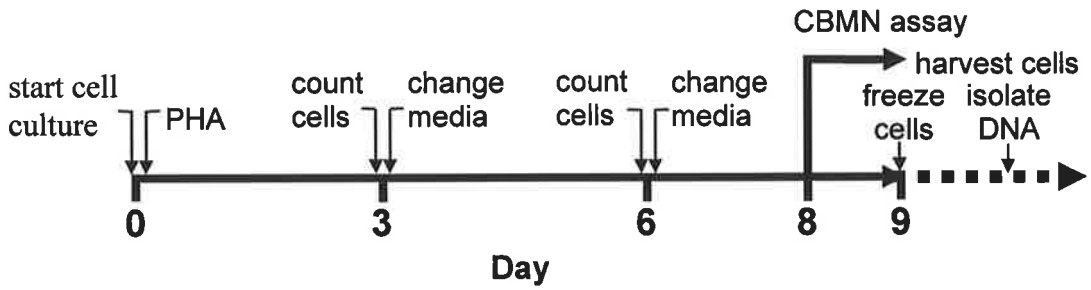
warmed to 37°C prior to returning the cells. This process of counting and re-culturing cells was repeated six days post-PHA treatment.

At eight days post-PHA, a 750 µl aliquot of each culture was removed to 6 ml culture tubes for the cytokinesis block micronucleus assay as described in Section 2.2.2.

Coded slides were scored for the presence of micronuclei, nucleoplasmic bridges and nuclear buds as well as apoptotic and necrotic cells (see Section 2.2.4.). Slides from TTs (n= 10) and control group 1 (CCs, n= 10) were scored for these cytotoxicity/genotoxicity markers.

Nine days after PHA, cell number and viability were determined before cells were transferred to cryovials (Nalgene, Rochester, NY, USA) in approximately 1 ml of medium and stored at –80°C. The growth data presented in Figure 6.3 are a projection of cell growth because only 5 x 10^6 cells were returned to each flask in fresh medium on days 3 and 6. The curves were generated by calculating the percentage increase in (viable) cell number and applying it to the number of cells present at the end of the previous three-day period.

Figure 6.2 Nine-day cell culture protocol



6.2.3 DNA isolation protocol

DNA was isolated from frozen samples containing approximately 5 – 10 x 10^6 lymphocytes in approximately 1 ml of RPMI 1640 medium using phenol-chloroform extraction as detailed below.

1. Cell samples were thawed and 1ml $T_{10}E_{10}$ (2.5 ml 1M Tris HCl, 5 ml 0.5 M EDTA, 242.5 ml H_2O) was added to the cryovial.
2. The cell lysate was transferred to 2 ml eppendorf-type tubes (generic) and the cryovial was rinsed with another 500-600 µl of $T_{10}E_{10}$ before refrigeration at –20°C for 30 mins.
3. Tubes were then spun at 13,000 rpm for 10 mins and supernatant was discarded. A smear/pellet should be visible in the bottom of the tube.
4. 0.9 ml $T_{10}E_5$ (2.5 ml 1M Tris HCl, 2.5 ml 0.5 M EDTA, 245 ml H_2O) was added to the tube before vortexing to resuspend and break up pellet.
5. 90 µl of 10% sodium dodecyl sulphate was added and mixed by gentle inversion until no clumps remained in the solution.
6. 20 µl RNase A and 20 µl RNase T1 (500 U/ml each, Roche Diagnostics, Mannheim, Germany) were added (final concentration of 10 U/ml in the reaction mix) and tubes were incubated at 37°C for 1 hr.
7. 20 µl proteinase K (500 U/ml, Roche Diagnostics, Mannheim, Germany) was added (final concentration of 0.5 mg/ml) and tubes were incubated at 37°C for 2 hrs. (It is important to note that higher temperatures may cause deamination of cytosine to uracil). All white precipitates and clumps should be digested at the end of this incubation.
8. An equal volume (1000 µl) of Tris-saturated Phenol (Sigma, St. Louis, MO, USA) was added and tubes were mixed by inversion until an emulsion formed. Tubes were then spun at 10,000 rpm for 15 mins.
9. The upper aqueous phase containing the DNA (lower organic phase is phenol) was removed into a fresh tube using a 1 ml pipette with the end cut off of the tip (opening of tip had a diameter of approximately 1.5 - 2 mm).
10. The extraction was repeated with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) and then with chloroform: isoamyl alcohol (24:1)(both from Sigma, St. Louis, MO, USA).

11. One-tenth volume (of aqueous phase) of 3 M sodium acetate and twice the volume of cold (-20°C) ethanol were added and tubes were mixed by inversion until the DNA precipitated.
12. DNA was washed once with ethanol and resuspended in ethanol before being transferred to 2 ml cryovials (Nalgene®, Rochester, NY, USA) and stored at -20°C until shipping.

DNA was shipped (on dry ice) to the laboratory of Prof. Bruce Ames, Berkeley, CA, USA where I measured the uracil content of samples by gas chromatography- mass spectrometry.

13. On arrival, DNA was washed once in ethanol and resuspended in Milli-Q water (pH 7.5 – 8) before being quantified by measuring absorbance at 260 nm in a spectrophotometer (Shimadzu UV 160U, Japan). It was assumed that a solution containing 10 μg/ml DNA has an absorbance of 1.0 at 260 nm.

6.2.4 Determination of DNA-uracil content by GC-MS

The uracil content of DNA was measured using the method of Blount and Ames (1994). Ten μg of DNA was dried in a speed-vac (Savant, SC110A-120 fitted with a Savant refrigerated vapour trap, RVT400, Farmingdale, NY, USA) and resuspended in 40 μl of TE buffer. Meanwhile, 1 μl (one unit) of uracil DNA glycosylase (UDG, Epicentre Technologies, Madison, WI, USA) was filtered per sample in a Microcon® centrifugal filter unit (YM-10, Millipore, Bedford, MA, USA) and eluted in TE to give a concentration of 1 unit/10 μl. Ten μl of filtered UDG was added to the DNA and tubes were incubated at 37°C for 1 hr in a water bath. After incubation, 100 pg of internal standard, labelled uracil ($^{13}C_4H_4O_2{}^{15}N_2$. Cambridge Isotope Laboratories, Andover, MA, USA), was added and tubes were dried in a speed-vac. Uracil and internal standard were then derivatised by adding 61 μl of 3,5-Bis(trifluoromethyl) benzyl bromide (BTFMBzBr, Aldrich Chem. Co, Milwaukee, WI, USA): acetoniltrile: triethyl amine (1:50:10) and incubating at 30°C in a shaker (G24 Environmental Incubator Shaker, New Brunswick Scientific Co., New Brunswick, NJ, USA) for 30 mins. Fifty μl of milli-Q water was then added to each tube and the derivatised uracil and internal standard were extracted into 100 μl of isooctane. Tubes were vortexed for at least 30 secs and a maximum of 70 μl of isooctane was removed into an auto-sampler vial that was capped immediately.

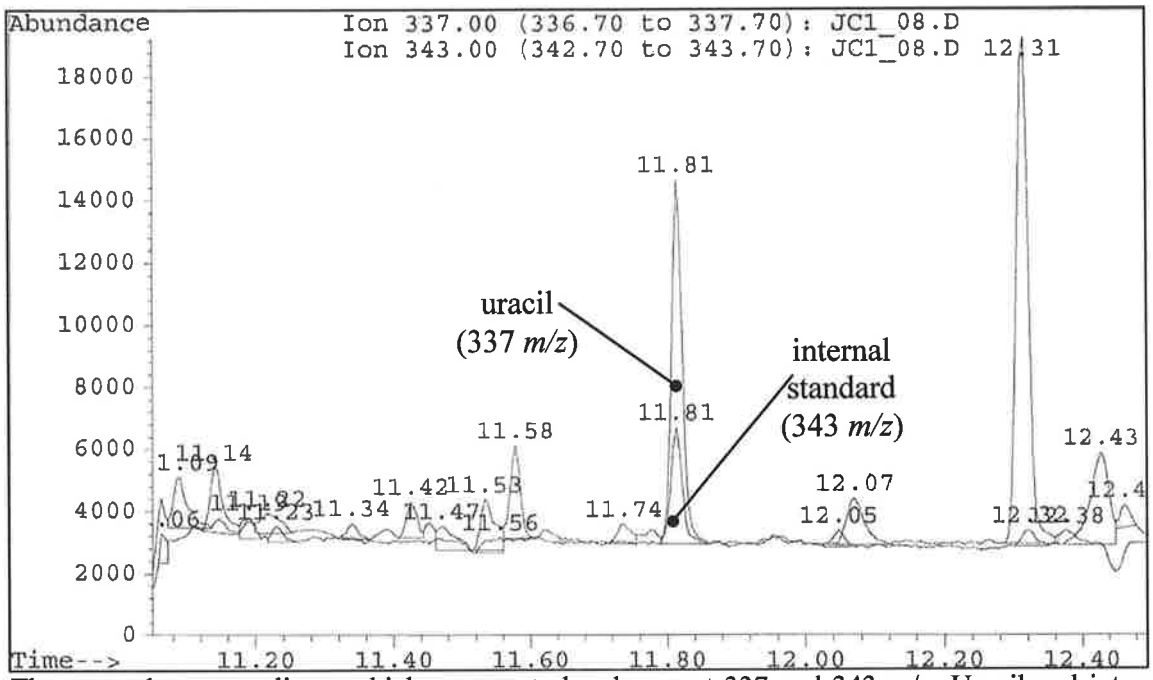
One µl of this isooctane was then injected into a Hewlett-Packard 5890-Series II Gas chromatograph using a Hewlett-Packard 7673 auto-sampler. The injection port was maintained at 280°C. Separation was achieved using a Hewlett-Packard HP-5ms column (30m x 0.25 mm internal diameter x 0.25 µm film thickness) kept at 100°C for 1 min then ramped to 280°C at 25°C/min and held for 5 mins. The GC-MS interface temperature was 300°C. A Hewlett-Packard 5989A mass spectrometer (Quadropole temperature, 100°C; electron energy, 200 eV; ion source temperature, 280°C; negative chemical ionisation mode) was used in single ion monitoring mode to detect uracil and internal standard peaks at 337 and 343 *m/z* respectively. Methane was maintained at 2 Torr.

The concentration of uracil (pg/10 µg DNA) in samples was determined by comparing the ratio of abundances at 337 and 343 *m/z* to a standard curve of 337/343 ratios generated from standards containing 0, 2.5, 5, 25, 50 or 100 pg uracil. R^2 values for the curve were 0.94 or higher (data not shown). One unit of heat denatured UDG and 12.5 µg of human placenta DNA (Sigma, St. Louis, MO, USA) was added to make standards as similar to samples as possible. An example of a chromatograph is given in Figure 6.2.

6.2.5 Statistical analyses

One-way ANOVA was used to compare DNA-uracil content while repeated measures one-way ANOVAs were used to compare all other markers of chromosome damage at various folic acid concentrations (irrespective of gender or genotype). In both cases a Tukey's post-hoc test was used to identify which concentrations were different from each other. Two-way ANOVA was used to determine the effect of folic acid concentration and genotype on the above-mentioned biomarkers. Unpaired t-tests were used in addition to two-way ANOVA to compare levels of uracil between groups at each folic acid concentration. Two-tailed Pearson correlations were used to analyse relationships between two variables. All data is expressed as mean ± SEM. The above statistical calculations were performed using GraphPad Prism v 2.01 (GraphPad Inc., San Diego, CA, USA) while a correlation matrix was constructed using Statsoft Statistica 99 v 5.5 (Statsoft, Tulsa, OK, USA). Significance was accepted at $P< 0.05$.

Figure 6.3 Typical GC-MS chromatogram for the uracil assay



The trace shows two lines, which represent absorbance at 337 and 343 m/z. Uracil and internal standard both appear at 11.81 minutes. Abundance on the Y-axis is in arbitrary units.

