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Title

INVESTIGATIONS INTO FOLATE METABOLISM AND THE EFFECTS OF METHOTREXATE ON FOLATE METABOLISM IN RELATION TO THE MTHFR C677T MUTATION IN CULTURED MAMMALIAN CELLS

A thesis presented for the degree of Doctor of Philosophy (PhD.) at the University of Dublin, Trinity College.

By

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June, 2004

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To my loving parents Marion and Gerry,
and my dear Andrew.

Declaration

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ABBREVIATIONS

10-CHO-THF = 10-formyltetrahydrofolate

5,10-CH^{+}-THF = 5,10-methenyltetrahydrofolate

5,10-CH_2-THF = 5,10-metheylenetetrahydrofolate

5-CH_3-THF = 5-methyltetrahydrofolate

5-CHO-THF = 5-formyltetrahydrofolate

6-CH_2OH-H_2pterin = 6-hydroxymethy-7,8-hydropterin

ADC = 4-amino-4-deoxychorismate

AdoCbl = adenosyl cobalamin

AICAR = aminoimidazolecarboxamide

AICAR = aminoimidazolecarboxamide ribonucleotide

ALL = Acute lymphoblastic lymphoma

ANOVA = analysis of variance

ATP = adenosine triphosphate

CBS = cystathionine β-synthase

CH_2 = methylene

CH_3 = methyl

CHO = formyl

CV = coefficient of variation

dCMP =deoxycytidylic acid

DHF = Dihydrofolate

DHFR = Dihydrofolate reductase

DMRAD = disease modifying anti-rheumatic drug

DNA = deoxyribonucleic acid

DPM = disintegrations per minute

dTMP = deoxythymidine monophosphate

dUMP = deoxyuridine monophosphate

ECGS = Endothelial Cell Growth Supplement

FAICAR = 5-formyl aminoimidazolecarboxamide

FBP = folate binding protein

FCS = Foetal Calf Serum

FGCP = folypoly-γ-glutamate carboxypeptidase

FIGLU = formimino-l-glutamate

FMN = flavin mononucleotide

FP = fluorescence polarisation

FPGS = folypoly-glutamate synthetase

FR = folate receptors

GAR = glycinamide ribotide

GGH = γ-glutamyl hydrolase

H_2pteroate = dihydropteroate

HCF = heavy chain ferritin

HCY = homocysteine

HPLC = High Performance Liquid Chromatography

HUVECs = Human Umbilical Vein Endothelial Cells

IC-S = Individual Cell Scanner

LC = liquid chromatography

LCF = light-chain ferritin

MeCbl = methylcobalamin

MS = mass spectrometry

MS = Methionine Synthase

MTHFD = Human Trifunctional Enzyme

MTHFR = methylene tetrahydrofolate reductase

MTHFS = Methenyltetrahydrofolate synthetase

MTRR = Methionine Synthase Reductase

MTX = Methotrexate

MTXPG = Polyglutamated MTX

NADP = nicotinamide adenine diphosphate

NTD = Neural Tube Defect

OH-Cbl = hyroxycobalamin

pABA = *p*-aminobenzoic acid

pABG = *p*-aminobenzoylglutamate

PBS = Phosphate Buffered Saline

PLP = pyridoxal 5′-phosphate

PSM = prostate specific membrane antigen

PtO_2 = platinum oxide

RA = Rheumatoid arthritis

RFC = reduced folate carrier

RNA = ribonucleic acid

SAH = S-Adenosylhomocysteine

SAM = S-adenosylmethonine

SD = standard deviation

SEM = standard error of the mean

SHMT = serine hydroxmethyl transferase

SMX = sulfamethoxazole

SSRP = simple-sequence repeat polymorphisms

TC = transcobalamin

TDT = transmission disequilibrium test

THF = Tetrahydrofolate

TMP = Trimethoprim

TNF= tumour necrosis factor

TRIS = tris[hydroxymethyl]aminomethane hydrochloride

TS = Thymidylate Synthase

HEPES = 2-Hydroxyethyl)piperazine-1-ethanesulfonic acid,N-2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonicacid)

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Chapter 1: Introduction

1.1 Structure, nomenclature, stability of folates

An English physician in 1920 identified an anaemia seen frequently in late pregnancy which responded well to treatment with a yeast extract called marmite (Wills, 1931). She called this anaemia "pernicious anaemia of late pregnancy" because of its similarities with pernicious anaemia, the difference being that this anaemia disappeared once the pregnancy ended. Mitchell later identified the active ingredient in the yeast as being folic acid/folate (Mitchell *et al.,* 1988). In 1941 Mitchell *et al.,* were the first to isolate and purify folic acid from spinach leaves (Mitchell *et al.,* 1988).

The name folic acid is derived from the Latin word folium or "leaf", and is the recommended trivial name for the compound (IUPAC, IUB, 1967). The term "folates" is applied to the related group of species *i.e.* naturally occurring folypolyglutamates found in food and the synthetic form of folic acid. The common features of all folates is the *p*-aminobenzoic (pABA) part of the molecule, attached to a pteridine ring on the NH_2 end and one or more glutamic acids in an amide linkage at the carboxyl end, see figure 1.1. The numbering system for the folate molecule is based on the numbering of the pteridine ring system. The active centre of folate is between the N5 and N10 site, see figure 1.1. Folate derivatives are formed by differing one-carbon subgroups that are added to the active site. Naturally occurring folates in food are largely 5,6,7,8-tetrahydrofolates (THF), in which the pteridine ring is reduced. They generally have a polyglutamate side chain of typically less than 7 glutamate residues in the γ-peptide linkage (Shane *et al.*, 1993).

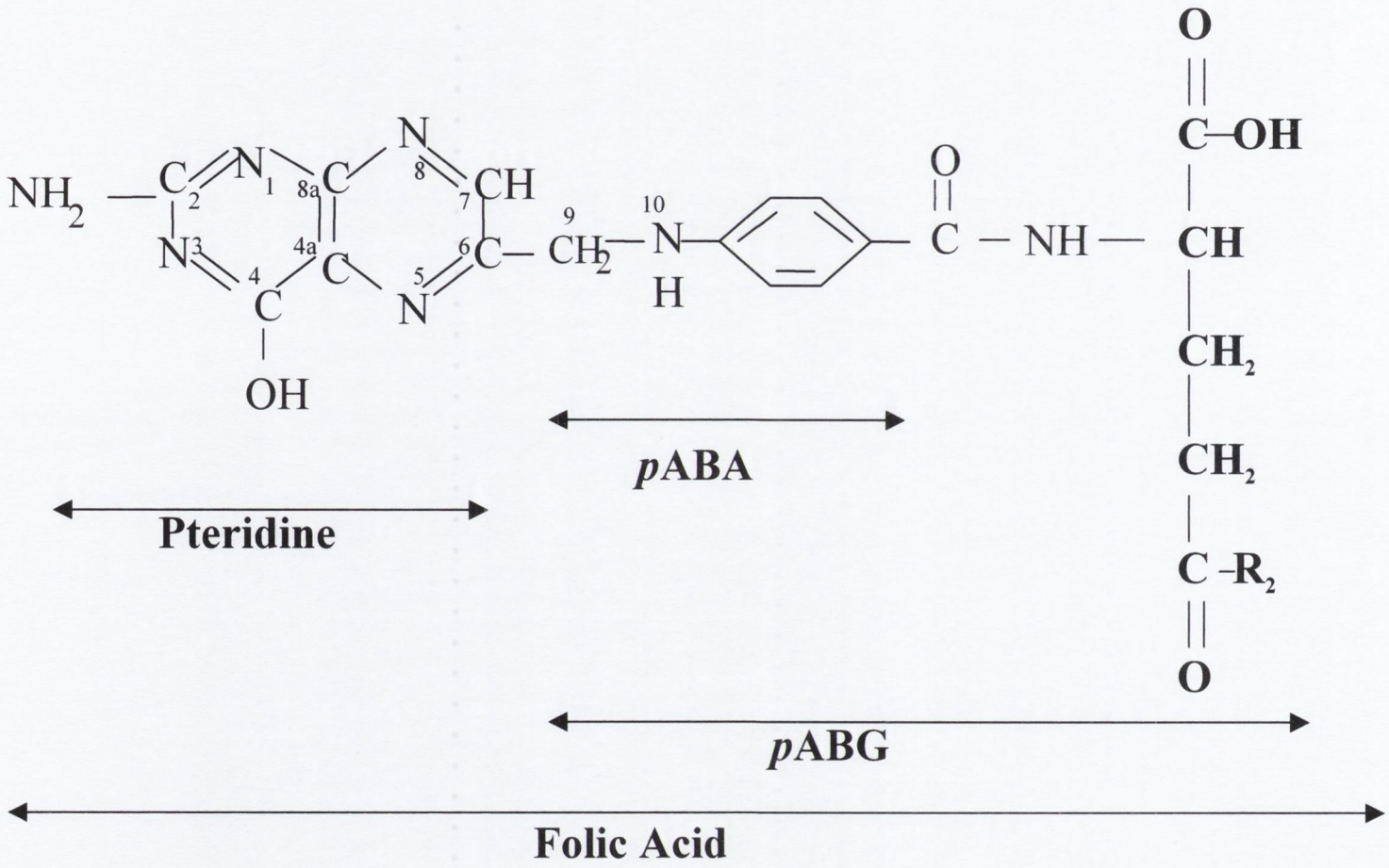


Figure 1.1: Structure of folic acid

Reduced forms of folate are very unstable and easily cleaved at the C9 and N10 bond to give p-aminobenzoylglutamate (*p*ABG) and pteridine and thus losing their biological activity. Substituting N5 and N10 with one-carbon units reduces the tendency to cleave but these forms are also susceptible to oxidative chemical rearrangements, which also results in the loss of activity. Substitution of the THF pteridine with a methyl (CH_3) group produces 5-CH_3-THF which has a significant stabilising effect (Blakley, 1969).

Both dihydrofolate (DHF) and THF are readily oxidised by air. Decomposition of DHF at pH 4 to 7 is retarded by thiols, transition metal cations or ascorbate and accelerated by EDTA (Blakley *et al.,* 1967). The instability of DHF makes storage of the compound over long periods impossible without considerable degradation. The solid may be stored in a vacuum desiccator over platinum oxide (PtO_2) and solid potassium hydroxide (KOH) at 0°C in the dark or as a suspension in 5mM HCl at 1°C for a period of one week without appreciable alteration in properties (Blakley *et al.,* 1969). Storage as a frozen suspension in 1mM HCl containing 10mM mercaptoethanol (Schrecker and Huennekens, 1964), or as a suspension in 10% potassium ascorbate, pH 6.0 at 4°C (Bertino *et al.,* 1965) have also been recommended. Suspensions of DHF in dilute HCl containing 2-mercaptoethanol are stable for several weeks if frozen (Scrimegeour, 1980).

THF is rapidly oxidised by air in neutral solution with the formation of *p*ABG and a number of pterins (Blakley *et al.,* 1967). Oxidative degradation of THF in neutral solution results in a rapid change in the absorption spectrum and is greatly retarded by

2,3-dimercaptopropanol and to a lesser extent by mercaptoethanol (Blakley, 1960). This effect of thiols does not seem due to binding of heavy metals since other sequestering agents are relatively ineffective, and it has been postulated that a stable thiol-THF complex of unknown structure is formed (Zakrzewski, 1966). Ascorbate is even more effective than thiols in protecting THF from oxidative degradation (O'Broin *et al.,* 1975), and two-thirds of the THF in such protected solutions remains after 4 days storage at pH 7.4 (the pH for maximum stability) and 0-5°C (Blakley, 1969). The protective action of ascorbate may be due in part to the removal of dissolved oxygen, but both thiols and ascorbate may protect by reducing quinonoid-dihydrofolate which is the probable primary product of oxidation (Blakley *et al.,* 1967).

Although unstable, solutions of 10-CHO-THF are more stable than solutions of THF. At pH 7.0, 10-CHO-THF is slowly converted to 5-CHO-THF with only 10% degradation being observed in 9hr (O'Broin *et al.,* 1975). Oxidation of 10-CHO-THF produces 10-CHO-DHF which can be easily converted back to 10-CHO-THF by a change in the redox state such as would be caused by ascorbic acid. However further loss of a hydrogen produces 10-CHO-FA which is thought to be biologically inactive (Blakley,1969; Baggott *et al.,* 1995).

THF can react non-enzymically with formaldehyde in dilute solution, the unique stability of the adduct suggests that formaldehyde forms a methylene bridge between the 5 and 10 position to give 5,10-CH_2-THF (Blakley and Benkovic, 1984). 5,10-CH_2-THF is considerably more stable to oxidation than THF, particularly in the presence of sufficient excess formaldehyde to prevent significant dissociation of the

adduct to THF (Osborn *et al.,* 1960). Stability is also increased at high pH and decreased by amines or ammonium salts (Ramasastri and Blakley, 1964). 5,10-CH_2-THF easily loses its methylene group to give formaldehyde and THF, with THF being easily oxidatively cleaved. The result of the cleavage is a pterin which is subsequently converted to several different products and *p*ABG.

5,10-CH^+-THF rearranges to 10-CHO-THF and slowly to 5-CHO-THF (Baggot *et al.,* 1995). Substitution at the N5 position also gives greater stability. Therefore, 5-CHO-THF is the most stable of the folates apart from folic acid.

Folic acid does not occur in nature but arises in the biological material owing to the chemical oxidation of reduced natural forms. The pteridine ring is not reduced and therefore it very resistant to chemical oxidation (Blakley, 1969). The vitamin is converted to the biologically active THF in the cell by dihydrofolate reductase (DHFR). Folic acid is stable to alkali under anaerobic conditions, but alkaline hydrolysis under aerobic conditions cleaves the side chain to yield *p*ABG and pterin-6-carboxylic acid. Nitrous acid reacts in the cold with folic acid to give a 10-nitrous derivative and under drastic conditions also diazotises at the 2-amino position to yield the 2-hydroxy-10-nitroso analogue of folic acid (Blakely,1969). The nitroso group can be removed from the latter to yield a 2-hyroxy analogue. 10-Formyl folic acid (10-CHO-FA) can be readily formed from folic acid by the action of 98% formic acid (Gordon *et al.,* 1948). The formyl group can be readily removed by alkali at room temperature (Blakely, 1959).

1.2 Folate co-factor preparation

Initially folate co-factors were difficult to buy commercially with only folic acid and 5-CHO-THF being available to purchase. Today the majority of folate co-factors are available to buy commercially from several sources (*e.g.* Schircks Laboratory, Switzerland and Epovra, Switzerland). As a result of the lack of commercially available folate co-factors, methods were developed to prepare these folate co-factors from the commercially available FA and 5-CHO-THF. These methods are described below:

5,10-CH^+-THF can be prepared by making a solution of 5-CHO-THF 0.1M with respect to HCL and standing overnight at 4°C overnight. The antioxidant 2-mercaptoethanol is added to the solution to give a final concentration of 0.5% (v/v) containing little or no impurities (Scott 1980).

10-CHO-THF is obtained by adjusting the 5,10-CH^+-THF prepared above to pH 7.0 with NaOH and allowing the resulting solution to stand at room temperature for 12hr (Scott, 1980). 10-CHO-DHF can be prepared by diluting 5,10-CH^+-THF into tris[hydroxymethyl]aminomethane hydrochloride (TRIS HCl) so as to achieve a pH of approximately 7.0 in the absence of 2-mercaptoethanol and standing for 60min at pH 7.0 (Scott, 1980). These methods have since been modified by Baggott *et al.,* (1995) and the standards obtained quantified (Baggott *et al.,* 1995; Baggott, 2000).

10-CHO-FA is prepared by heating folic acid in excess 98% formic acid at 60°C for 2hr. The reaction solution is placed at 4°C overnight, and formic acid is removed by ether. The product is recrystallized from hot water to a constant UV spectrum (Baggott, 2000).

THF is synthesized by the reduction of folic acid (Scott, 1980). Folic acid is added to a deareated solution of glacial acetic acid and platinum oxide (PtO_2) and is flushed with hydrogen. The mixture is stirred continuously for 4hr at room temperature in the dark, flushed with oxygen-free nitrogen and filtered through a sintered-glass funnel. The filtered solution, while still under nitrogen, is removed, capped and lyophilised. The dry THF is resuspended in a solution of deaerated 0.2M 2-mercaptoethanol in distilled water, the pH adjusted to 7.0 with KOH, placed under a vacuum and stored at 4°C without significant degradation over a period of months (Scott, 1980).

DHF is prepared by the reduction of folic acid by the following technique. Folic acid is dissolved in deaerated 0.1M KOH followed by the addition of 10% potassium ascorbate and pH adjusted to 6.0. Sodium hydrosulfite is added and the reaction proceeds under nitrogen for 5min. Following cooling, the pH is adjusted to 2.8 with HCL. DHF precipitates and is collected by centrifugation and re-dissolved in 10% potassium ascorbate at room temperature and re-precipitated by adjusting the pH to 2.8. The precipitate is washed several times with 0.005M HCL under vacuum at 4°C (Scott, 1980). The precipitate can be further purified by dissolving the precipitate in 1% potassium ascorbate and after appropriate dilution running through a QAE Sephadex column (Scott, 1980).

5,10-CH_2-THF can be prepared from THF whose preparation is described above using the following method. To 40mg of THF 3.6mg of TRIS base is added, the pH is adjusted to pH 8.0 and the volume adjusted to 2ml. To this 7.5mg formaldehyde is added. The mixture is made 1% with respect to mercaptoethanol and the volume adjusted to 2.5ml with distilled water and allowed to stand at room temperature in the dark for 15min under nitrogen. The product of this reaction is mostly 5,10-CH_2-THF but further purification can be carried out by running the mixture through a DEAE column (Scott, 1980).

5-CH_3-THF can be synthesized either by the formylation and reduction of THF prepared from above or directly from folic acid. 5,10-CH_2-THF is prepared as above and is further reduced by adding 7.6mg sodium borohydride in 1ml of Tris-HCl buffer, pH 8.0 and incubating under nitrogen at 50°C for 60min. 5.0M acetic acid is added drop wise under nitrogen to remove the borohydide, 1.0ml mercaptoethanol is added and the mixture is kept under a vacuum. The mixture can then be run through a QAE sephadex column to purify the standard (Scott, 1980). An alternative method in which FA is reduced by borohydride to produce THF followed by condensation with formaldehyde and further reduction also produces 5-CH_3-THF (Scott, 1980).