6.3 Results

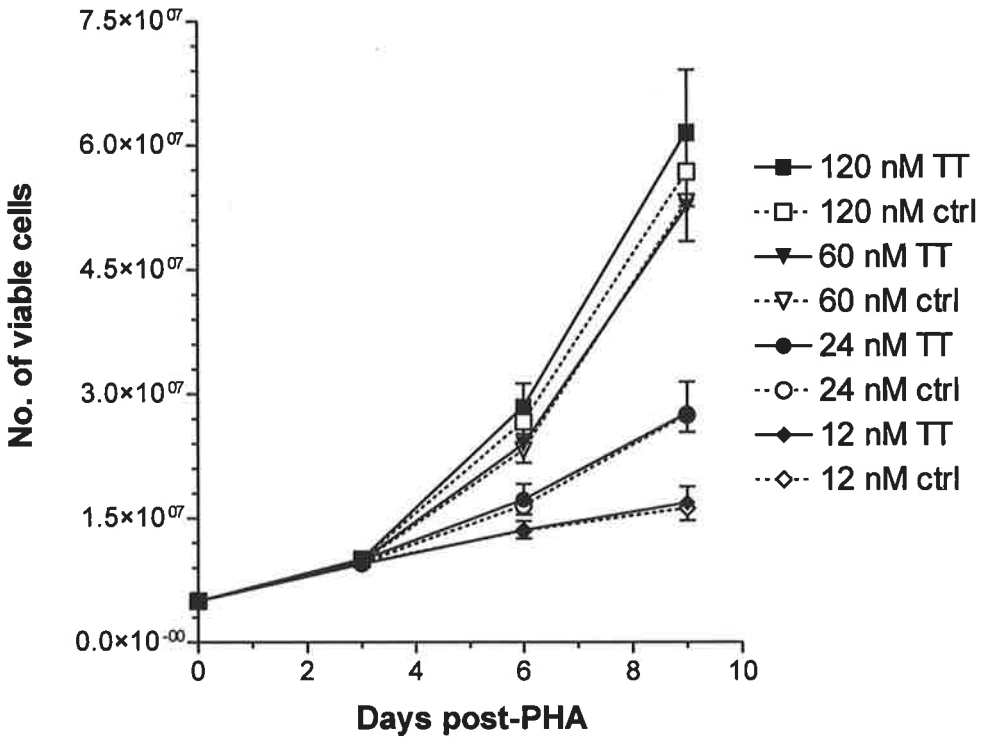
Figure 6.4 shows the cell growth in media with various concentrations of folic acid. There was a steady increase in cell number over the nine-day period with the maximum cell growth being achieved in the 120 nM folic acid medium. Two-way ANOVAs were performed to determine the effect of genotype (TT vs. Controls) and folic acid concentration on cell number at days 3, 6 and 9. At each time point folic acid was a significant determinant of cell number ($P< 0.0001$) while genotype was not ($P= 0.9$, 0.51 and 0.71 for days 3, 6 and 9 respectively).

Age did not correlate with DNA-uracil content ($P= 0.2 – 0.86$) or MN frequency ($P= 0.16 – 0.91$) at any folic acid concentration. The gender of volunteers also did not effect DNA-uracil content (Two-way ANOVA; Gender $P= 0.12$, [folic acid] $P< 0.0001$) or MN frequency (Two-way ANOVA; Gender $P= 0.61$, [folic acid] $P< 0.0001$).

Uracil data are shown in Figure 6.5. When data from all volunteers were combined (n=29), there was a highly significant negative correlation between DNA-uracil content and folic acid concentration (Pearson r^2= 0.192, P< 0.0001). Furthermore, as reported in Table 6.2, uracil content at 12 and 24 was significantly higher than at both 60 and 120 nM folic acid (ANOVA P< 0.0001, post-test P < 0.05). There was no difference in the DNA-uracil content between TTs and the two control groups (Two-way ANOVA; Genotype/Group P= 0.4, [folic acid] P< 0.0001). Furthermore, when data from TTs and CTs were combined (n= 14), there was no difference in DNA-uracil content to the CCs (n= 15)(See Table 6.2. Two-way ANOVA; Genotype P= 0.752, [folic acid] P< 0.0001). When TTs, CTs, and CCs were considered separately, there was also no difference in DNA-uracil content (Two-way ANOVA; Genotype P= 0.12, [folic acid] P< 0.0001). No difference between genotypes was detected when genotypes were compared two at a time (e.g. CC v CT by t-test) or at each folic acid concentration by one-way ANOVA (CC v CT v TT for all four concentrations). When baseline uracil levels (120 nM results) were subtracted from the remaining three data sets (to give a change from baseline result) there was also no significant difference between TTs and controls (Two-way ANOVA, Genotype P= 0.125; [folic acid] P= 0.007).

The effect of the MTHFR A1298C and MS A2756G polymorphisms on DNA-uracil content was also determined (See Table 6.2). When considering the MTHFR A1298C polymorphism, data from 11 heterozygotes (AC) was compared to 18 wildtypes (AA). Two-way ANOVA revealed no diffences due to the polymorphism (Genotype P= 0.362, [folic acid] P<0.0001) however, when data from each concentration was compared seperately using an unpaired t-test, ACs exhibited a larger DNA-uracil content than wildtypes in deficient medium containing 12 nM folic acid (51.5 ± 9.4 and 31.9 ± 4.1 pg/ 10 μg DNA respectively. P= 0.038).

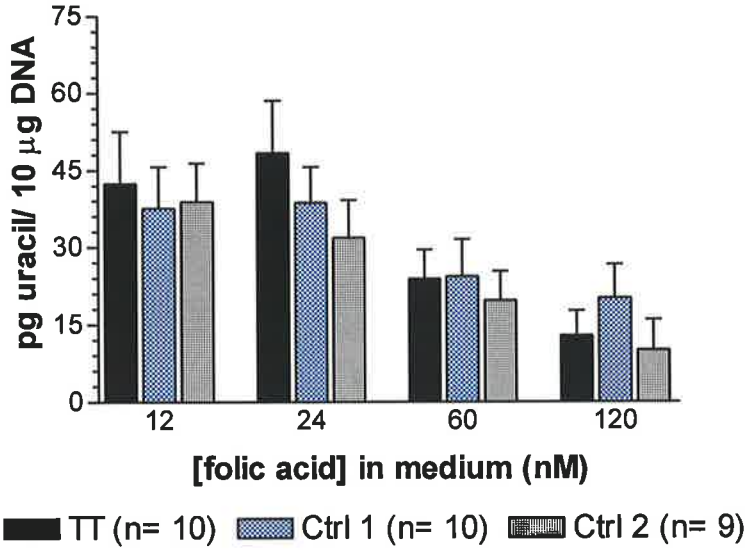
Figure 6.4 Growth curves for primary human lymphocytes grown in media with varying concentrations of folic acid.



n= 10 TTs and n= 20 controls (Ctrl, n= 14 CCs, n= 6 CTs). Media changed at day 3 and 6. Cultures initiated with $5x10^6$ cells and $5x10^6$ cells were returned to each flask after changing to fresh medium on days 3 and 6. Two way ANOVA at days 3, 6 and 9: [folic acid] $P< 0.0001$, genotype $P>> 0.05$.

Similarly, two-way ANOVA revealed no differences in DNA-uracil content between volunteers heterozygous for the MS A2756G polymorphism (AG, n=6) and wildtypes (AA, n= 23) (Table 6.2). However, when treatments were compared separately using an unpaired t-test, AGs were found to have a significantly higher DNA-uracil content than AAs when their cells were grown in replete medium containing 120 nM folic acid (28.8 ± 10.0 and 10.8 ± 3.0 pg/10 μg DNA respectively. P= 0.025).

Figure 6.5 Effect of medium folic acid concentration and MTHFR C677T polymorphism on DNA-uracil content of primary human lymphocytes *in vitro*.



Two-way ANOVA: [folic acid] P< 0.0001, Genotype/Group P= 0.4.

Table 6.2 Effect of MTHFR and MS polymorphisms on DNA-uracil content of primary human lymphocytes *in vitro*

			DNA-uracil content (pg/ 10 µg DNA)					
			[Folic acid] in medium (nM)				Two-way ANOVA P	
	Group	n	12	24	60	120	[folic acid]	Genotype
MTHFR C677T	**CC**	15	41.7 ± 6.4	37.2 ± 5.8	22. ± 5.3	16.6 ± 5.4	< 0.0001	0.75
	CT/TT	14	35.9 ± 6.2	42.8 ± 7.5	23. ± 4.4	12.3 ± 3.5		
t- test P			0.55	0.56	0.84	0.53		
MTHFR A1298C	**AA**	18	31.9 ± 4.1	42.1 ± 6.7	22. ± 4.6	14.1 ± 4.0	< 0.0001	0.36
	AC	11	51.5 ± 9.4	36.4 ± 6.4	23. ± 5.6	15.2 ± 6.1		
t- test P			**0.038**	0.57	0.94	0.87		
MS A2756G	**AA**	23	39.7 ± 5.6	41.7 ± 5.3	23. ± 4.2	10.8 ± 3.0	0.008	0.89
	AG	6	38.0 ± 8.6	33.0 ± 11.4	19. ± 5.6	28.8 ± 10.0		
t- test P			0.88	0.47	0.59	**0.025**		
All volunteers (n=29)			39.3 ± 4.7[1,2]	39.9 ± 4.8[1,2]	22.8 ± 3.5	14.5 ± 3.3	One-way ANOVA P< 0.0001	

[1] P< 0.001 v 120 nM, [2] P< 0.05 v 60 nM

Data for the CBMN assay are presented in Table 6.3 and a correlation matrix is presented in Table 6.4. When data for TTs (n=10) and CCs (control group 1, n= 10) were combined there was a strong negative correlation between folic acid concentration in the culture medium and all markers of chromosome damage. The r^2 value for MNi, buds and NPBs in binucleated cells and MNi in mononucleated cells was 0.55, 0.46, 0.39 and 0.25 respectively (P< 0.0001). These data indicate that DNA damage was minimised at a folic acid concentration of 120 nM.

Table 6.3 Results from CBMN assay

End-point	Gp	[folic acid] in medium (nM) 12	24	60	120	ANOVA P
MNed BNs	CC	30.9 ± 2.7	33.6 ± 2.5	11.7 ± 1.2	7.19 ± 0.7	[F]: <0.0001
	TT	27.2 ± 2.4	21.1 ± 3.2	10.5 ± 1.6	7.54 ± 0.6	Gp: 0.24
	All	29.0 ± 1.8[1,2,3]	22.2 ± 2.0[1,2]	11.1 ± 1.0	7.4 ± 0.4	<0.0001
MNed monos	CC	4.98 ± 0.5	4.1 ± 0.6	1.4 ± 0.3	2.4 ± 0.5	[F]: <0.0001
	TT	4.6 ± 0.6	4.6 ± 0.6	3.5 ± 0.4	1.4 ± 0.2	Gp: 0.63
	All	4.6 ± 0.4[1,3]	4.4 ± 0.5[1,2]	2.4 ± 0.3	2.1 ± 0.4	<0.0001
BNs + Buds	CC	9.0 ± 1.1	5.2 ± 0.8	1.7 ± 0.3	1.1 ± 0.2	[F]: <0.0001
	TT	9.2 ± 1.4	7.2 ± 1.2	2.2 ± 0.7	1.4 ± 0.3	Gp: 0.22
	All	9.1 ± 0.9[1,2,3]	6.2 ± 0.7[1,2]	2.1 ± 0.4	1.2 ± 0.2	<0.0001
BNs + NPBs	CC	7.7 ± 1.2	6.0 ± 0.5	2.4 ± 0.4	1.8 ± 0.5	[F]: <0.0001
	TT	7.2 ± 1.1	5.3 ± 1.0	2.4 ± 0.6	1.6 ± 0.6	Gp: 0.56
	All	7.4 ± 0.8[1,2,3]	5.6 ± 0.6[1,2]	2.4 ± 0.4	1.7 ± 0.4	<0.0001
% Apop.	CC	0.8 ± 0.2	0.9 ± 0.1	1.1 ± 0.2	0.5 ± 0.1	[F]: 0.06
	TT	0.7 ± 0.1	1.1 ± 0.1	1.0 ± 0.2	0.8 ± 0.1	Gp: 0.52
	All	0.8 ± 0.1	1.0 ± 0.1	1.1 ± 0.1[1]	0.7 ± 0.1	0.008
% Necr.	CC	14.1 ± 0.7	9.9 ± 0.5	12.3 ± 2.1	8.9 ± 0.5	[F]: 0.0007
	TT	12.8 ± 1.2	9.6 ± 0.9	10.0 ± 8.1	8.1 ± 0.6	Gp: 0.17
	All	13.4 ± 0.7[1,3]	9.7 ± 0.6	11.2 ± 1.3[1]	8.5 ± 0.5	<0.0001
NDI	CC	1.28 ± 0.01	1.41 ± 0.01	1.46 ± 0.01	1.47 ± 0.01	[F]: 0.0001
	TT	1.29 ± 0.01	1.38 ± 0.01	1.46 ± 0.01	1.47 ± 0.02	Gp: 0.96
	All	1.29 ± 0.01[1,2,3]	1.40 ± 0.01[1,2]	1.46 ± 0.01	1.47 ± 0.01	<0.0001

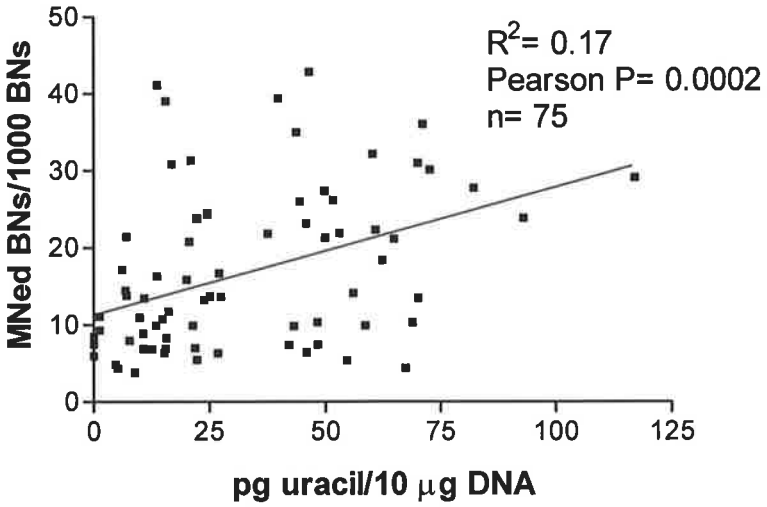
%Apop., % of cells that are apoptotic; BNs + Buds, binucleated cells with nuclear buds; BNs + NPBs, binucleated cells with nucleoplasmic bridges; NDI, nuclear division index; %Necr., % of cells that are necrotic; [F], comparison across folic acid concentrations; Gp, comparison between groups (CC v TT). All= combined data from CCs and TTs (n= 10 + 10) which was compared using one-way ANOVA.

[1] P <0.05 v 120 nM, [2] P <0.05 v 60 nM, [3] P <0.05 v 24 nM.

The percentage of cells that were necrotic correlated negatively with folic acid concentration (P= 0.0023, r^2= 0.11) while apoptosis did not correlate. One-way ANOVA of the apoptosis data revealed that significantly more apoptosis occurred at 60 compared to 120 nM folic acid (P< 0.05). Nuclear division index was positively correlated with folic acid concentration (P< 0.0001, r^2= 0.38) and was maximised at a concentration of 60 nM (NDI at 60 v 120 nM not different, P> 0.05).

The DNA-uracil content of cells from these cultures was significantly and positively correlated with the number of BNs containing MNi (P= 0.0002, r^2= 0.17, n=75. Figure 6.6), buds (P= 0.0002, r^2= 0.17, n= 75) and NPBs (P= 0.003, r^2= 0.12, n= 75). MNed BNs, MNed mononucleates, NPBs, Buds and necrosis were all significantly and positively correlated with each other, however the strongest correlation was between MNed BNs, NPBs and Buds (r^2= 0.56 – 0.59) (Table 6.4).

Figure 6.6 Correlation between DNA-uracil content and MN frequency



N= 20 volunteers x 4 folic acid concentrations. DNA samples not available for 5 samples.

Two-way ANOVA shows that homozygosity for the MTHFR C677T polymorphism does not affect MNed BNs (P= 0.24), MNed monos (P= 0.63), buds (P= 0.22) or NPBs (P= 0.56) (Figure 6.7). The P-value for the effect of folic acid concentration in each of these analyses was less-than 0.0001. Similarly, apoptosis, necrosis and NDI were not affected by MTHFR genotype.

Table 6.4 Correlation matrix

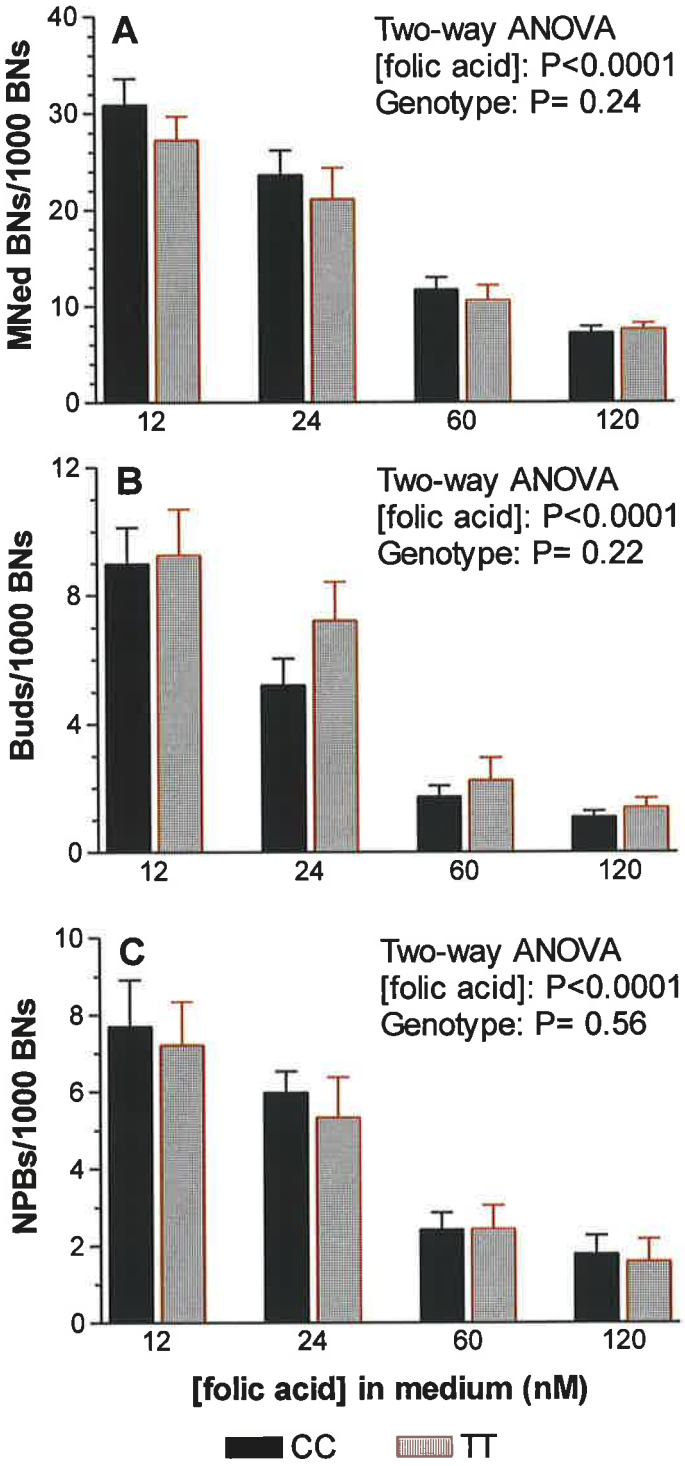
		[folic acid]	DNA uracil	MNed BNs	MNed mono	NPBs	Buds	%Apop	%Necr
NDI	P	< 0.001	0.019	< 0.001	< 0.001	< 0.001	< 0.001	0.96	0.002
	r	0.62	-0.27	-0.74	-0.39	-0.41	-0.61	-0.01	-0.35
%Necr.	P	0.002	0.094	< 0.001	0.03	< 0.001	< 0.001	0.78	
	r	-0.33	0.19	0.47	0.24	0.39	0.44	0.03	
%Apop.	P	0.29	0.14	0.67	0.50	0.69	0.69		
	r	-0.12	0.17	0.049	-0.08	0.04	0.04		
Buds	P	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001			
	r	-0.68	0.41	0.76	0.48	0.75			
NPBs	P	< 0.001	0.003	< 0.001	< 0.001				
	r	-0.63	0.34	0.77	0.43				
MNed monos	P	< 0.001	0.22	< 0.001					
	r	-0.49	0.14	0.61					
MNed BNs	P	< 0.001	< 0.001						
	r	-0.74	0.42						
DNA uracil	P	< 0.001							
	r	-0.42							

6.4 Discussion

The results of this study provide further evidence that folic acid deficiency causes an imbalance of intracellular nucleotide pools, which facilitates the excessive incorporation of uracil into DNA. The results also indicate a positive correlation between uracil content and MNed cell frequency, which is in agreement with the results of Blount *et al.* (1997). However, the correlation factor between uracil and MNed BNs suggests that only 17% of the variance in these chromosome damage markers can be explained by excessive uracil in DNA. This suggests that other mechanisms such as the loss of DNA methylation may have contributed to the induction of MN and NPB formation.

The three lower concentrations of folic acid used in this experiment (12 – 60 nM) closely reflect those seen *in vivo*. For example, Jacob *et al.* (1998) report that baseline plasma folic acid concentrations (in postmenopausal women) were 19.5 ± 4.2 nM and fell to 9.3 ± 1.8 nM after a five week folic acid depletion period. Furthermore, plasma concentrations are reported to rise to almost 50 nM with eight weeks of folic acid supplementation (400 μg per day) (Bronstrup *et al.*, 1998). Data from this laboratory also show that baseline plasma folic acid concentrations range from approximately 21 to 36 nM depending on age, gender and diet (Fenech and Rinaldi, 1994; 1995). It is unclear whether *in vivo*

Figure 6.7 Effect of folic acid concentration and MTHFR C677T polymorphism on MNi, buds and NPBs.



plasma concentrations of 120 nM could be sustainable by supplementation.

The data from this experiment clearly show that all of the markers of chromosome damage tested were minimised at a folic acid concentration of between 60 and 120 nM. There was a two to five-fold higher frequency of these markers in cells grown in medium with 24 nM folic acid relative to 120 nM. Because 'normal' plasma folate concentrations generally range from approximately 21 to 36 nM (Fenech and Rinaldi, 1994; 1995) these results support the view that there may be some benefit, with respect to genetic stability, in raising blood folate levels. Indeed, studies have shown that relatively short-term folate supplementation on its own or in combination with vitamin B_{12} can significantly lower baseline levels of genetic damage in humans (Blount *et al.*, 1997; Fenech *et al.*, 1998).

The amount of uracil measured in the DNA samples from this study ranged from approximately 10 - 48 pg/10 μg DNA or approximately 34,600 – 167,400 uracils/diploid cell. These figures are in good agreement with the 7 – 30 pg/10 μg DNA observed in rat liver DNA (Blount and Ames, 1994) and the 62,220 – 256,200 uracils/diploid cell (17 – 70 uracils/10^6 thymidines) observed for cultured primary murine erythroblasts (Koury *et al.*, 1997). However, these values are considerably lower than the 500,000 – 4,000,000 uracils/diploid cell reported by Blount *et al.* (1997) in blood DNA from folic acid replete and deficient splenectomized humans respectively.

One factor that may help explain this large difference in uracil values is the length of folate deficiency involved. The two animal studies (Blount and Ames, 1994; Koury *et al.* 1998), as well as this current study of cultured human cells, involved short-term folate deficiency of approximately one to four weeks. In contrast, the study of human splenectomized patients reported by Blount *et al.* (1994) used samples from individuals who were often severely anaemic, with marked macrocytosis and grossly megaloblastic erythropoiesis, which is indicative of chronic, extended folate deficiency (i.e. several months or years). Furthermore, micronuclei were shown to increase significantly with age and the folate deficient group tended to be elderly (mean = 60.4 years, ± 13.4 years) (Personal communication with Ben Blount). These factors could have interacted with a long-term, poor diet to exacerbate uracil incorporation into DNA. It is interesting to note that there was great inter-individual variation of uracil between folate-deficient

individuals (approaching 50-fold). This indicates the possibility of underlying complex genetic differences in folate metabolism that could cause such highly variable responses to folate deficiency. Further human studies should be done to clarify this issue, and differences in age as well as length of folate deficiency should be considered in the comparative analyses. Improvements to the uracil assay and an examination of whether methodological factors may have contributed to the differences in the different above-mentioned studies are currently being undertaken in the laboratory of Prof. Ames.

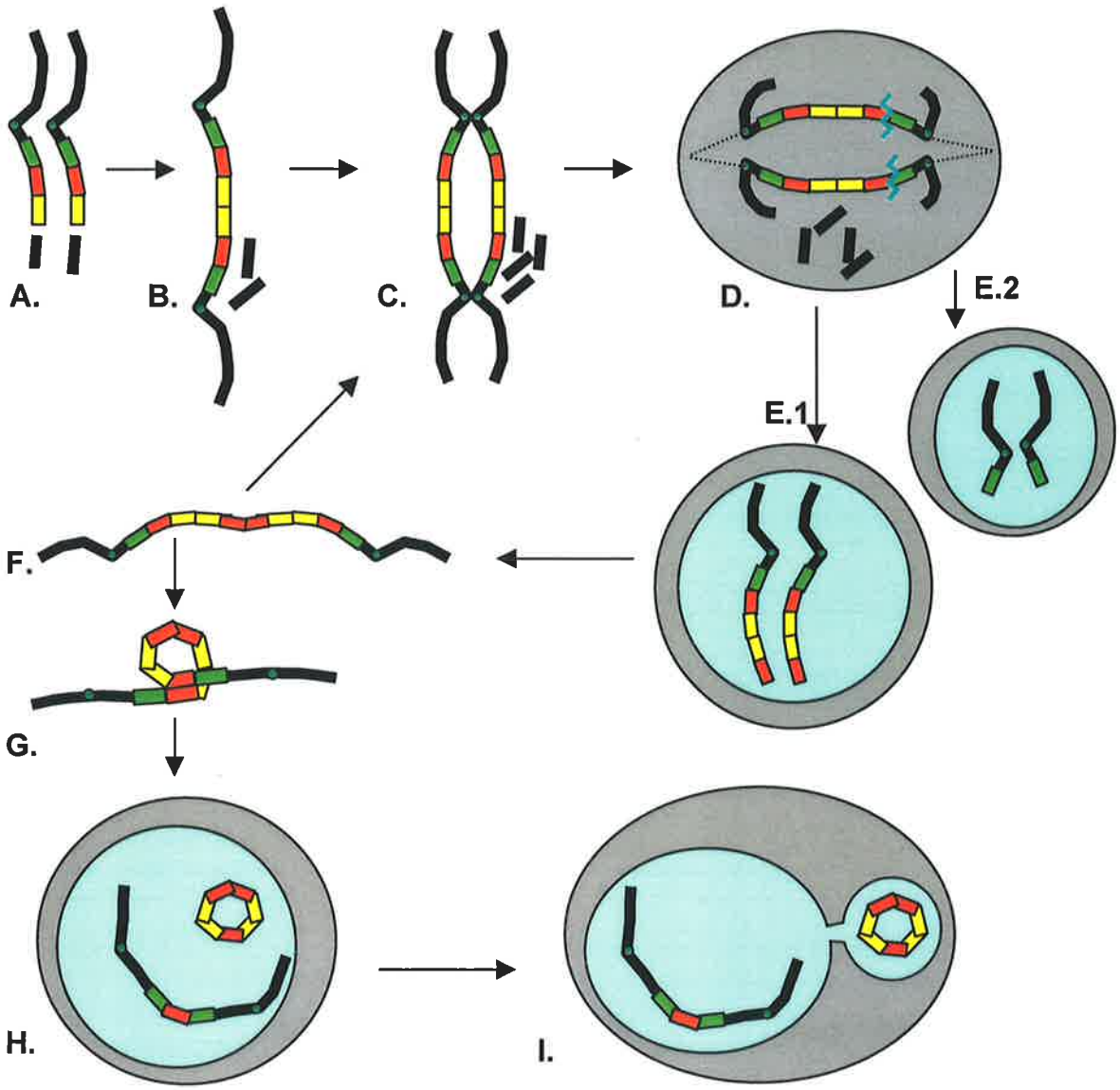
This thesis describes and validates the presence of nuclear buds (Figure 2.3.) as a novel biomarker for folic acid deficiency. In this system, nuclear buds correlate well with folic acid concentration in the culture medium (r^2= 0.46, P< 0.0001). It has now become evident that amplified genes may be eliminated via nuclear budding and MN formation. Shimizu and colleagues (1998; 2000) showed that amplified DNA is selectively localised to specific sites at the periphery of the nucleus and eliminated via nuclear budding to form MNi during S phase of mitosis. Furthermore, DNA synthesis inhibitors such as hydroxyurea were shown to increase the rate of elimination of amplified DNA via this process. It was suggested that amplified DNA is eliminated through recombination between homologous regions within amplified sequences forming mini-circles of acentric and atelomeric DNA (double minutes) which localise to distinct regions within the nucleus or through the excision of amplified sequences after segregation to distinct regions of the nucleus. The above suggests that the nucleus has a capacity to sense excess DNA that does not fit well within the nuclear matrix indicating a higher order DNA repair or nuclear housekeeping process. Furthermore, Miele *et al.* (1989) showed that amplified dihydrofolate reductase genes in methotrexate-resistant V79 cells were accumulated in nuclear buds, nucleoplasmic bridges and MNi. It is also reported that MNi are formed by a budding process following exposure to γ–irradiation (Haaf *et al.*, 1999). In this process, Rad 51-recombination protein complexes are detectable throughout the entire nucleus three hours after irradiation and then concentrate into distinct foci before being extruded from the nucleus into MNi. Using end-labelling these MNi were shown to exhibit DNA fragmentation analogous to that observed in apoptotic nuclei (Haaf *et al.*, 1999).