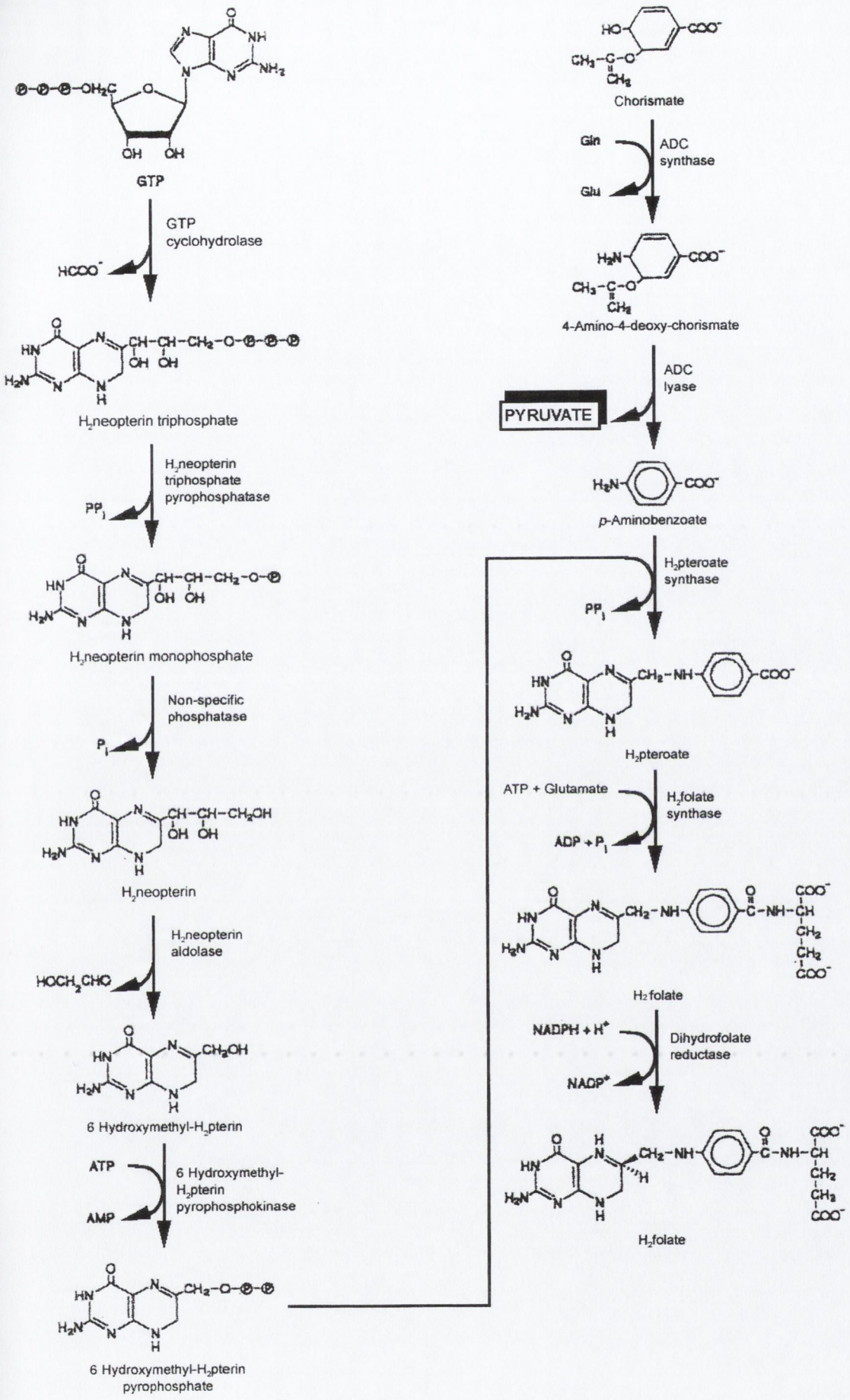
Folic acid and many of its derivatives can easily be reduced by a variety of agents to the corresponding dihydro or tetrahydro derivatives. Catalytic hydrogenation of folic acid over platinum or palladium yields either DHF or THF as the main product, depending on the conditions (Blakley, 1957). In glacial acetic acid or in neutral aqueous solution two molecules of hydrogen are rapidly consumed with the formation of THF, but there is also a slow further uptake of hydrogen with the formation of

unknown products (Blakley, 1967). Catalytic reduction of folic acid in 0.1N NaOH yields mainly DHF (O'Dell *et al.,* 1947) but the uptake of hydrogen beyond one mole proceeds at a considerable rate so that it is not possible to prepare pure DHF by this method (Blakely *et al.,* 1967).

1.3 Biogenesis of folate in microorganisms

In humans, folate is a dietary requirement but many micro-organisms and plants posses the ability to synthesise folates *de novo.* The end product of *de novo* biosynthesis of folate is dihydrofolate (DHF). DHF is not active in the one-carbon cycle and so needs to be reduced to the level of tetrahydrofolate (THF) in order to be incorporated into the one-carbon cycle. Folate biosynthesis can be divided into two parts as shown in figure 1.2. The first involves the conversion of the aromatic precursor chorismate to pABA and the second is the production of the pteridine portion of folate from GTP in a series of reactions that require six enzymes (Neidhardt *et al.,* 1987).

PABA is one of seven aromatic products derived from chorismate in *E.coli.* Chorismate is converted to pABA in 2 steps through the action of a three gene product PabA, PabB and PabC. 4-amino-4-deoxychorismate (ADC), a heterodimer of PabA and PabB convert chorismate and glutamine to ADC and glutamate. ADC lyase aromatizes ADC, releasing pyruvate and generating pABA. Next pABA and 6-hydroxymethy-7,8-hydropterin (6-CH_2OH-H_2pterin) pyrophosphate (which has been derived from GTP) are coupled to form dihydropteroate (H_2pteroate) by H_2pteroate synthase and the bifunctional protein encoded by *folC*, dihydrofolate



(Neidhardt and Curtis, 1996)

Figure 1.2: The sequence of reactions involved in the biosynthesis of dihydrofolate from GTP and chorismate.

synthetase/folypolyglutamate adds the initial glutamate molecule to form DHF. DHF is then reduced to THF by dihydrofolate reductase (DHFR).

1.4 Human Absorption of Folate

Folate is absorbed primarily from the proximal third of the small intestine, although it can be absorbed from the entire length of the small bowel (Rosenberg, 1976). Polyglutamated folates are the predominant form of folates found naturally and cannot be absorbed across the gut directly and so must be firstly be hydrolysed to monoglutamate forms. Pteroylpolyglutamate hydrolases have been identified in a variety of tissues and their function is to convert intracellular polyglutamyl folates to their monoglutamyl derivatives (Silink *et al.*, 1975). Chandler *et al.*, (1986) obtained human jejunal mucosa from patients undergoing elective jejunal illeal bypass surgery and purified a hydrolase folypoly-γ-glutamate carboxypeptidase (FGCP) from the jejunal brush border membrane and a separate intracellular lysosomal FGCP in human jejunal mucosa. The two distinct intestinal mucosal pteroylpolyglutamate hydrolases differed in pH optima, inhibition characteristics and molecular size (Wang *et al.*, 1986).

The role of the intracellular pteroylpolyglutamate hydrolase in folate metabolism is still unclear. A possible involvement of the intracellular pteroylpolyglutamate hydrolase in the absorption of dietary folate is not ruled out but this would require transport of all or part of the pteroylpolyglutamates into the cell prior to hydrolysis. The human brush border pteroylpolyglutamte hydrolase plays a major role in the digestion and intestinal absorption of dietary folates (Chandler *et al.*, 1986). Wang *et*

al., (1985) compared the activity of intestinal brush border pteroylpolyglutamate hydrolases found in the mucosa of humans, pigs, rats and monkeys. In both the human and pig the intestinal brush border pteroylpolyglutamte hydrolase was active from pH 4.5- 8.5 and the activity was increased by zinc acetate. In the mucosal brush border of rat and monkey the folate hydrolase activity was negligible. Intracellular folate hydrolase was maximally active at pH 4.5 in the human, pig and monkey, whereas it was equally active from pH 4.0-7.5 in rat (Wang *et al.,* 1985). Brush border pteroylpolyglutamte hydrolase exhibited similar properties in both humans and pigs and therefore it was suggested that the pig maybe a suitable experimental model to further study folate hydrolysis and absorption in humans (Wang *et al.,* 1985).

Following the identification of two separate folate hydrolases in intracellular and brush border membrane fractions of pig jejunal mucosa, Chandler *et al.,* (1986) demonstrated that each had properties identical to those found in human jejunum (Chandler *et al.,* 1986). A monoclonal antibody Mab-3 to the purified pig jejunual brush border FGCP detected a 120-kDA subunit protein that was localised by immunoreactivity to the jejunal brush-border site of the *in vivo* hydrolysis of folypoly-γ-gluatamates (Chandler *et al.,* 1991). Halsted *et al.,* (1998) transfected PC3 cells with a supercoiled plasmid DNA containing the cDNA of pig FGCP or human prostate specific membrane antigen (PSM). Human PSM was also used as a study by Pinto *et al.,* (1996), who demonstrated that PC3 cells transfected with the human PSM cDNA were capable of hydrolysis with an exopeptidase activity mechanism similar to that previously described for human jejunal FGCP (Pinto *et al.,* 1996). Halsted *et al.,* (1998) investigated the enzyme activity associated with FGCP transfected PC3 cells and concluded that the intestinal absorption of dietary folypoly-γ-gluatamates

involved a two step process of γ-linked glutamy residues by FGCP at the jejunal brush-border membrane, releasing monoglutamyl folate derivatives for subsequent membrane transport by genetically distinct reduced folate carieer (RFC) (Halsted *et al.*, 1998). The monoglutamyl folates are resynthesised to their polyglutamate forms once inside the cells by folypolyglutamate synthetase (FPGS), this step is discussed in detail in section 1.6.