Because folic acid deficiency is known to cause gene amplification (Melnyk *et al.*, 1999) and chromosome damage such as DSBs (Blount *et al.*, 1997), it is likely that both of these

events contribute to the formation of nuclear buds in this system. Gene amplification is thought to be a key event in cellular resistance to drugs like methotrexate (Biedler and Spengler, 1976) and also in tumour progression (Brison, 1993). Several plausible models of gene amplification have been proposed (reviewed in Windle and Wahl, 1992; Stark 1993), however the presence of NPBs in the cytokinesis-blocked cells provides support to the 'breakage-fusion-bridge (BFB) cycle' model adapted from the seminal work of McClintock in maize (1942). According to this theory, sister chromatids which have both undergone DSB fuse at a distal position (possibly telomeric) forming a dicentric chromosome which has two copies of one ore more homologous genes positioned between the two centromeres. During anaphase these dicentric chromosomes are drawn towards opposite poles and form (nucleoplasmic) bridges. During cytokinesis these dicentric chromosomes, which span the distance between both daughter nuclei, are thought to break unevenly and may form a chromosome with two copies of one or more genes and a chromosome with no copies of these genes. The chromatids with multiple copy number of these genes may fuse again during interphase forming a dicentric chromosome (doubling again the gene copy number within the chromosome), which is then replicated during the next nuclear division leading to the next bridge-breakage-fusion cycle and further gene amplification (Figure 6.8).

The BFB cycle model has been validated as a mechanism for gene amplification in various systems including 1) coformycin-induced amplification of the adenylate deaminase gene in Chinese hamster cells (Toledo *et al.*, 1992), 2) N-(phosphonyacetyl)-L-aspartate-induced amplification of the CAD gene in human fibrosarcoma cells which is enhanced by the expression of *Vpr*, a HIV accessory gene (Shimura *et al.*, 1999), 3) methotrexate-induced amplification of the dihydrofolate reductase gene in Chinese hamster cells (Ma *et al.*, 1993) and 4) actinomycin D-induced amplification of the multi-drug resistance 1 gene in Chinese hamster cells through the induction of fragile sites which determined the initiation and size of amplicons (Coquelle *et al.*, 1997). Folic acid deficiency-induced fragile site expression and DNA hypomethylation may have also contributed to the promotion of gene amplification and resulted in elimination of this DNA by nuclear budding in our system. The induction of hypomethylation by 5-aza-2'deoxycytidine has been reported to enhance N-(phosphonylacetyl)-L-aspartate-induced amplification of the CAD gene in Syrian hamster kidney cells (Perry *et al.*, 1992).

Figure 6.8 Gene amplification via Breakage-Fusion-Bridge cycles

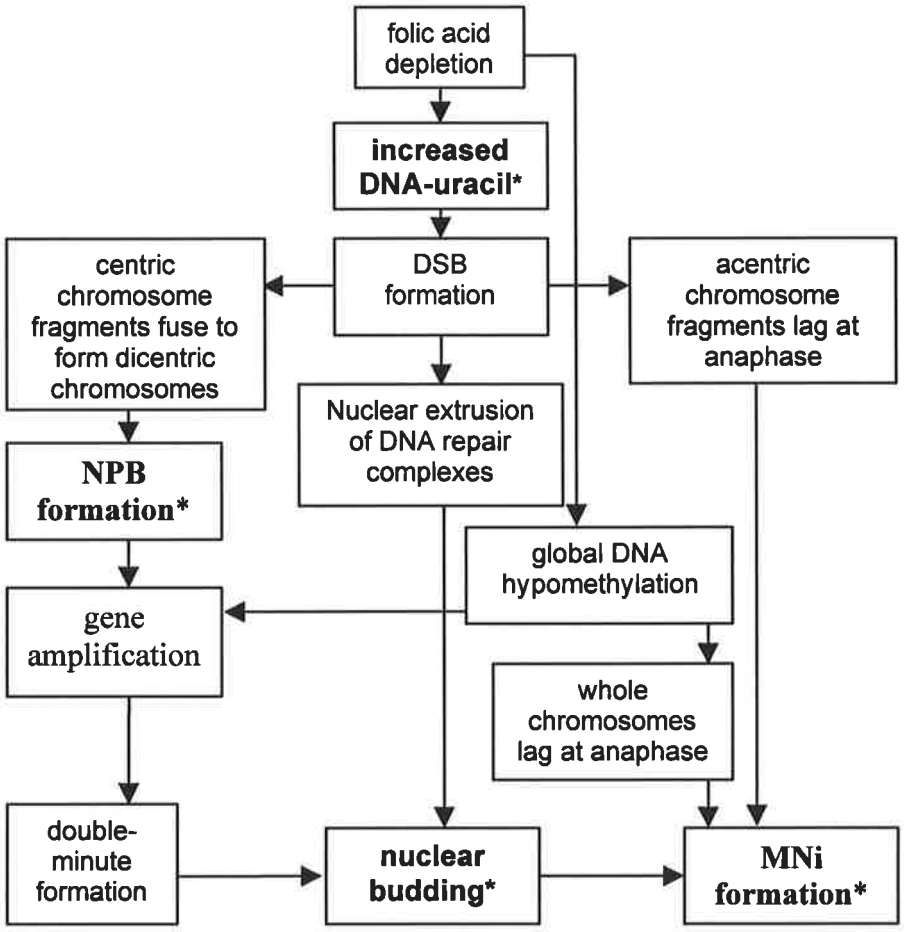


Broken sister chromosomes (A) (for mechanism of breakage see figure 2.1) fuse at the 'sticky' ends to form a dicentric chromosome (B). The dicentric chromosome is replicated in S-phase (C) and drawn to the cell's poles during anaphase (bridge) (D). In this example, the dicentric chromosomes break in a non-central region and one daughter cell will receive chromosomes that contain two copies of the yellow and red genes (E.1) while the other cell's chromosomes have a deletion in these genes (E.2). The chromosomes with multiple copy number may fuse again (F) to propagate the amplification cycle (back to step C). Alternatively, recombination may occur between homologous sequences (G) and result in the 'looping-out' of a circular acentric DNA fragments or double minute (H) which are subsequently extruded from the nucleus via budding (I).

The scoring of NPBs and nuclear buds is valuable because it has the potential of adding considerably more information to the cytokinesis block micronucleus assay. For example the presence of NPB provides a measure of chromosome rearrangement and implies the induction of chromosome breaks, which cannot be assumed directly when scoring MNi only, because MNi may arise from both chromosome breaks and chromosome loss. In addition this study verifies the importance of nuclear buds as a biomarker of DNA damage (gene amplification) because of the strong positive correlation with NPBs and MNi and the negative correlation with folic acid concentration. These correlations fit well with the concept that folic acid deficiency may cause gene amplification by initiating BFB cycles in dividing cells. A summary of the events surrounding gene amplification that may be occurring in the micronucleus assay system used in our experiments is presented in Figure 6.9.

As described earlier, measures of cytotoxicity are now incorporated into the CBMN assay to give a more thorough indication of the cellular response to the treatment being tested, whether it be nutrient deficiency or exposure to a known mutagen. In this system apoptosis did not appear to be related to folic acid concentration. Although apoptosis was higher at folic acid concentrations of 60 nM than 120 nM, the magnitude of this difference is rather small i.e. four cells in 1000, and it is unclear whether this is a physiologically relevant difference. Necrosis was not minimised at 60 nM, but at 120 nM, with a small but significant 3% difference. NDI exhibited a strong positive correlation with folic acid concentration but reached a plateau at 60 nM (60 v 120 nM, $P > 0.05$). When markers of cytotoxicity and chromosome damage are considered it is clear that the most favourable or healthy cell profile is generated at a folic acid concentration of between 60 and 120 nM, which is more than double those currently considered normal. Further research is required to determine whether the DNA damage markers described here are at the lowest possible level when the plasma folate concentration is around 30 nM or whether the profile can be improved by increasing folic acid intakes and intracellular folate concentrations. The requirement for higher than 'normal' *in vivo* concentrations of folate and vitamin B_{12} to minimise the MN index was demonstrated by an intervention study in young adults taking 700 μg of folic acid and 7 μg vitamin B_{12} daily (350 % Australian RDA) (Fenech *et al.*, 1998).

Figure 6.9 Summary of genotoxic events that may be induced by folic acid depletion in this *in vitro* system



* indicates endpoints measured in this paper. Double-minutes are widely accepted as markers for gene amplification (Schimke, 1988).

As shown in Tables 6.2 and 6.3, two-way ANOVA has revealed that there is no significant difference in any of the endpoints measured between CCs and TTs. These results suggest that lowered uracil incorporation and DNA strand breakage may not contribute significantly to the reduced cancer risk in TTs compared to CCs. The study compared CCs and TTs over a broad physiological folic acid concentration range to maximise the chance of detecting differences between the two groups, however it is possible that the actual MTHFR activity of the two groups may not be sufficiently different in this system. The C677T polymorphism is thought to decrease the binding affinity of flavin adenine dinucleotide (FAD) to MTHFR and may increase the rate of dissociation of FAD from the enzyme (Guenther *et al.*, 1999). Recent evidence suggests

that riboflavin (vitamin B_2), the immediate precursor to FAD, is an independent determinant of plasma homocysteine levels, but only in individuals with the C677T polymorphism (Hustad *et al.*, 2000). Therefore, it is possible that an abundance of riboflavin may correct or improve MTHFR activity in people with the polymorphism. This may serve to negate or lessen any influence that the C677T polymorphism may have on uracil incorporation into DNA. Although not a definitive indicator of intracellular concentrations, the riboflavin concentration of RPMI-1640 medium is 530 nM, which is considerably higher than the 12 – 13 nM found in plasma (Hustad *et al.*, 2000) and may therefore raise intracellular FAD levels. It is possible that high riboflavin in the culture medium, which increases FAD concentrations, may cause our *in vitro* results to differ from the normal situation in humans *in vivo* (Ames *et al.*, in preparation). Further research is clearly needed to investigate the interaction of riboflavin, folic acid and MTHFR polymorphisms on uracil misincorporation into DNA *in vivo*.

If the lack of difference between CCs and TTs seen in this experiment can be explained by the fact that high riboflavin concentrations do improve MTHFR activity and abolish the protection afforded by the C677T polymorphism, this begs the question of whether low riboflavin and FAD concentrations may be beneficial in TTs due to a preservation of the polymorphism's protective effect. It may be interesting to explore the concept of whether current riboflavin RDAs for TTs may need to be carefully evaluated to preserve the protective effect of the C677T polymorphism against some cancers. Similarly, a lower riboflavin intake in CCs may reduce MTHFR activity to a level that produces a net benefit with respect to uracil incorporation into DNA.

It is also possible that changes in DNA methylation may be an alternative explanation for the impact of the C677T polymorphism on cancer risk. There is now preliminary evidence (Stern *et al.*, 2000) that the C677T polymorphism causes global DNA hypomethylation in normal somatic cells. However, global DNA hypomethylation is believed to be associated with increased cancer risk (Laird and Jaenisch, 1994) and cancer progression (Cravo *et al.*, 1994; Kim *et al.*, 1994)). One plausible explanation is that the MTHFR mutation may specifically protect against the hypermethylation-induced silencing of tumour suppressor genes. Hypermethylation of tumour suppressor genes is a common event in many cancers (Robertson and Jones, 2000).

The C677T polymorphism has been associated with a reduced risk for colorectal cancer and acute lymphocytic leukaemia (ALL)(Slattery *et al.*, 1999; Chen *et al.*, 1996; Ma *et al.*, 1997; Skibola *et al.*, 1999). It is, however, probable that the polymorphism has different effects in different types of cells. In contrast to reports of a reduced risk for some cancers, the polymorphism has no apparent benefit with respect to acute myeloid leukaemia (Skibola *et al.*, 1999) and has been associated with a 2.8-fold increased risk of endometrial cancer (Esteller *et al.*, 1997). In this instance, the authors suggest that compromised DNA methylation, as a result of reduced MTHFR activity, was responsible for the elevated risk. It is important that future experiments test the effects of MTHFR polymorphisms and folic acid deficiency on various different types of cells, in particular stem cells originating from the colon and bone marrow that have the potential of becoming cancer cells *in vivo*.

In the study by Skibola *et al.* (1999) which shows a reduced risk for ALL associated with the C677T polymorphism, it is also reported that people heterozygous for the A1298C polymorphism have a 3-fold reduced risk for ALL. The preliminary data presented in Table 6.2 does not support the hypothesis that reduced uracil incorporation into DNA is the mechanism responsible. In fact, at the deficiency concentration of 12 nM folic acid, heterozygotes (AC) incorporated significantly more uracil into DNA than wildtypes (51.5 ± 9.4 and 31.9 ± 4.1 pg/10 μg DNA respectively. T-test, P= 0.038).

Also, heterozygotes for the MS A2756G polymorphism incorporated approximately 3-fold more uracil into DNA at the 120 nM concentration than wildtypes (28.8 ± 10.0 and 10.8 ± 3.0 respectively. T-test, P= 0.025)(Table 6.2). It is unlikely that this observed increase in uracil incorporation is due to methyl folate trapping and an increase in the ratio of dUMP to dTMP because there is no evidence that this polymorphism is associated with reduced MS activity. Rather, two reports describe significantly lower plasma homocysteine levels in people homozygous for the mutation (Harmon *et al.*, 1999; Tsai *et al.*, 2000). These data do not support the hypothesis that altered uracil incorporation into DNA is a main causative factor of the altered cancer risk in those with common polymorphisms in MTHFR and MS.

Another factor that may cause differences in uracil misincorporation is the presence of other polymorphisms in folic acid metabolising genes. To date, five polymorphisms in the MTHFR gene have been identified (Goyette *et al.*, 1994, 1995 and 19996; Frosst *et al.*, 1995; Kluijtmans *et al.*, 1998). Coupling these with polymorphisms in genes coding for other folic acid metabolising enzymes, such as methionine synthase, methionine synthase reductase and thymidine synthase gives a large combination of polymorphisms, all of which could conceivably influence uracil incorporation into DNA

The concentration of methionine in the medium may also influence MTHFR activity in this system. The concentration of L-methionine in the RPMI (not considering FBS) was 100 μM which is more than the 20 to 30 μM normally found *in vivo* (Ubbink *et al.*, 1996). It is possible that as a result of high methionine levels, intracellular levels of the next metabolite in the methionine cycle, S-adenosyl methionine (SAM), may have become elevated. SAM is a known inhibitor of MTHFR (Kutzbach and Stokstad, 1971) and it is possible that this effect may have lowered the MTHFR activity of control cells to a level similar to that of TT cells.

In conclusion it has been shown that markers of genetic damage, including DNA uracil-content, MNi, nuclear buds and NPBs, correlate strongly with the folic acid content of culture medium over a broad physiological concentration range. The link between uracil incorporation and micronucleus formation is also confirmed. Because of the importance of DNA damage as a cause of cancer and other diseases of old age, a new paradigm has been suggested for determining recommended dietary allowances based on optimising genomic stability (Fenech, 2001). This experiment, and others like it, may prove to be an important preclinical step in redefining RDAs from a level that prevents deficiency disease to a level that maximises genomic stability and therefore minimises the risk for cancer and other DNA damage-related diseases.

The results of this experiment suggest that MTHFR C677T genotype does not influence levels of DNA-uracil-content or chromosome damage as measured by the cytokinesis block micronucleus assay. Furthermore, these results suggest that the hypothesis that reduced cancer risks associated with MTHFR polymorphisms are due to a diversion of 5,10-MnTHF to thymidine synthesis may be an over-simplification of real events.

Clearly, further research is needed to define the effect of this polymorphism in combination with folic acid, riboflavin, and methionine deficiency on DNA-uracil incorporation and DNA methylation (global and specific) in a variety of cell types, particularly those originating from tissue in which there is evidence for a change in the risk of pathology (e.g. colon, endometrium, cervix, breast, leukocyte stem cells).

6.5 Publications arising from this chapter

Crott,J.W., Mashiyama,S.T., Ames,B.N. and Fenech,M.F. (2001) Methylenetetrahydrofolate reductase C677T polymorphism does not alter folic acid deficiency-induced uracil incorporation into primary human lymphocyte DNA *in vitro*. *Carcinogenesis* [Accelerated Paper], **22**(7), 1019-1025.

Crott,J.W. Mashiyama,S.T., Ames,B.N. and Fenech,M. Folic acid deficiency increases chromosome breakage and rearrangement, gene amplification and DNA uracil-content in human lymphocytes *in vitro*: effect of the MTHFR C677T polymorphism. Cancer Epidemiology Biomarkers and Prevention. In Press.

Chapter 7: General Discussion. Folic acid deficiency-induced chromosome damage: an integrated scheme.

Folate is an essential vitamin that is involved in the transport and transfer of one-carbon units to various intracellular reactions. These reactions include the synthesis of thymidine and methionine and hence the methylation of DNA. Because of folate's role in these reactions, folate deficiency causes deleterious changes to DNA which have been associated with the development of cancer and other diseases of old age. This thesis describes a series of experiments that investigated how folic acid deficiency and inborn errors in folate metabolism affect genetic stability *in vitro*.

Because the issue of folic acid in genetic stability was addressed from a number of different angles, an integrated scheme that draws on the results presented here, as well as evidence from the literature, could be constructed to illustrate the wide-ranging effects of folic acid deficiency on cellular health (see Figure 7.1).

Homocysteine

It is well documented that one of the side effects of folate deficiency is that blood homocysteine concentrations become elevated (Jacob *et al.*, 1998; Kang *et al.*, 1987). In the 1960s and 70s it became apparent that homocysteine, when present at high concentrations, may be involved in the pathogenesis of cardiovascular disease (McCully, 1969). Since then, increased plasma homocysteine concentrations have been positively associated with Alzheimer's disease (Clarke *et al.*, 1998) and elevated MN expression (Fenech *et al.*, 1997a, 1998). What is not clear is whether homocysteine, at high concentrations, can induce damage to cellular structures independent of an underlying folate or vitamin B_{12} deficiency, both of which are known to cause chromosome damage (Chang *et al.*, 1996; Fenech *et al.*, 1997a, 1998; Titenko-Holland *et al.*, 1998). The first experiment described in this thesis aimed to clarify this issue by testing the hypothesis that high concentrations of homocysteine are genotoxic to human lymphocytes *in vitro* (see Chapter 3).

The results from this experiment suggest that homocysteine only marginally increases MN frequency in human lymphocytes compared to similar concentrations of methionine *in vitro*. In contrast to previous evidence that homocysteine (100 μM to 10 mM) is a potent inducer of necrosis (Wall *et al.*, 1980; Starkembaum and Harlan, 1986), the results presented herein show that, in comparison to methionine, homocysteine was also not a strong inducer of necrosis. Because of the absence of a strong affect on MN or necrosis induction, it remains to be clarified whether homocysteine can participate in the pathology of disease *in vivo* or whether it is simply a marker of an underlying folic acid and/or vitamin B_{12} deficiency in previous studies. Apart from the possibility of elevated homocysteine simply being a marker for B-vitamin deficiency, it may be possible that alternate forms of homocysteine are damaging to cellular structures *in vivo*. As reported by Jacobsen (1998), 99% of homocysteine in plasma is found in various oxidised forms such as homocystine, some of which may be cytotoxic and/or genotoxic but may not be present in cell culture systems.

An improved CBMN assay

One of the main aims of this thesis was to thoroughly characterise the effects of *in vitro* folic acid deficiency, over a physiological concentration range, on markers of chromosome damage using the cytokinesis-block micronucleus assay format. Because folic acid deficiency causes chromosome damage through the incorporation of uracil into DNA (Figure 7.1), multiple rounds of replicative DNA synthesis are required for chromosome damage to be expressed. Therefore it became apparent that the conventional micronucleus assay, which involves a culture duration of three days or only enough time for one or two rounds of cell division to occur, would need to be modified and extended to allow the expression and detection of folic acid deficiency-induced chromosome damage.

The development of a longer-term comprehensive micronucleus assay is described in Chapter 5. Early experiments revealed that the addition of interleukin-2 enhanced cell proliferation and permitted the culture of primary lymphocytes for up to nine-days with only a single addition of the mitogen at the initiation of cell culture. Time-course experiments then showed that nine days was sufficient time to observe substantial

increases in MN frequency in response to folic acid depletion whereas the effects of folic acid depletion on cell proliferation became apparent after only six days. These experiments also showed that besides MNi, the frequency of nuclear budding, a novel endpoint in the cytokinesis-block micronucleus assay, was increased in response to folic acid deficiency. Together with nucleoplasmic bridges, nuclear buds were validated as biomarkers of folic acid deficiency in this system. Nucleoplasmic bridges and nuclear buds are indicative of chromosome rearrangement and gene amplification respectively. Nuclear budding has previously been reported in cancer cells (Gissellson *et al.*, 2001; Shimizu *et al.*, 1998), methotrexate-resistant cells (Meile *et al.*, 1989) and Bloom syndrome cells (Yakiwski *et al.*, 2000) however, to the best of my knowledge this is the first report of nuclear budding in primary normal human lymphocytes from healthy people in response to folic acid deficiency. In addition, NPBs have been associated with nuclear buds in cancer (Gissellsson *et al.*, 2001) and methotrexate-resistant cells (Meile *et al.*, 1989) but as yet there are no reports of NPBs being associated with folic acid concentrations.

The end result of these experiments is a comprehensive technique for studying the genotoxic and cytotoxic effects of folic acid deficiency to human lymphocytes *in vitro*. A major benefit of this system is that it allows biochemical measures on the composition of cellular DNA to be made in cells from the same population of cells that chromosome damage is measured in. For example, in this project the DNA-uracil content of cells was measured in parallel to the cytogenetic endpoints of the CBMN assay. Many other highly informative measures could be coupled to this system in addition the uracil assay. Analysis of global and specific DNA methylation, mitochondrial deletions and gene/protein arrays may provide further insight into how folic acid depletion causes perturbations at the molecular and chromosome level of DNA and can translate into the cytogenetic endpoints of the CBMN assay.

Experiments with this new comprehensive assay indicated that 60 – 120 nM folic acid is required to minimise chromosome damage rate and uracil content in DNA *in vitro*. These concentrations are approximately double to triple plasma folate concentrations in the normal range (20 – 30 nM) for healthy non-anaemic individuals (Fenech and Rinaldi, 1994; 1995), however it is reported that plasma folate concentrations of up to 50 nM are sustainable by folic acid supplementation (Bronstrup *et al.*, 1998). These experiments, as

well as future experiments that use 5-MeTHF as the folate source, may prove to be an important pre-clinical step in the process of revising and increasing the recommended daily intake of folate to a level that maximises *in vivo* chromosome stability (Fenech, 2000).