1.5 Cellular uptake of folates

There are three major pathways by which folates can be transported across mammalian cell membranes (Fan *et al.*, 1992). These are carrier mediated transport (Sirotnak *et al.*, 1985), receptor mediated transport (Freisheim *et al.*, 1992) or by passive diffusion. The pathway of entry of folates is likely to be distinct in different cells, depending on the relative efficiency of carrier-mediated and receptor mediated mechanism and also the intracellular and extracellular concentration of folates.

1.5.1 Carrier Mediated Folate Transport

Carrier mediated folate transport is saturable, pH and energy dependent and has a greater preference for the transport of reduced folates, methotrexate (MTX) and other anti-folates rather than oxidised folates (Antony, 1992). Influx of folates by this reduced folate carrier (RFC) is sodium independent, inhibited by anions and is either insensitive to or increased by sodium azide (Sierra *et al.*, 1995). Transmembrane α-

helices of the RFC are believed to form channels through which substrates pass (Brigle *et al.,* 1995).

1.5.2 Receptor Mediated Folate Transport

Receptor mediated folate transport occurs via different isoforms of folate receptors (FR) (Anderson *et al.,* 1992; Fan *et al.,* 1992). Folate receptors are glycosylphosphatidylinositol anchored receptors that internalise folates via an endocytotic process (reviewed in Henderson, 1990). These isoforms are FRα , FRβ , FRγ , FRγ′, which have tissue specific expression. FRs are crucial to the assimilation, distribution and retention of food folates and have been identified in various cells, extracellular fluid and tissues in mammals (Selhub, 1994). Folate receptors have a preference for oxidised folates as opposed to the reduced folates and have a high affinity for folate in the nanomolar range. They are separated into soluble and membrane bound FR forms, which are structurally related but differ in function (Selhub, 1994; Antony, 1996). The exact function of soluble FRs is not yet known but they are thought to serve multiple functions; they can protect the bound reduced folates from oxidation, act as a mechanism by which folates are concentrated and also act as a storage protein to conserve folates (Antony, 1996). Membrane FRs can accumulate folate at the cell surface and mediate the transport of folate compounds in cells.

1.5.3 Folate transport in pregnancy

Folate requirements in pregnant women increase 5 to 10 fold during pregnancy for growth of the fetus, placenta and maternal tissue (Antony, 1996). FRs are very important in the transport of folates from mother to developing child. The placenta

contains both FRα and FRβ (Shen, 1994). There are two steps involved in the transport of folate from the mother to the developing fetus. The first involves the binding of 5-CH_3-THF to the placental FR on the maternally facing chorionic surface. The second involves the transfer of folates to the fetal circulation along a downhill concentration gradient (Antony, 1996). It is unclear how folate is transported prior to the development of the placenta but a similar pathway is thought to occur.

1.6 Folate Metabolism

1.6.1 Folate Polyglutamation

In order for a cell to retain folates it has to be in its polyglutamate form (Shane, 1989). It was originally thought that polyglutamation of the folates was a means of storing the vitamin in the cell. It is now apparent that the intracellular conversion of folates to their polyglutamate form is important for the normal function and regulation of one-carbon metabolism (Schirch and Strong, 1989). The conversion of folate monoglutamates to polyglutamates is carried out by an enzyme folypoly-glutamate synthetase (FPGS) (Rosenblatt, 1995).

FPGS is essential for the synthesis and cellular retention of functional folate co-factors. Its activity is increased in proliferating tissues (Garrow *et al.,* 1992). It is present in the mitochondria and cytosol of the cell (Lowe *et al.,* 1993). Low levels of mammalian FPGS enzymes and unstable proteins have made their isolation and purification in significant quantities very difficult. Pig liver FPGS is the only mammalian FPGS enzyme purified to homogeneity (Cichowicz and Shane, 1987). It was shown to have similar properties to human FPGS and its activity was maximal at

pH 9.5 (Cichowicz and Shane, 1987). Under conditions that resembled the expected substrate and enzyme concentrations in pig liver, THF was metabolised to long glutamate chain length derivatives with the hexaglutamate being the major *in vivo* folate derivative present (Cichowicz and Shane, 1987).

Cook *et al.,* (1987) carried out *in vitro* metabolism studies using this purified pig liver FPGS and suggested that the changes in the polyglutamate distributions reflect substrate availability for FPGS. A further suggestion was made that the modulation of the glutamate chain length would occur at a slow rate and therefore limit the regulatory usefulness of any such changes (Cook *et al.,* 1987). In order to investigate the physiological importance of *E.coli* and human folypolyglutamate synthesis, chinese hamster ovary cell (CHO) mutants AUXB1 lacking FPGS activity and therefore requiring exogenous purines, thymidine and glycine for growth were used as models (Osborne *et al.,* 1993; Lowe *et al.,* 1993; Lin *et al.,* 1993, Chen *et al.,* 1996).

AUXB1 cells were transfected with both *E.coli* and human FPGS gene constructs that targeted the expression of the FPGS protein to the cytosol or mitochondria (Lowe *et al.,* 1993; Lin *et al.,* 1993). The *E.coli* studies demonstrated that cytosolic FPGS activity is required for cytosolic folate accumulation and purine and thymidylate biosynthesis, while the mitochondrial FPGS is required for mitochondrial folate accumulation and glycine synthesis (Lin *et al.,* 1993). Expression of *E.coli* FPGS solely in the mitochondria of AUXB1 cells restored normal folate accumulation and metabolism in the cytosol as well as in the mitochondria. Results also demonstrated that pteroyltriglutamates synthesised in the mitochondria can be released into the

cytosol of the chinese hamster ovary cell (CHO) mutant AUXB1 which lack cytosolic FPGS activity (Lin *et al.*, 1993).

Garrow *et al.*, (1992) isolated human FPGS cDNA and expressed this construct in CHO AUXB1 cells. The transfected cells had restored cytosolic thymidylate and purine synthesis, but remained auxotrophic for glycine. These results suggested the absence of FPGS activity and a folate pool in the mitochondria (Garrow *et al.*, 1992). Freemantle *et al.*, (1995) suggested the human FPGS cDNA used previously (Garrow *et al.*, 1992; Osborne *et al.*, 1993; Lowe *et al.*, 1993; Lin *et al.*, 1993) lacked the 5′ region of the mitochondrial leader sequence. The expression of the complete cDNA containing the entire mitochondrial leader sequence restored glycine prototrophy of these cells (Freemantle *et al.*, 1995).

Chen *et al.*, (1996) over-expressed the complete FPGS cDNA in *E.coli* to allow the purification of the human enzyme to homogeneity and its properties characterised. Human FPGS was shown to utilise THF and other folates as substrates with different affinities, preferring unsubstituted reduced folates (Lin *et al.*, 1993). The effect of varying FPGS levels on folate accumulation was investigated using the CHO AUXB1 cells expressing various levels of human and *E.coli* FPGS activity (Lowe *et al.*, 1993). In cells grown in low folate media folate accumulation was limited by influx and was independent of FPGS activity except in cells expressing low levels of FPGS. All the transported folates were metabolised to retain polyglutamated derivatives whose chain lengths varied in accordance with the level of FPGS activity (Lowe *et al.*, 1993). The cellular accumulation of folate in cells grown in high folate media became proportional to FPGS activity and the chain length of the intracellular folates

decreased. The high folate concentration lead to competition between the substrates for FPGS which limited the extent of polglutamation and as a result less than 5% of transported folates were retained by the cell (Lowe *et al.,* 1993). The conclusion from the results obtained from the studies discussed above suggest that the cellular accumulation of folate, which requires its conversion to polyglutamated derivatives of chain length at least three, is dependent on FPGS activity levels and folate levels (Chen *et al.,* 1996).

Reduced levels of FPGS activity has been identified as a mechanism for cellular resistance to antifolates especially methotrexate (MTX) (Kim *et al.,* 1993). Following the extension of the MTX glutamate chain length the level FPGS activity drops. In pig liver and CHO cells a major drop in enzyme activity occurs at the di-glutamate chain length stage whereas in human cells a major drop occurs at the tri-glutamate chain length stage. This improved substrate activity of the MTX di-glutamate makes human cells especially sensitive to MTX because for MTX to accumulate in mammalian cells it is required to have a chain length of at least three glutamates (Chen *et al.,* 1996). It has been demonstrated by Kim *et al.,* (1993) that CHO cells expressing human FPGS activity accumulate MTX more effectively than wild type CHO cells expressing the same levels of FPGS activity and are more sensitive to the antifolate (Kim *et al.,* 1993). The cellular mechanism of action of MTX is discussed further in section 1.11.