Micronuclei

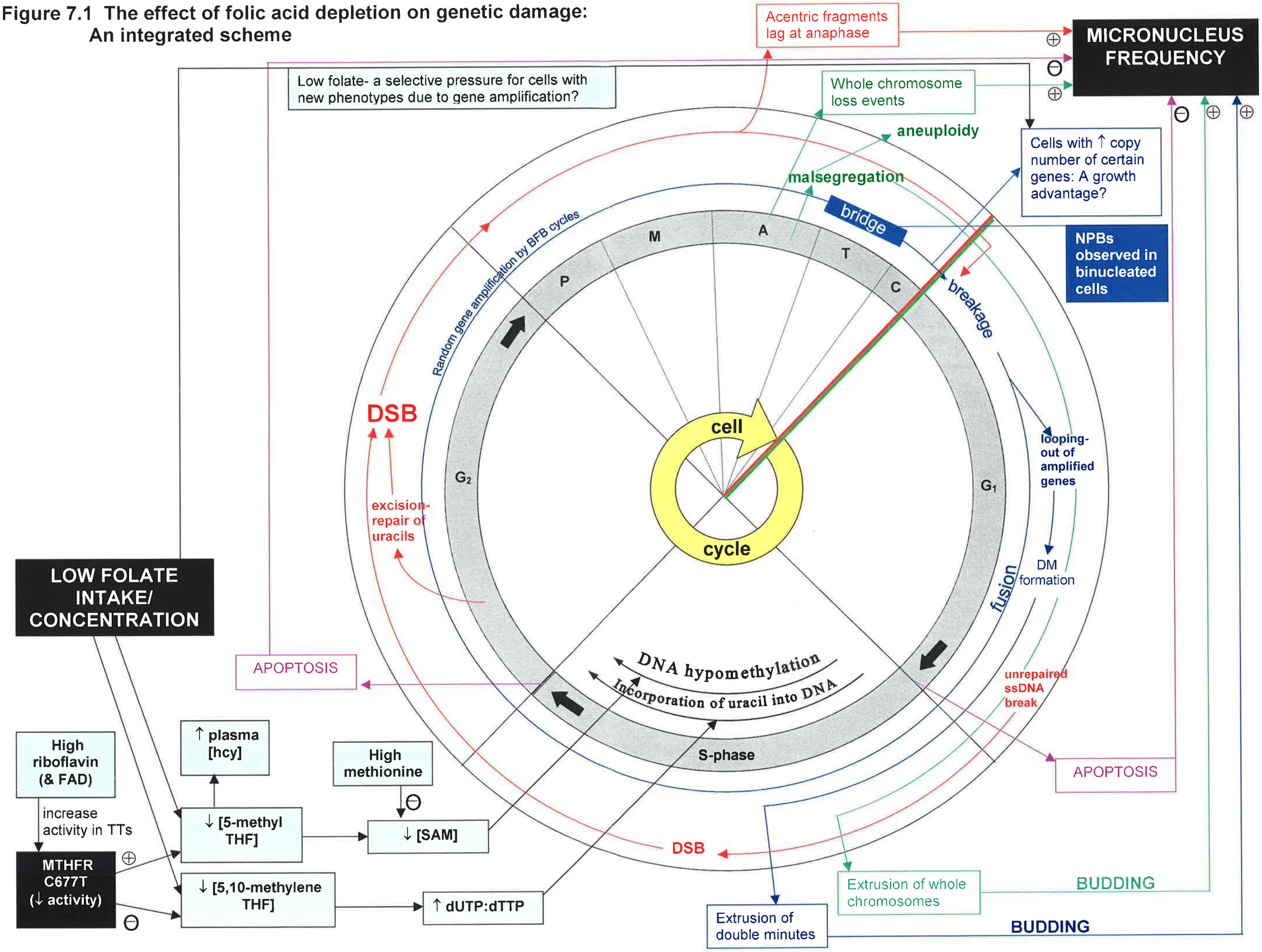
Folate deficiency is known to induce a variety of deleterious changes to the structure and composition of DNA, including the incorporation of excessive amounts of uracil into DNA (Blount *et al.*, 1997) and the hypomethylation of DNA (Jacob *et al.*, 1998). These molecular changes to DNA are thought to cause chromosome aberrations and translate into a number of structural abnormalities of the nucleus, such as MNi, NPBs and nuclear buds (Figure 7.1). The results presented in Chapter 6, which show that DNA uracil-content increases with folic acid deficiency and that DNA uracil-content is significantly and positively correlated with these cytogenetic endpoints supports the hypothesis that DNA changes at the molecular level translate into nuclear abnormalities. Furthermore Gissellsson and colleagues (2001) confirm the link between abnormal nuclear morphology and damage at the chromosome level by showing that the frequency of MNi, NPBs or buds was significantly and positively correlated with chromosome aberrations such as ring (double minutes) and dicentric chromosome structures in a variety of soft tissue tumours.

As mentioned above, the results suggest that folic acid concentrations currently considered to be in the 'normal range' for healthy non-anaemic individuals (i.e. 12 nM) are not sufficient to prevent a significant increase in MN frequency in this *in vitro* system. Furthermore, the results also show that a folic acid concentration between 60 and 120 nM is required to minimise chromosome damage *in vitro*. The experiments described in this thesis utilise folic acid as the sole folate source to support cell growth. This differs somewhat from the *in vivo* situation where the predominant species of folate in plasma and that is therefore absorbed by cells is 5-MeTHF. It seems likely that for experiments studying the effects of folate deficiency on chromosome damage, similar results would be obtained with both folic acid and 5-MeTHF given that all cofactors required for folate interconversions, such as vitamin B_{12} and FAD are in abundance and also that the relevant

Figure 7.1 The effect of folic acid depletion on genetic stability: An integrated scheme

This figure summarises the genotoxic effects of folic acid deficiency at the molecular, chromosome and cellular level. More specifically this figure illustrates when in the cell cycle the damage occurs and is expressed. **A**, anaphase; **C**, cytokinesis; **DM**, double minute; **DSB**, double stranded (DNA) break; **FAD**, flavin adenine dinucleotide; **hcy**, homocysteine; **M**, metaphase; **MTHFR**, methylene tetrahydrofolate reductase; **NPB**, nucleoplasmic bridge; **P**, prophase; **SAM**, S-adenosyl methionine; **ss**, single stranded; **THF**, tetrahydrofolate; **TT**, person homozygous for the MTHFR C677T polymorphism.

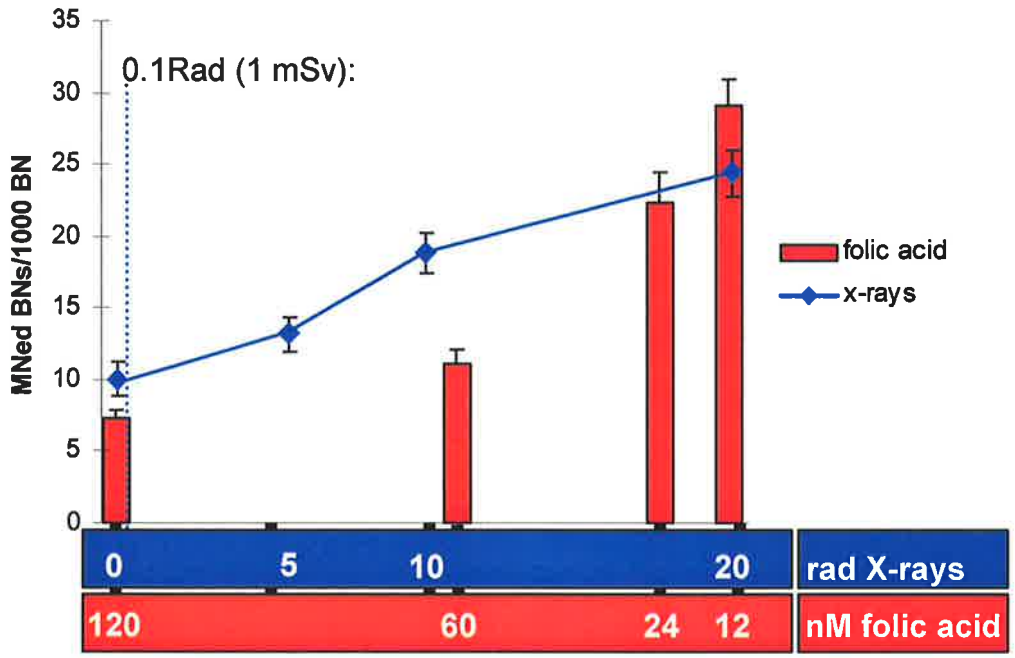
Figure 7.1 The effect of folic acid depletion on genetic damage: An integrated scheme



folate metabolising enzymes are functioning normally. However, it is likely that when investigating the effect of vitamin B_{12} deficiency of chromosome damage, the folate source in medium may be an important determinant of whether an effect of the vitamin B_{12} deficiency is observed. I speculate that the effect of vitamin B_{12} deficiency on chromosome breakage may only be expressed if the folate source requires a vitamin B_{12}-dependent conversion before it can participate in thymidine synthesis. This may explain why no effect of vitamin B_{12} deficiency was seen in this system where folic acid was the sole folate source. Folic acid is converted directly to dihydrofolate, tetrahydrofolate and then to 5,10-MnTHF (the methyl group donor for thymidine synthesis) with each step being independent of vitamin B_{12}. In this case, the use of 5-MeTHF may have facilitated the observation of an effect of vitamin B_{12} deficiency because it must first be converted to DHF by vitamin B_{12}-dependent methionine synthase (and then to THF and 5,10-MnTHF) before it can participate in thymidine synthesis.

The data presented in Chapter 6 show that the MN frequency of cultures grown in medium containing 12 nM folic acid is approximately 18 MNed BNs (per 1000 BNs) higher than in cultures with 60 nM folic acid. Both of these concentrations are within the physiological range. To put this increase into perspective, 20 rad of X-rays (0.2 Gy) is reported to induce an increase of approximately 14 MNi from baseline (see Figure 7.2)(Fenech and Morely, 1986). This dose of X-rays is equivalent to approximately 200 millisieverts, or the dose absorbed by approximately 2000 chest x-rays (0.1 mSv each). Furthermore 20 rad (200 mSv) is 200 times higher than the safe annual exposure limit from all sources for the general public set by the International Atomic Energy Agency (1 mSv). Figure 7.2 shows the MN data from this experiment superimposed onto MN frequencies induced by X-irradiation of human lymphocytes. It is clear from this comparison that *in vitro* folic acid depletion to a level within the normal physiological range can induce MN formation as effectively as doses of X-rays that are well above safe annual allowances.

Figure 7.2 Comparison of rates of micronucleus induction by folic acid deficiency and X-irradiation in human lymphocytes *in vitro*



MN frequency in human lymphocytes in response to X-irradiation (blue) and folic acid depletion (red). X-ray data are reproduced from Fenech and Morley (1986). Dose rate= 50 rad/min. The dashed line at 0.1 rad (1mSv) is the annual safe exposeure limit to radiation set by the International Atomic Energy Agency. 100 rad X-rays = 1 Gy ~ 1 Sv. Data expressed as mean ± SEM.

The MN frequency at 60 and 120 nM folic acid was 11.1 ± 1.0 and 7.4 ± 0.4 MNed BNs/1000 BNs respectively for the main experiment (Table 6.3), values similar to or lower than previously reported values obtained from people in a similar age bracket using the standard three day protocol (Fenech and Rinaldi, 1994). This indicates that in this system, multiple rounds of cell division can be supported with a level of fidelity in DNA replication that translates to a MN frequency which does not deviate from frequencies observed with only one round of cell division (when folic acid is in abundance).

In this thesis, it was found that as well as MNi, which arise from chromosome breakage and loss, nuclear budding and nucleoplasmic bridges, which may be indicative of gene

amplification and chromosome rearrangement respectively, are induced by folic acid deficiency.

Nucleoplasmic bridges

Nucleoplasmic bridges were found to be significantly and negatively correlated with the concentration of folic acid in culture medium and were therefore validated as a novel biomarker of *in vitro* folic acid deficiency. Nucleoplasmic bridges are formed when the centromeres of dicentric chromosomes are pulled to opposite poles of the cell during division. Double-stranded DNA breakage in two chromosomes allows the formation of dicentric chromosomes.

The frequency of NPBs in cell cultures followed the same trend as MN counts, although at a lower frequency. For the main experiment the ratio of BNs with MNi to BNs with NPBs is between 3.9 and 4.6 (3.9, 3.9, 4.6 and 4.3 for 12, 24, 60 and 120 nM respectively). Because NPBs can only be formed due to chromosome breakage and MNi can be formed by chromosome breakage or loss, the ratio of BNs with MNi: NPBs in a cell population may give some indication of the mechanism of MN formation. For example if the ratio of BNs with MNi: NPBs is very high, this might imply that the majority of MNi are formed due to chromosome loss events rather than breakages. On the other hand if the number of NPBs is close to that of MNi and the ratio of BNs with MNi: NPBs is smaller, it could be implied that the majority of the MNi were formed due to chromosome breakage. Because NPBs require two broken chromosomes to fuse, it would not be expected for a population of cells to ever exhibit as many BNs with NPBs as MNi, i.e. cells with only one broken chromosome may express a MN but not a NPB. Although this theory requires validation in studies that score kinetochore positive and negative MNi as well as NPBs, it seems likely that the ratio of four BNs with MNi to every one BN with a NPB in this system may be indicative of a situation where the predominant mechanism of micronucleus formation is chromosome breakage. Given that not every cell will have more than one chromosome break and also that there is a good probability that two broken chromosomes will not come into contact and fuse before anaphase, a ratio of 4:1 seems quite low and may indicate MN formation predominantly by chromosome breakage. Furthermore, the high methionine concentrations in the culture medium of this system

(100 μM compared to 20-30 μM *in vivo*) may preclude excessive chromosome loss caused by hypomethylation of peri-centromeric DNA because methionine is the precursor to the methyl group donor for DNA methylation (SAM).

Also similar to the MN data, there is a three-fold lower frequency of NPBs in cultures grown in medium with 60 nM (high physiological) compared to 12 nM (low/normal physiological) folic acid ($P< 0.05$), indicating that *in vivo* folic acid concentrations that are currently considered normal may not be sufficient to minimise the occurrence of NPBs *in vitro*. NPBs may be the initiating step in gene amplification by breakage-fusion-bridge cycles (see below).

Nuclear budding: a marker of gene amplification and more!