1.6.2 Protein Bound Folates

There are five folate binding proteins (FBPs) identified to date. Three are found in the cytosolic fraction and two in the mitochondrial fraction of the cell (Appling, 1991). The cellular concentration of folate-binding proteins exceeds that of folate derivatives and therefore the concentration of free folate in the cell is negligible (Suh *et al.,* 2001). This implies that folate dependent biosynthetic pathways must compete for folate co-factors (Herbig *et al.,* 2002). Protein bound folates are less susceptible to degradation. FBPs have a high affinity for monoglutamyl folates (Antony, 1992) and have been identified in human placenta (Elwood, 1989), rat kidney (Selhub and Franklin, 1984), pig kidney (Kamen and Caston, 1986) and human cancer cell lines (Lacey *et al.,* 1989).The nucleotide and amino acid similarities of FBPs suggest they may play a role in folate homeostasis through regulation of transport across intestinal, liver and kidney membranes (Sadasivan and Rothenberg, 1989; Lacey *et al.,* 1989, Da Costa and Rothenberg, 1988). Van Hoozen *et al.,* (1996) suggest that FBPs are essential for the membrane transport of monoglutamyl folates into liver and kidney but not into jejunal mucosa.

1.6.3 Intracellular folate compartmentalisation and one-carbon metabolism

As previously discussed in section 1.6.1 folates function in the cell in their reduced form conjugated to a polyglutamate chain. Their function is to accept one-carbon units from various donors and pass them on to various biosynthetic reactions. One-carbon units are only carried by folates when the co-enzyme is in the fully reduced tetrahydro state which is achieved by the enzyme dihyrofolate reductase (DHFR). THF carries a one-carbon unit as a formate derivative in its most oxidised and as a methyl derivative in its most reduced form. All these one-carbon subgroups can be

converted into each other. Cellular one-carbon metabolism is compartmentalised (Gregory *et al.,* 2000). Mitochondrial and cytosolic folate pools differ in their distribution of one-carbon substituents, susceptibility to nutritional deficiency and response to inactivation of methionine synthase (Wagner, 1996). Mitochondrial folate metabolism is necessary for the conversion of serine to glycine and formate (a one carbon unit); cytoplasmic folate metabolism utilises mitochondrial derived formate for the biosynthesis of purines (supplies the #2 and #8 carbons of the purine ring), thymidine by the conversion of deoxyuridine (dUMP) to deoxythymidine monophosphate (dTMP) and the generation of methionine from homocysteine (Herbig *et al.,* 2002) see figure 1.3.

Serine is a major source of one-carbon units but they can also be derived from glycine, formate and histidine. In mammals serine is derived from the diet and can be biosynthesised from glycolysis via phosphoglycerate. Serine plays a central role in intermediary metabolism as a contributor to phospholipid, sphingolipid and cysteine biosynthesis and as a primary source of glycine. As a consequence of its role in glycine production, serine is a major donor of folate-linked one-carbon units, which are used in biosynthesis of purines and dTMP and the methylation of homocysteine to methionine (Herbig *et al.,* 2002). There is a tremendous demand for glycine that probably exceeds dietary intake by 10-50 fold (Herbig *et al.,* 2002). For every glycine molecule derived from serine there is also a one-carbon unit formed.

Serine can be metabolised in the cytosol and mitochondria as both contain SHMT, which reversibly converts serine and THF to glycine and 5,10-CH_2-THF (Gregory *et al.,* 2000; Wagner,1996). The central role of SHMT in producing one-carbon-

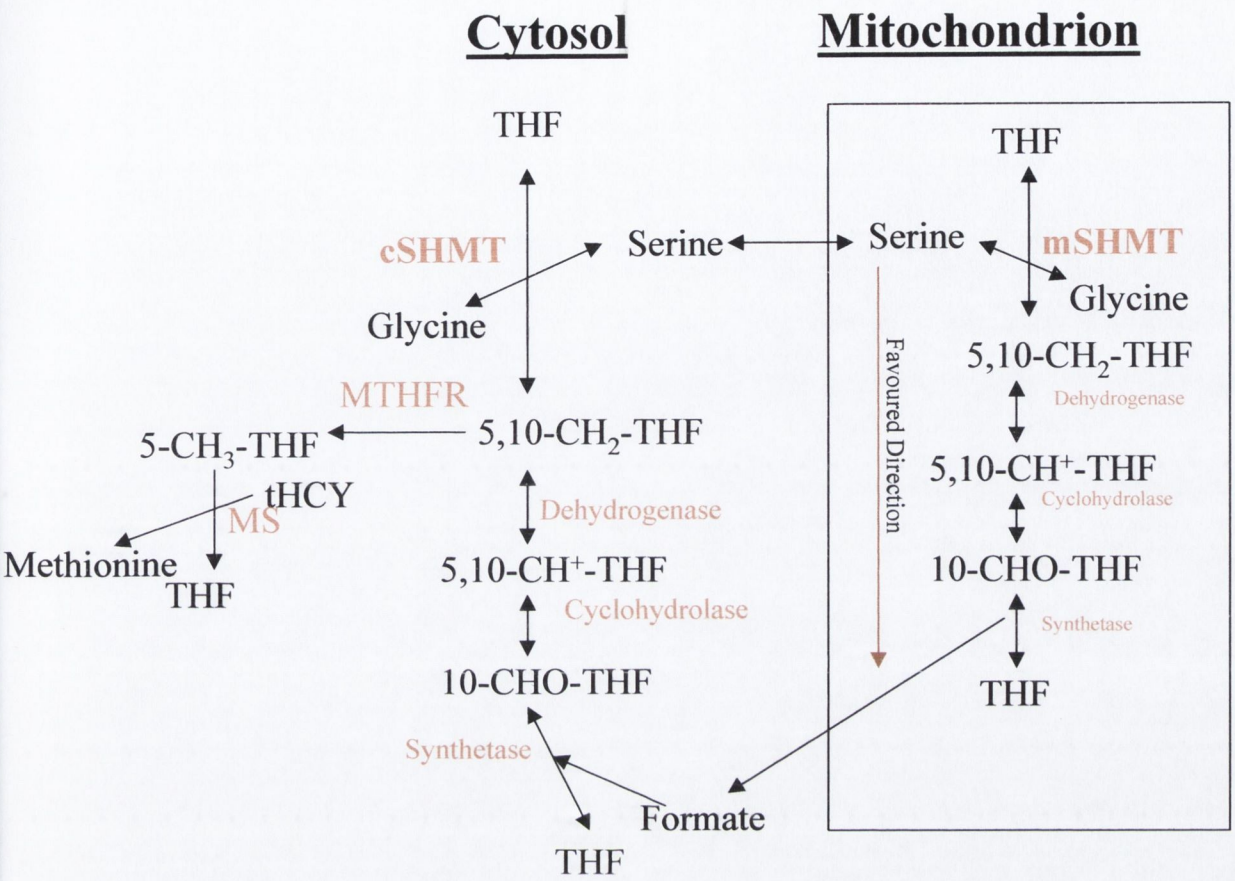


Figure 1.3: Compartmentation in one-carbon metabolism between cytosol and mitochondria. cSHMT, cytosolic serine hydroxymethyltransferase; mSHMT, mitochondrial serine hydroxymethyltransferase; THF, tetrahydrofolate; 10-CHO-THF, 10-formyltetrahydrofolate; 5,10CH^+-THF, 5,10-methenyltetrahydrofolate; 5,10CH_2-THF,5,10-metheylenetetrahydrofolate;5-CH_3-THF,5-methyltetrahydrofolate; 5-CHO-THF, 5-formyltetrahydrofolate; MS, methionine synthase; MTHFR, methylene tetrahydrofolate reductase.

substituted folate co-factors has suggested that the regulation of these enzymes may influence cell growth and proliferation (Stover *et al.,* 1997). Studies using CHO cells have shown that cells deficient in mSHMT activity accumulate 15-fold increased intracellular serine concentrations over wild type CHO cells. Despite the accumulation of intracellular serine, the net metabolic flux through the cytosolic SHMT (cSHMT) enzyme in CHO cells deficient in mithochondrian SHMT (mSHMT) activity is in the direction of serine synthesis. The cSHMT enzyme is in a prime position to regulate the metabolic competition between TS and MTHFR. The reaction catalysed by cSHMT is reversible *in vitro*, and therefore its capacity to affect this competition *in vivo* is expected to be dependent on the cellular concentration of glycine (Herbig *et al.,* 2002). It has been demonstrated that the cSHMT enzyme is regulated by heavy chain ferritin (HCF) and that HCF induced increases in cSHMT expression and enhanced *de novo* thymidylate synthesis (Oppenheim *et al.,*, 2001).

The serine derived one-carbon unit of mitochondrial 5,10-CH_2-THF is ultimately converted to THF and formate see figure 1.3. One carbon metabolism appears to favour the formation of formate as the ultimate mitochondrial product from carbon in the three position of serine, which suggests that cytosolic one carbon units originate largely from mitochondrial production of formate (Gregory *et al.,* 2000) see figure 1.3.

Formate derived one-carbon units are formed by the ATP-dependent enzyme formyl synethase which activates formate to 10-CHO-THF. One carbon units enter the active pool of one-carbons at the 5,10-CH_2-THF and 10-CHO-THF level with 5,10-CH_2-

THF being involved in thymidine biosynthesis and 10-CHO-THF a substrate for purine biosynthesis. Much of the net flow of substrate through cytosolic SMHT appears to be in the direction of glycine to serine (Girgis *et al.,* 1997), but the cytosolic formation of 5,10-CH_2-THF from serine and THF may also occur through the action of cSHMT as has been shown to be the case in both rat and human models (Martinez *et al.,* 2000; Gregory *et al.,* 2000).

Glycine derived one-carbon units are generated from the second carbon in glycine by a glycine cleavage system which results in the formation of 5,10-CH_2-THF by the action of glycine synthase (Herbig *et al.,* 2002). This reaction only occurs in the mitochondria of mammalian cells.

An alternate one-carbon source is formimino-l-glutamate (FIGLU) catabolism of histidine. A formimino group is transferred to THF releasing ammonia and 5,10-CH^+-THF by two enzymes, glutamate formiminotransferase and formino-THF cyclodeaminase. This represents a minor source of one-carbon units and may exist only in the liver and kidney. Excess one-carbon units are removed from the one-carbon pool by their oxidation to CO_2 by formylTHF dehydrogenase.

1.6.4 Folates in Purine Biosynthesis

One-carbon units from 10-CHO-THF are used in the biosynthesis of purines. THF is regenerated after the one-carbon group of 10-CHO-THF is transferred. THF is thus the cofactor for two different enzymes involved in the biosyntheses of carbon 2 and 8

of purine. In both cases the coenzyme activates a one-carbon fragment at the oxidation state of formate.

The first step involves the introduction of a carbon fragment by formylation of glycinamide ribotide (GAR) by 5,10-CH_2-THF using the enzyme GAR transformylase to give formyl GAR (Goldthwait *et al.*, 1954: Hartman and Buchanan, 1959a). The formyl group subsequently condenses with the amide nitrogen to form an imidazole ring and thus become the C8 of the final purine ring.

The second reaction involves the formylation of aminoimidazolecarboxamide riboucleoide (AICAR) with 10-CHO-THF and the enzyme AICAR transformylase to give 5-formyl AICAR (FAICAR), this further condenses to give inosinic acid (Hartman and Buchanan, 1959b). The AICAR transformylase activity is contained on a bifunctional enzyme which also contains IMP cyclohydrolase activity, the final reaction in purine synthesis (Akira *et al.*, 1997; Yamauchi *et al.*, 1995). The coupling of the AICAR transformylase and IMP cyclohydrolase activity on a single polypeptide allows the overall conversion of AICAR to IMP to be favourable by coupling the unfavourable formation of formylAICAR with the highly favourable cyclization (Wall *et al.*, 2000). Exogenous FAICAR has been shown to act as an inhibitor of AICAR transformylase or a substrate for IMP cyclohydrolase (Wall *et al.*, 2000). Kinetic studies carried out by Wall *et al.*, (2000) indicate that the release of FAICAR is a rate-limiting step, under steady state conditions in the bifunctional enzyme and channelling is not observed between AICAR transformylase and IMP cyclohydrolase. Therefore the location of the two enzymes on the same polypeptide is essential to ensure an efficient metabolic flux (Wall *et al.*, 2000).

Initially it was thought that 5,10-CH_2-THF and 10-CHO-THF were one-carbon donors for the formyltransformylase (Hartman and Buchanan, 1959b) but now it is considered likely that 10-CHO-THF is the sole carbon donor for the two reactions (Caperelli *et al.,* 1980). The reason for the original suggestion was that Hartman and Buchanan (1959b) had purified the formyltransferases on an enzyme complex containing the tri-functional enzyme activity of 10-CHO-THF synthase, 5,10-CH^+-THF cyclohydrolse and 5,10-CH_2-THF dehydrogenase, as well as SHMT (Caperelli *et al.,* 1980). The removal of the tri-functional enzyme activity prevented the use of 5,10-CH_2-THF as a substrate (Smith *et al.,* 1980). Analogues of 10-CHO-THF were also prepared that could not form 5,10-bridges but still acted as substrates for GAR transformylase (Caperelli *et al.,* 1980).

Purines are essential components of DNA and RNA. GAR transformylase and AICAR transformylase, as discussed in the previous paragraph, are two enzymes involved in purine synthesis and therefore designing inhibitors to these enzymes had been proposed to be an effective approach for antineoplastic intervention (Divekar and Hakala, 1975). Marsilje *et al.,* (2002) synthesized and evaluated a series of compounds as potential inhibitors of GAR transferase and AICAR transferase. From this study four compounds were identified (*N*-{4-[4-(2,4-Diamino-6(1*H*)-pyrimidinon-5-yl)-1-oxo-but-1-yl]benzoyl}-L-glutamic acid, *N*-{4-[5-(2,4-Diamino-6(1*H*)-pyrimidinon-5-yl)-1-(dimethylhydrazono)pent-2-yl]benzoyl}-L-glutamic acid, *N*-{4-[5-(2,4-Diamino-6(1*H*)-pyrimidinon-5-yl)-1-hydroxypent-2-yl]benzoyl}-L-glutamic acid and 10-Formyl-5,10-dideaza-acyclic-5,6,7,8-tetrahydrofolic acid) as having potent biological activity in the absence of growth medium purines, indicating

selective cytotoxicity through the inhibition of the purine *de novo* biosynthetic pathway (Marsilje *et al.,* 2002)

1.6.5 Folates in Pyrimidine Biosynthesis

Folates are not directly involved in the biosynthesis of pyrimidines but they are involved in the biogenesis of the methyl group of thymidine by thymidylate synthase (Blakley, 1957) and the hydroxymethyl group of 5-hydroxymethylcytosine (Flaks and Cohen, 1957). In both cases the folate derivative involved is 5,10-CH_2-THF. In the first reaction, deoxyuridine monophosphate (dUMP) is methylated to give deoxythymidine monophosphate (dTMP) and the reaction is accompanied by the concurrent oxidation of THF to DHF (Humphreys and Greenberg, 1958). The resulting DHF must be reduced by NADPH and DHFR before it can participate in thymidylate synthesis again. The level of thymidylate synthetase is related to the replication level of a cell (Humphreys and Greenberg, 1958).

During the S-phase of a cell cycle, a multienzyme complex called replitase is formed. Replitase contains at least eight enzymes involved in DNA precursor synthesis and DNA replication which interact with each other (Hammond *et al.,* 1989). Some of these enzymes included in the multienzyme complex are thymidylate synthetase, DHFR, DNA polymerase, thymidine kinase, and ribonucleotide reductase (Shane, 1989). The function of the replitase is to produce deoxynucleoside triphosphates and deliver them to the DNA polymerase enzyme (Hammond *et al.,* 1989). The active complex is only formed in the nuclear region of the cells when they are in the S-phase of the cell cycle (Preem veer Reddy and Pardee, 1982). Plucinski *et al.,* (1990) have demonstrated cross inhibition by the enzymes of the replitase complex *in vivo* using

chinese hamster embryo fibroblast cells. Cross inhibition occurs when the inhibition of one enzyme of the complex leads to the inhibition of a second unrelated enzyme. Allosteric interaction is thought to be responsible for this cross inhibition (Plucinski *et al.*, 1990).

The second reaction in which the folates are involved is the synthesis of 5hydroxymethyl cytidylic acid from deoxycytidylic acid (dCMP). No oxidation of THF occurs. This reaction is thought not to occur in the mammalian system.

1.6.6 Folate Function and the Methylation Cycle

5,10-CH_2-THF is irreversibly reduced to 5-CH_3-THF by methylene tetrahydrofolate reductase (MTHFR). This reaction is physiologically irreversible, committing the one-carbon of 5-CH_3-THF to the remethylation of homocysteine for methionine synthesis (Lathrop-Stern *et al.*, 2003). The methyl group of 5-CH_3-THF is transferred to homocysteine resulting in the formation of methionine and THF by means of a vitamin B_{12}-dependent enzyme called methionine synthase (MS). Methionine is activated by ATP to form S-adenosylmethonine (SAM), which in turn acts as a co-factor for many methyltransferases present in the cell. Methyltransferase enzymes use SAM as a source of methyl groups to methylate a wide range of compounds *i.e.* proteins, DNA, phospholipids and small molecules such as DOPA (Scott, 1999). The methylation cycle ensures that a cell always has a supply of SAM. When SAM donates its methyl group it becomes S-adenosylhomocysteine (SAH), which in turn is enzymatically, converted by a hydrolase back to homocysteine and adenosine (see figure 1.4 for folate biosynthetic pathway). This reaction is reversible and the direction of SAH production is thermodynamically favoured. SAH is a potent

competitive inhibitor of transmethylases. The direction of SAH hydrolysis predominates in the cell because of the remethylation of homocysteine and the removal of adenosine. Homocysteine can be remethylated back to methionine via the vitamin B_{12} dependent- methionine synthase or in the liver by betaine homocysteine methyltransferase. Betaine homocysteine methyltransferase utilises betaine which is a degradation product of choline in the liver and kidney. In effect the betaine acts as an alternate methyl donor for the synthesis of methionine and is a folate-independent pathway.

1.6.7 The role of 5-CHO-THF in folate metabolism

The metabolic role of 5-CHO-THF is not fully understood, however, it is an inhibitor of several folate-dependent enzymes including serine hydroxymethyltransferase (Suh *et al.,* 2000). 5-CHO-THF usually accounts for 3-10% of total intracellular folate in mammalian cells (Girgis *et al.,* 1997). *In vitro* 5-CHO-THF is synthesised from 5,10-CH^{+}-THF by both mitochondrion and cytoplasmic isozymes of SMHT. *E.coli* lacking this enzyme do not contain 5-CHO-THF which suggests that SMHT catalyses this reaction *in vivo* also (Stover and Schirch, 1990).