The presence of nuclear buds in this system was first observed in a preliminary folic acid dose-response experiment (see section 5.5.3 a). Although not quantified at this stage, nuclear buds seemed to be more prevalent in cell populations that had a high MN frequency. From this time the scoring of nuclear buds was incorporated into the CBMN assay because nuclear budding is reported to be a mechanism for the removal of double-minute chromosomes form the nucleus and therefore is a marker of gene amplification (Shimizu *et al.*, 1998; 2000). Gene amplification is thought to be a key event in tumour progression (Brison, 1993) and resistance to drugs like methotrexate (Biedler and Spengler, 1976).

The presence of NPBs in this system supports gene amplification by the bridge-fusion-breakage model of gene amplification. According to this model, which is based on McClintock's work in maize (1942), the fusion of broken sister chromosomes (forming a nucleoplasmic bridge) followed by unequal breakage of the resulting dicentric chromosome leads to an increased gene copy number on one chromosome (gene amplification) and a gene deletion on the other. In this model multiple rounds of cell division are required to increase gene copy number (Figure 6.8). In chromosomes that have undergone gene amplification, homologous recombination can occur between amplified sequences and result in the looping out of amplified genes into acentric circular DNA structures called double- minutes (DM). It is known that DMs are formed due to

gene amplification (Schimke, 1988) and that DMs are extruded from the nucleus via nuclear budding into MNi (Shimizu *et al.*, 1998; 2000).

It is unclear how DMs are recognised as material that should be removed from the nucleus, however it is possible that these extraneous chromosome fragments are 'muscled' out of the nucleus when they encroach upon the territory of another chromosome. Researchers hypothesise that each chromosome has its own region or territory within the nucleus and that this dynamic spatial organisation of chromosomes within the nucleus may play a role in the regulation of gene expression (Cremer and Cremer, 2001). It seems possible that if foreign chromosomes or DMs intrude into these specific territories, which are occupied by other chromosomes, that they may be forced out of that territory and out of the nucleus by a yet undefined process.

In addition to the potential role of chromosome territories in the extrusion of DMs, these territories may dictate the frequency at which amplified genes translocate to other chromosomes. In other words, the close proximity of a chromosome to another that contains amplified genes may make it the most likely candidate to host amplified sequences after translocation. For example, Wong and colleagues (1998) show that in response to low folic acid concentrations, there is an increase in the copy number of the reduced folate carrier (RFC) gene (originating on chromosome 21) to an average of 4-5 copies per cell, and that amplified copies were most frequently found on chromosomes 2, 13, 15 and 16. Furthermore, these RFC gene copies were frequently localised to DMs.

It is possible that given sufficient time, cells that have gained an increased copy number of certain genes could be selected for or against by a low folate environment. For example, cells grown in low folic acid conditions a variant was isolated which had an increased copy number of the reduced folate carrier gene, the product of which allows the uptake of more folate from the culture medium (Wong *et al.*, 1998). This suggests that genetic instability in cells, a situation that generates new genetic variants, is a (inadvertent?) mechanism for survival in a toxic or stressing environment such as folic acid deficient culture conditions.

Besides being associated with gene amplification in normal cells, nuclear budding is a frequent event in fibroblasts of people with Bloom syndrome (BS), a rare cancer-

predisposing disorder (Yankiwski *et al.*, 2000). Bloom syndrome is characterised by a high frequency of sister chromatid exchanges and elevated rates of somatic mutation. In BS cells nuclear buds contain centromeres and telomeric sequences, indicating that it is whole chromosomes that are extruded from the nucleus. Furthermore BS fibroblasts have an elevated MN frequency, with rates of 18-25% in BS fibroblasts compared with 2-6% in normal fibroblast cell lines. Because this chromosome loss is an active process that occurs during S phase (Yankiwski *et al.*, 2000), it is distinct from the passive act of chromosomes lagging behind at the equator of a cell during anaphase (Figure 7.1). It is unclear whether intracellular folate or nucleotide pools can influence this process in normal cells. However, it seems possible that if a cell gains an extra chromosome through malsegregation in cell division, the extra chromosome may be extruded from the nucleus by budding because it does not fit well within the predetermined spatial arrangement of the nucleus (Figure 7.1). It is known that low concentrations of folate can cause chromosome loss into MNi *in vivo* (Titenko-Holland *et al.*, 1998). Theoretically, it is possible that a chromosome lagging at the equator of a cell may be still packaged into a daughter nucleus rather than being packaged into a MN. If this chromosome, which has not been split during anaphase, does reach a daughter nucleus then that nucleus/cell will contain three copies (3N) of a certain chromosome rather than the normal two copies (2N). Prior to division, the cell will now be 6N as opposed to the normal 4N. The extra chromosome may then be extruded from the nucleus by budding because it is intruding on the territory of another chromosome and does not fit well within the spatial arrangement of the nucleus.

DNA uracil content

The DNA uracil-content of cells from the same cultures that were sampled for the CBMN assay was also measured in response to folic acid depletion. It was found that uracil levels in DNA increased in response to decreasing folic acid in the medium. It was found that there was a 42% reduction in the uracil content of cells grown in 60 nM compared to cells grown in 12 nM folic acid ($P < 0.05$). The results also show that at 120 nM, the highest folic acid concentration tested, there is still a detectable amount of uracil present in DNA, however it is unclear whether this is due to the incorporation of uracil into DNA or due to the deamination of cytosine resulting in the formation of uracil (Stryer, 1995).

It is unclear whether cell culture periods of longer than nine days in folic acid-deficient medium would facilitate higher DNA-uracil contents than those measured here. The studies of Blount and colleagues (1997) report values much higher than those shown in Chapter 6. DNA uracil-content ranged from 34,600 - 167,400 uracils per diploid cell (10 – 48 pg uracil/ 10 μg DNA). In the report of Blount and colleagues (1997), the minimum uracil contents were three-fold higher than the maximum reported in this study, with a range of 500,000 – 4,000,000 uracils/diploid cell. It is suggested that the volunteers who participated in the above mentioned study (Blount *et al.*, 1997) had chronic extended folate deficiency for several months or years. It seems logical that the more times cells replicate in a folate deficient environment, the greater the accumulation of uracil in DNA. The question is of course, how much uracil can a cell tolerate? A longer-term experiment with cultures of up to several months may be required to answer this question.

The DNA-uracil content of cells correlated significantly and positively with MN expression in cells from the same cultures, a result that supports the hypothesis that the removal of uracil from DNA can cause double-stranded DNA breakage. Similarly, the uracil content of DNA correlated significantly and positively with the frequency of NPBs and buds. This supports a role for uracil-associated strand breakage in NPB formation, the subsequent random amplification of genes by the breakage-fusion-bridge cycle and the extrusion of amplified DNA by nuclear budding. The correlation between DNA-uracil content and NPB frequency was however weaker than expected and uracil could explain only 17% of the variance in NPB. This is surprising since NPBs can only be formed through chromosome breakage. The most likely reason for this relatively weak correlation is the inaccuracy of the uracil assay. Although GC-MS is a highly sensitive method for detecting uracil, the limiting factor for the sensitivity of the assay is that it relies on DNA quantitation, a procedure that is somewhat less precise using classical spectrophotometric techniques.

Inborn errors in folate metabolism

As well as studying how folic acid deficiency affects genetic stability, experiments were conducted to investigate if and how inborn errors in folate metabolism impact on genetic stability. It is thought that errors in folate metabolism may, in some cases, result in the

depletion of folate species that are required for thymidine synthesis and DNA methylation and thereby increase the rate of uracil and hypomethylation-associated chromosome damage.

Chapter 4 describes an experiment that tested whether methionine-dependence, a putative marker of methionine synthase deficiency, affects MN frequency in humans. It was hypothesised that methionine-dependence is caused by methionine synthase deficiency and would therefore result in methyl folate trapping, a depletion of 5,10-MnTHF and a subsequent increase in MNi due to uracil-induced DSB (see section 4.1). The results of these experiments showed that the relative methionine-dependency of cells was not related to MN expression *in vitro*. It is possible that the methionine-dependence phenotype is only expressed *in vitro* and may not be relevant to the *in vivo* situation, hence the absence of an association of *in vitro* methionine-dependence with baseline MN frequency. Furthermore, it now appears likely that for the effects of methionine-dependency to be expressed *in vitro* the folate source and culture duration may be important determinants of whether an effect on MN expression is observed. In a similar fashion to the way in which using folic acid as the folate source may preclude the observation of any effect of vitamin B_{12} deficiency, folic acid may not allow the effects of methionine-dependence to be fully expressed. This is because, unlike 5-MeTHF, folic acid does not require any methionine synthase-mediated interconversion before it can participate in thymidine synthesis. It is also likely that the new long-term CBMN protocol, in which cells can undergo multiple rounds of cell division, may facilitate the expression of any differences in MN expression between tertiles of methionine-dependence that would have otherwise been undetectable in the standard three-day protocol used in this experiment.

Interestingly, methionine-dependence was related to increased rates of cell division in medium supplemented with methionine. As discussed, it is possible that increased polyamine production may have been responsible for the altered division. It remains to be determined whether methionine-dependency is a physiologically relevant phenotype *in vivo* in terms of chromosome damage and cell proliferation rates.

In addition to methionine-dependence, the effect of the MTHFR C677T polymorphism on uracil incorporation into DNA was studied. There is good evidence to suggest that this

polymorphism alters folate metabolism because of reports of lowered MTHFR activity *in vitro* and increased plasma homocysteine levels *in vivo* (Frosst *et al.*, 1995). It was hypothesised that the less active MTHFR enzyme in homozygous mutants (TTs) would cause a diversion of folate towards thymidine synthesis, thus lowering the rates of uracil incorporation into DNA and chromosome damage. Despite the known reduction in enzyme activity associated with the C677T polymorphism, the results presented herein suggest that TTs incorporate a similar amount of uracil into DNA and have similar amounts of chromosome damage, as measured by the CBMN assay, compared to CCs over a broad physiological concentration range of folic acid *in vitro*. This *in vitro* result does not support a role for lowered uracil-associated chromosome breakage as a mechanism for the reduced risk of colorectal cancer and acute lymphocytic anaemia enjoyed by TTs. However, these results should be considered preliminary because it is unclear whether the actual MTHFR activity of cultured cells did vary significantly between the two groups. It is possible that the MTHFR activity of TTs was improved by a high concentration of riboflavin in the medium and also that the MTHFR activity of CCs was reduced by high concentrations of methionine in the medium. There is evidence to suggest that high serum riboflavin levels lower blood homocysteine concentrations in TTs by improving the activity of MTHFR (Hustad *et al.*, 2000). It is thought that because the C677T mutation decreases the affinity of MTHFR for its essential cofactor, FAD, a high concentration of riboflavin, which is the precursor to FAD, can alleviate the effect of the polymorphism on enzyme activity (Hustad *et al.*, 2000). It is possible that a similar situation occurred in this system because the riboflavin concentration of the medium was approximately 500 nM, a concentration that is 40 times higher than the 12-13 nM normally found in plasma. In addition, because methionine is the precursor to S-adenosyl methionine (SAM), high methionine concentrations in the culture medium (100 μM compared to 20-30 μM in plasma) may have caused an increase in SAM concentrations. If this did occur, it is possible that the MTHFR activity of CCs was reduced to a level similar to that of TTs because SAM is a known inhibitor of MTHFR (Kutzbach and Stoksatd, 1971). This scenario may be the less likely of these two mechanisms because SAM would also inhibit MTHFR in TTs unless the polymorphism affects feedback inhibition by SAM.

These hypothesised mechanisms by which MTHFR activity is modulated to conceal the relative effect of the C677T polymorphism present an intriguing topic for future research. It seems likely that the net MTHFR activity of a cell is dependent not only on the presence of the C677T polymorphism but also methionine, riboflavin and folate concentrations in the culture medium. In any case there is a clear need to determine whether these molecules are determinants of MTHFR activity *in vitro* and whether they can confound the results of studies such as these.

To supplement or not to supplement

The results presented herein conclusively show that folic acid concentrations within the normal plasma range for folate (mainly 5-MeTHF) in healthy non-anaemic individuals (12 and 24 nM) are insufficient to minimise the frequency of various markers of genetic stability in this *in vitro* system. It could be extrapolated from these results that there may be some benefit in attempting to raise plasma folic acid concentrations in the general population from the current average (10 – 30 nM) to somewhere around 60 nM. The rationale for this would be to slow the normal age-related increase in genetic damage by fuelling efficient thymidine synthesis and methylation reactions thereby lowering the risk of diseases caused by the accumulation of mutations such as cancers and other diseases of old age. These results should be confirmed in experiments where the folate source is 5-MeTHF, the predominant folate species in plasma.

The results presented here show that homocysteine marginally induces MN expression at high concentrations. Even supra-physiological homocysteine concentrations (400 μM), which are twenty-fold higher than high physiological concentrations (20 μM), failed to induce an unequivocal increase in the MN frequency of cells. These data suggest that the benefits associated with minimising plasma homocysteine concentrations through supplementation may be more to do with the risk of cardiovascular disease than with genetic stability however these studies need to be repeated with longer-term exposure to various homocysteine derivatives.

Future research

Homocysteine genotoxicity

The current experiments suggest that homocysteine is only a weak inducer of MN at high concentrations. Future research should aim to reproduce the *in vivo* spectrum of homocysteine derivatives, which are mostly oxidised (99%)(Jacobsen 1998), when studying the potential genotoxic effects of homocysteine. In other words, it is possible that there are alternate derivatives of homocysteine present *in vivo,* which may be able to induce MN formation but are not produced *in vitro*.