Methenyltetrahydrofolate synthetase (MTHFS) is the only known enzyme to metabolise 5-CHO-THF and catalyse the conversion of 5-CHO-THF to 5,10-CH^{+}-THF *in vivo* (Maras *et al.,* 1994), see figure 1.4. MTHFS is located in the cytoplasm of rabbit liver, whereas in humans it is located both in the cytoplasm 85% and in the mitochondria 15% (Girgis *et al.,* 1997). It has been suggested that the combined enzymatic activities of MTHFS and SHMT constitute a futile pathway that may buffer

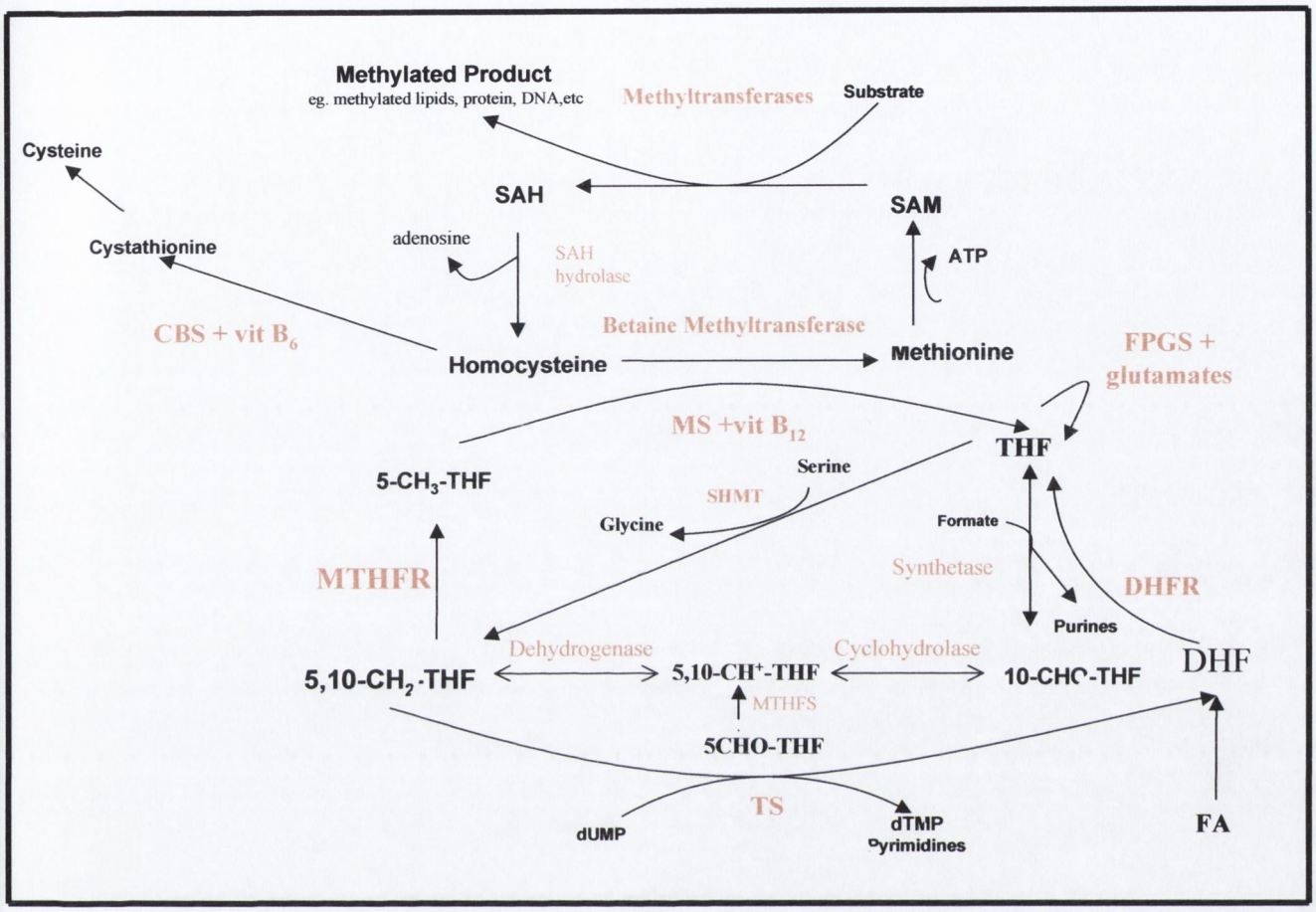


Figure 1.4: Folate one-carbon metabolism and methylation cycle. SHMT, serine hydroxymethyltransferase; THF, tetrahydrofolate; 10-CHO-THF, 10-formyltetrahydrofolate; 5,10CH^{+}-THF, 5,10-methenyltetrahydrofolate; 5,10-CH_2-THF, 5,10-metheylenetetrahydrofolate; 5-CH_3-THF, 5-methyltetrahydrofolate; 5-CHO-THF, 5-formyltetrahydrofolate; MS, methionine synthase; MTHFR, methylene tetrahydrofolate reductase; MTHFS, Methenyltetrahydrofolate synthetase; TS, thymidylate synthase; THF, tetrahydrofolate; SAH , s-adenosylhomocysteine; SAM, s-adenosylmethonine; HCY, homocysteine; FPGS , folypoly-glutamate synthetase; dTMP, deoxythymidine monophosphate; dUMP, deoxyuridine monophosphate; DHF, dihydrofolate; DHFR, dihydrofolate reductase.

cellular concentrations of 5-CHO-THF and regulate SHMT activity (Stover and Schirch, 1993), see figure 1.4.

5-CHO-THF is a potent inhibitor of several folate-dependent enzymes including AICAR and SHMT and may regulate folate-dependent metabolic pathways (Stover and Schirch, 1993). 5-CHO-THF polyglutamates are slow tight binding inhibitors of SHMT and therefore may have a significant physiological relevance because slow binding inhibitors are generally more effective enzyme inhibitors *in vivo* (Stover and Schirch, 1991). Girgis *et al.,* (1997) suggested that the inhibition of SHMT *in vivo* may be a mechanism whereby cells regulate not only folate–dependant glycine synthesis but also the supply of one-carbon units required for purine and thymidylate synthesis as well as homocysteine remethylation (Girgis *et al.,* 1997).

To investigate the effect 5-CHO-THF has on cell proliferation, intracellular serine, glycine and methionine concentrations, and the relative distribution of folic acid one-carbon derivatives MTHFS was over-expressed in 5Y neuroblastoma cells (Girgis *et al.,* 1997). Analysis of the one-carbon pools demonstrated that overexpression of MTHFS in the cytoplasm depleted all cellular 5-CHO-THF, suggesting that 5-CHO-THF accumulates in the cell due to limiting MTHFS activity. Overexpression of the MTHFS cDNA resulted in accumulation of MTHFS enzyme in the cytoplasm. The possibility that mitochondrial MTHFS activity was also increased could not be ruled out as MTHFS activity could not be detected in the purified mitochondria from these cells. Analysis of the intracellular free amino acid pools suggested that depletion of 5-CHO-THF increased both serine and glycine concentrations, suggesting that 5-

CHO-THF polyglutamates do inhibit cSHMT activity *in vivo*, consistent with previous *in vitro* studies (Girgis *et al.*, 1997).

Low levels of 5-CHO-THF in the cell appears to play a role in folate dependent one carbon metabolism. The need for folate-dependent serine synthesis in the cytoplasm is not clearly understood because serine synthesis from glycolytic intermediates also occurs in the cytoplasm. The ability of 5-CHO-THF to influence 5-CH_3-THF concentrations and homocysteine remethylation suggests that the regulation of the 5-CHO-THF futile pathway may be critical to maintain one-carbon homeostasis especially during rapid proliferative stages in development (Girgis *et al.*, 1997).

1.6.8 Proposed role for 10-formyldihydrofolate (10-CHO-DHF) in folate metabolism

The pteridine ring of folate exists at three oxidation levels; the fully oxidised, the 7,8-dihydro and the 5,6,7,8-tetrahydro states. The tetrahydro states create an asymmetric centre as carbon 6. The configuration at carbon 6 is designated 6R or 6S depending on the one-carbon substituents, which are attached to the N5 or N10 of the folate molecule (Baggot *et al.*, 2001). It had been believed that all the enzymes which catalyse folate-dependent one-carbon transfer reactions or other metabolism of folates had been considered active only with the naturally occurring isomers of THF folates. In 1986, Baggot *et al.*, demonstrated that 10-CHO-DHF served as a substrate for the folate-dependent purine nucleotide biosynthetic enzyme AICAR transformylase (Baggott *et al.*, 1986). Following this discovery they studied this phenomenon in bacterial cells, mammalian cell lines and in human subjects to establish if there might be a co-factor role for 10-CHO-DHF in folate metabolism. It had been assumed

previously that 10-CHO-DHF was simply a substrate analogue, not found in-vivo and should not be utilised well by the enzyme AICAR transformylase. Using unpurified AICAR transformylase from human leukaemia cells it was shown that 10-CH0-DHF was the preferred substrate when compared with 10-CHO-THF (Baggot *et al.,* 1995). In 1998, Baggot *et al.,* showed that 10-CHO-DHF supported the growth of human leukaemia cells in a culture medium containing methotrexate which is a DHFR inhibitor. Although, it was not as active as 5-CHO-THF it suggested that 10-CHO-DHF enters the pool of metabolically active folates and reverses methotrexate cytotoxicity, thus supporting cell growth. They suggested that an alternative route involving the direct reduction of 10-CHO-DHF to 10-CHO-THF was unlikely as 10-CHO-DHF is a poor substrate for DHFR (Baggot *et al.,* 1998).