There is a clear need for further investigation into the genotoxic potential of homocysteine in tissues where there is evidence of a role for homocysteine in the pathology of disease. The experiments described herein utilised primary human lymphocytes as a model for testing the genotoxic potential of homocysteine due to the ease of obtaining, culturing and measuring chromosome damage in these cells. Future experiments should focus on the cells of the cardiovascular epithelium, due to the well-established link between hyperhomocysteinemia and the development of atherosclerosis. Furthermore there is evidence that the cells of the cardiovascular epithelium may be more sensitive to hyperhomocysteinemia than other cell types due to a reduced expression of cystathione β-synthase (Chen *et al.*, 1999). Cystathione β-synthase is an enzyme that converts homocysteine to cystathione and therefore a deficiency in this enzyme compromises the ability of a cell to process homocysteine.

Furthermore, experience gained from this thesis may guide the design of future experiments. The homocysteine experiments were designed based on the assumption that the best control for a homocysteine challenge was a similar concentration of methionine. Although this may have some merits, in practice these experiments (Chapter 3 and 4) have shown that *in vitro* methionine-dependence is a common phenotype that reduces the ability of lymphocytes to proliferate when methionine is substituted by homocysteine and consequently hinders the scoring of MNi because the frequency of dividing cells is low. It now seems clear that dose-response experiments for homocysteine should be conducted using culture medium that contains a physiological concentration of methionine. These experiments could involve using the new comprehensive long-term CBMN assay (described in Chapters 5 and 6) because an increased duration of exposure of cells to high

homocysteine concentrations may increase the chance of detecting an effect on chromosome damage rates. The combination of necrosis as an endpoint in the CBMN assay as well as measures of cell viability (made on days 3, 6 and 9) would certainly aid in clarifying whether the effects, if any, of high homocysteine concentrations are predominantly on cell membrane integrity or on genomic stability.

Methionine-dependence

As discussed above, there was no significant association between *in vitro* methionine-dependence of cells and baseline MN frequencies. Because the methionine-dependence phenotype may be caused by a reduced methionine synthase activity it now appears likely that no effect on MN induction was detected because folic acid was the folate source. Folic acid does not require a methionine synthase-mediated conversion before it can participate in thymidine synthesis and therefore may prevent 5,10-MnTHF depletion due to a reduced methionine synthase activity- the postulated mechanism for increased MN frequencies with methionine-dependence. Future experiments should address this issue and use both 5-MeTHF and folic acid as the folate source in parallel cultures. The extended culture duration of the new comprehensive nine-day CBMN protocol may also allow the expression of any chromosomal changes associated with methyl folate trapping in methionine-dependent cells.

Cell culture medium

Perhaps one of the primary requirements of further *in vitro* studies in this area is that cell culture medium be tailored to match blood vitamin concentrations as closely as possible. In this thesis, folic acid, vitamin B_6 and vitamin B_{12} were matched to plasma values reported in the literature.

In preliminary experiments, which tested the effect of vitamin B_{12} deficiency on MN induction, no increase in MN frequency was observed even with the complete omission of vitamin B_{12} from the medium. The likely reason for this null result is that the folate source used here, folic acid, does not require any vitamin B_{12}-dependent interconversions before it can participate in thymidine synthesis. Furthermore, little effect of vitamin B_{12} would be expected on DNA methylation because there is a relatively high concentration of methionine in the culture medium. Under low methionine conditions there may be some effect of low vitamin B_{12} deficiency on MN induction. Theoretically the reduced

recycling of homocysteine to methionine by vitamin B_{12}-dependent methionine synthase would result in lowered methionine and S-adenosyl methionine concentrations. The subsequent hypomethylation of centromeric DNA may then induce MN expression through the loss of whole chromosomes during anaphase. Future experiments should investigate whether the use of 5-MeTHF, a folate that requires a vitamin B_{12}-dependent conversion before participating in thymidine synthesis, and low concentrations of methionine allow the effects of vitamin B_{12} deficiency on chromosome damage to be expressed.

In addition to these experiments, it is likely that riboflavin concentrations in culture medium may be an important issue for further investigation. In this system it is intriguing that no effect of homozygosity for the MTHFR C677T (TT) polymorphism was seen on DNA uracil-content or chromosome damage. This is despite strong evidence that the enzyme activity is significantly reduced *in vitro* and *in vivo* (Frosst *et al.*, 1995). As discussed above, the most likely reason for this null result is that high riboflavin concentrations in the culture medium caused an elevation in the intracellular concentration of FAD and thereby increased MTHFR activity in TTs (Guenther *et al.*, 1999). Future research should therefore address whether low riboflavin concentrations are required to allow the expression of reduced MTHFR activity in *in vitro* systems. These studies should firstly quantify the actual enzyme activity of cellular extracts under the actual cell culture conditions to establish what folate and riboflavin concentrations are required to allow the expression of the reduced enzyme activity. Once a reduced enzyme activity is established, the experiments described in this thesis, whereby cells from people with and without the C677T polymorphism are compared for rates of chromosome damage and uracil incorporation into DNA, could be repeated.

Other polymorphisms

There is an emerging need to study the effects of various common polymorphisms on chromosome damage rate in an effort to identify potential gene diet interactions that could be exploited to minimise the rate of chromosome damage in humans. Furthermore, early identification of individuals who require greater than normal concentrations of folate and related vitamins/cofactors to maintain genomic stability may reduce the incidence of various DNA damage-associated diseases in these people. This thesis describes an experiment that was designed to specifically test the effect of the C677T polymorphism in

MTHFR on folic acid deficiency-induced chromosome damage. Future experiments may use the new comprehensive CBMN protocol to investigate the influence of polymorphisms in other key folate metabolising genes such as methionine synthase, thymidylate synthase and cobalamin reductase not to mention other polymorphisms in MTHFR. An especially interesting experiment would test the effect of combinations of polymorphisms. This is light of a report of a person who had severe methionine synthase deficiency with out the normally associated severe megaloblastic anaemia. Methionine synthase deficiency causes megaloblastic changes in haematopoietic cells by inhibiting thymidine synthesis. Because this person was also homozygous for the MTHFR C677T polymorphism, the reduced MTHFR activity was suggested to prevent the trapping of folate as 5-MeTHF because 5,10-MnTHF was being diverted to thymidine synthesis (Kvittingen *et al.*, 1997). It would significantly enhance our understanding of folate metabolism to reproduce this situation in an *in vitro* system and measure changes at the molecular level of DNA.

DNA methylation

Future research should also investigate how the MTHFR C677T polymorphism affects DNA methylation under folate deficient and replete conditions. Preliminary evidence suggests that TTs have relatively hypomethylated DNA compared to CCs (Stern *et al.*, 2000). As is described, this mutation is very much a 'double-edged sword' and may be beneficial during adequate folate intake due to maximised thymidine synthesis while being detrimental during low folate intakes due to compromised DNA methylation. It would be interesting to investigate at what folate concentrations (folic acid and 5-MeTHF) the mutation is protective against uracil-associated chromosome damage and at what concentrations the mutation is promotive of hypomethylation-associated damage. In addition, it would also be very interesting to investigated whether a high concentration of methionine can abolish the detrimental effects of the polymorphism during folate deficiency by fuelling DNA methylation. The information gained from these experiments may aid in designing RDAs for TTs, which may be different to RDAs in CCs, to optimise *in vivo* genetic stability.

5-methyl cytosine and uracil assay

In order to facilitate studies in this area, there is a clear need for an assay that can simultaneously detect the presence of 5-methyl cytosine, cytosine, uracil and thymidine.

Such an assay, besides giving a measure of both important endpoints relevant to the MTHFR C677T polymorphism, will be more sensitive than current assays which rely on knowing how much DNA is present in the sample. The process of DNA quantitation is time-consuming and adds another variable that may reduce the sensitivity of the assay. In the uracil assay used for this system, there is some concern that the limiting factor for the sensitivity of the assay is the quantitation of DNA. Removing this element completely by designing an assay that simply gives ratios of each base may significantly aid future research in this area. Furthermore, measuring all of these endpoints in one sample will substantially reduce the amount of DNA and number of cells needed.

Nuclear buds

This thesis describes the validation of nuclear buds as a biomarker for folic acid deficiency in this system. It is hypothesised that these buds contain amplified DNA that is being extruded from the nucleus. Future research may address whether these nuclear buds actually contain DNA and more specifically whether they contain certain sequences more often than others. It is proposed that randomly amplified sequences are being extruded from the nucleus, however given sufficient selective pressure and time, certain genes may begin to appear in buds more frequently than others due to the selection of new favourable genetic variants by the low folic acid conditions. For example, in folate deficiency one may expect cells that have randomly amplified genes associated with folate uptake and usage to be selected for. Although the increased copy number of genes may increase the ability of cells to survive under certain conditions, amplified sequences may still be removed from the nucleus by recombination between homologous sequences. The resulting double-minute chromosomes are removed by budding. The content of nuclear buds could be assessed by fluorescent in situ hybridisation. Firstly, probes could be targeted at telomeric DNA sequences, with telomeric-positive buds being unlikely to contain amplified DNA but likely to contain chromosome fragments formed by DSB. This is because the model for the looping out of amplified DNA predicts that the ends (and telomeres) of a chromosome remain with the parent chromosome during DM formation by homologous recombination (see Figure 6.8). Furthermore, assuming that some selection for cells that have an increased tolerance to folic acid depletion will occur, probes could be targeted at sequences within genes that code for specific folate-metabolising enzymes. The most obvious place to start in this instance would be to probe for the genes encoding the reduced folate carrier and dihydrofolate reductase, given that

there is evidence that these genes are amplified in response to folic acid depletion (Wong *et al.*, 1998) and methotrexate treatment (Miele *et al.*, 1989) respectively.

It is thought that nuclear buds eventually break away from the nucleus to form MNi, however it is unclear what percentage of MNi are formed by this mechanism relative to MNi formed by chromosome breakage and loss. Because budding is known to occur in S-phase, observing cells when they are in this stage of the cell cycle may shed light on the relative prevalence of each mechanism of MN formation.

Conclusions from this thesis

1. Homocysteine marginally increases micronucleus formation and is not a strong inducer of necrosis in comparison to similar (supra-physiological) concentrations of methionine in human lymphocytes *in vitro*.

   This result does not support a strong causative role for homocysteine in MN induction *in vivo,* however longer-term studies using alternate and oxidised homocysteine derivatives need to be performed to clarify the potential genotoxicity of homocysteine.

2. *In vitro* methionine-dependency, a putative marker of methionine synthase deficiency, is a common phenotype in healthy individuals and is not related to baseline MN frequency. Methionine-dependency cannot be explained by common polymorphisms in MTHFR or methionine synthase but is associated with elevated rates of cell division in serum-free medium when methionine is in abundance.

   These results suggest that methionine-dependency is a common phenotype that is present in cells from normal healthy individuals and may be a pre-existing phenotype in those who develop methionine-dependent tumours. The physiological relevance of methionine-dependency, in terms of chromosome damage rate and risk for mutagenesis, remains to be clarified.

3. Proliferation of primary normal human lymphocytes can be supported for nine days in RPMI culture medium containing 10 U/ml Il-2 and 5% v/v FCS.

   In this project these extended culture conditions for primary lymphocytes have proven useful for studying folic acid deficiency and may have utility in further studies into the potential genotoxic effects of homocysteine.

4. *In vitro* folic acid deficiency (within a physiological concentration range) causes an increase in DNA uracil-content, chromosome breakage and rearrangement and gene amplification. The latter three events can be unequivocally detected using the new comprehensive CBMN assay, which includes the scoring of MNi, NPBs and nuclear buds in binucleated cells.

The development of the nine-day CBMN assay has allowed the effects of *in vitro* folic acid deficiency over a physiological concentration range to be expressed and detected in a convenient lymphocyte culture system.

5. Nucleoplasmic bridges and nuclear buds have been validated as biomarkers of chromosome damage that are sensitive to folic acid concentration.

   The validation of these endpoints has facilitated a more comprehensive evaluation of the genotoxic effects of folic acid deficiency on human lymphocytes using the CBMN assay.

6. A folic acid concentration of between 60 and 120 nM is required to minimise DNA uracil-content, MNi, NPBs and nuclear buds in human lymphocytes *in vitro*.

   This result raises the question of whether there may be some benefit to the community, in terms of lowering the rate of chromosome damage in dividing cells, of increasing plasma folate concentrations above the range that is currently considered normal for healthy non-anaemic people (10-30 nM).

7. Excessive incorporation of uracil is supported as a mechanism for double-stranded DNA breakage due to the correlation between NPBs, an aberration that can only result from DSB, and DNA uracil-content.

8. MTHFR C677T polymorphism has no effect on DNA uracil-content or chromosome damage in human lymphocytes cultured in medium containing physiological concentrations folic acid when riboflavin and methionine are in abundance.

   This result indicates that the MTHFR activity of lymphocytes taken from people homozygous and nullizygous for the C677T polymorphism is not significantly different in this *in vitro* system. It is suggested that high riboflavin and methionine concentrations in the medium may have caused an equalisation of the MTHFR activities between these two groups of people.

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Appendix: Paper Reprints

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