In vitro studies showed that micromolar concentrations of Fe^{3+} in a citrate buffer catalyses the oxidation of 10-CHO-THF to 10-CHO-DHF (Baggott *et al.,* 1998). Cytochrome c also reacts with 10-CHO-THF to form 10-CHO-DHF folate *in vitro* (Baggott *et al.,* 2001). The second order rate constant for this non-enzymatic reaction is 1.3 x $10^4 M^{-1} sec^{-1}$, which is one of the largest reported for a non-enzymatic cytochrome c reaction. It is impossible, however, to remove cytochrome c from humans or laboratory animals to test whether it participates in this oxidation. *S. faecalis* lacks cytochromes when grown in normal media but can biosynthesise cytochromes, when it is grown in a medium containing haemin (Baggott *et al.,* 2001). When *S. faecalis* was grown in medium without haemin, it responded to a racemic mixture of 5-CHO-THF as if it is only 50% active, whereas the organism responds to the racemic mixture as it is 100% active when grown in haemin containing medium. These results are consistent with the hypothesis that cytochromes oxidise 10-CHO-

THF to 10-CHO-DHF. It is unclear whether these findings obtained from bacteria can be extrapolated to an *in vivo* condition in humans.

In recent years pure isomers of various THF folate derivatives have become commercially available and so a study using human subjects was carried out (Baggot *et al.,* 2001). It involved giving the subjects oral doses of the unnatural and natural isomer of 5-CHO-THF. The study concluded that the rapid oxidation of 10-CHO-THF to 10-CHO-DHF, the enzymatic conversion of 10-CHO-DHF to DHF by AICAR transformylase and the metabolism of DHF to the natural isomer of 5-CH_3-THF would explain the presence of naturally occurring 5-CH_3-THF within the cells when the unnatural form of 5-CHO-THF is used (Baggot *et al.,* 1998; 2001). Following on from these studies Baggot *et al.,* (2001) suggest the traditional map of folate metabolism should be revised to incorporate this new pathway (Baggott *et al.,* 2001).

1.7 MTHFR and folate pathway

1.7.1 5,10-Methylene-Tetrahydrofolate Reductase (MTHFR)

In 1982, Daubner and Matthews purified MTHFR from porcine liver and showed the enzyme contained noncovalently bound FAD and used NADPH as a reductant (Daubner and Matthews, 1982). Using the peptide sequence from this enzyme oligomers were designed and a clone for the human MTHFR gene was identified and sequenced (Goyette *et al.,* 1994). The catalytic domains of the human enzyme were shown to have extensive sequence homology with MTHFR from *E.coli* (Goyette *et*

al., 1994). MTHFR is expressed at very low levels in *E.coli* (Guenther *et al.*, 1999), constructs of *E.coli* strains which overproduced *E.coli* MTHFR were designed, which allowed the purification and characterisation of the enzyme (Guenther *et al.*, 1999).

The purified *E.coli* MTHFR protein was shown to be a flavoprotein capable of catalysing the NADH-linked reduction of 5,10-CH_2-THF to 5-CH_3-THF without added enzymes or co-factors (Sheppard *et al.*, 1999). The reduction of 5,10-CH_2-THF by MTHFR is the only route for synthesis of the 5-CH_3-THF that is utilised by methionine synthase to convert homocysteine to methionine (Guenther *et al.*, 1999). The bacterial enzyme is a (α_4) tetramer of identical subunits, each of which contains bound FAD if the enzyme is isolated without storage at dilute concentrations. The enzyme readily loses its activity and flavin co-factor when present in dilute form (Guenther *et al.*, 1999). The loss of FAD is associated with the conversion of the tetrameric enzyme to a dimer (Guenther *et al.*, 1999).

In 2001, Yamada *et al.*, developed a baculovirus expression system, which allowed the expression of recombinant human MTHFR at levels suitable for biochemical characterization. Using this system several differences between the *E.coli* MTHFR and the mammalian MTHFR were identified. Mammalian MTHFR are dimers of approximately 70kDa subunit with each subunit containing non-covalently bound FAD. The polypeptide contains an N-terminal catalytic domain and a C-terminal regulatory domain where the allosteric inhibitor SAM binds and regulates the enzyme activity in response to the level of methionine in the cells (Sumner *et al.*, 1986). The dissociation of the enzyme into monomers preceded flavin release suggesting that allosteric interactions between the regulatory domains of one subunit and the catalytic

domain of another modulate the affinity of the flavin cofactor. *E.coli* MTHFR lacked this regulatory domain and used NADH as a reductant compared with the mammalian MTHFR enzyme, which utilises NADPH.

Severe deficiencies of MTHFR are the most common inborn errors of folate metabolism with approximately 50 cases reported world-wide. It is an autosomal recessive disorder. Patients with severe MTHFR deficiency (residual enzyme activity of 0-20%) show a wide range of clinical symptoms such as developmental delay, severe mental retardation, perinatal psychiatric disturbances and later-onset neurodegenerative disorders (Rosenblatt, 1995). Symptoms usually present during infancy or adolescence. At least 19 severe mutations have been identified with some patients presenting three or four mutations (Goyette *et al.,* 1995).

1.7.2 MTHFR C677T mutation

Reduced MTHFR activity, due to a thermolabile form of the enzyme, was first identified by Kang *et al.,* 1988. Upon isolation of the human cDNA for MTHFR (Goyette *et al.,* 1994), the *in vitro* properties of the thermolabile enzyme were found to be due to a homozygous cytosine-to-thymine transition at nucleotide C677T, resulting in an alanine-to-valine substitution in the protein (Frosst *et al.,* 1995). Kang *et al.,* (1988) identified the MTHFR C677T functionally in lymphocyte cell extracts by a reduction in the enzyme stability following *in vitro* incubation at 46°C for 5min. It is believed that 5-20% of the population possess this MTHFR thermolabile gene (Pepe *et al.,* 1998).

The MTHFR C677T mutation affects conserved residues in the catalytic domains of the enzyme. Initially human MTHFR could not be expressed at high enough levels to permit purification and biochemical analysis and therefore the analysis of the mutant MTHFR C677T was undertaken in *E.coli* using constructs of *E.coli* strains which overproduced *E.coli* MTHFR (Guenther *et al.,* 1999). The human MTHFR C677T mutation was mimicked by constructing a corresponding A177V mutation in *E.coli* in order to compare the biochemical properties of the wild and mutant type MTHFR proteins. The results obtained suggested that the mutation lead to a thermolabile enzyme that loses its flavin eleven times more readily than the wild type enzyme (Guenther at al, 1999). Following the addition of folate derivatives both genotypes demonstrated reduced rates of flavin release and activity loss which suggests, that the protection afforded by the folates resulted from an increased affinity of MTHFR for FAD in the ternary complexes of the enzyme (Guenther *et al.,* 1999). They further suggested that the activity of both wild and mutant enzymes may be controlled by intracellular folate and FAD concentrations, and the C677T mutation simply shifts the balance point at which the enzyme activation occurs. The *E.coli* enzyme is markedly less stable than its eukaryotic counterpart and readily loses it activity and flavin co-factor when present in dilute solution (Sheppard *et al.,* 1999).

The development of the baculovirus expression system by Yamada *et al.,* (2001) for the MTHFR enzyme allowed the comparison of the phenotypes conferred by the human MTHFR C677T polymorphism. The properties of the C677T mutation were generally consistent with earlier *E.coli* studies (Guenther *et al.,* 1999) suggesting the validity of such types of model studies. But the phenotypic differences between the wild type and mutant human enzymes were more subtle, consistent with the retention

of the allele in the human population (Yamada *et al.,* 2001). The homozygous MTHFR TT genotype release FAD approximately three fold faster than the wild type. The enzyme dissociates into monomers before the flavin is released which suggests that allosteric interactions between the regulatory domains of one subunit and the catalytic domain of another modulate the affinity for the flavin cofactor. SAM binds to the regulatory domain of the enzyme (Sumner *et al.,* 1986) suppressing the release of the flavin after dilution. When the folate status of MTHFR TT homozygotes is low there is a decrease in the flux of homocysteine to methionine which is associated with the destabilisation of MTHFR and leads to further flavin release and loss of activity. Therefore MTHFR TT homozygotes are more at risk if their folate or riboflavin status is low (Yamada *et al.,* 2001; Lathrop-Stern *et al.*, 2003).

It would be expected that a decrease in MTHFR activity *in vivo* would result in a lower rate of reduction of 5,10-CH_2-THF to 5-CH_3-THF, resulting in increased availability of 5,10-CH_2-THF for oxidation to the formylated forms. Therefore, 5-CH_3-THF levels would be proportionally lower and nonmethylated folates such as formyl folates would be proportionally higher, see figure 1.5. This proved to be the case in red blood cells as individuals with the wild-type genotype contained 100% 5-CH_3-THF polyglutamates where as homozygous mutant individuals contained 71% 5-CH_3-THF polyglutamates and the remaining folates were formylated THF polyglutamates (Bagley and Selhub, 1998). Stern *et al.,* (2000) investigated if the conversion of 5-CHO-THF to 5-CH_3-THF could be impaired in folate-adequate persons homozygous for the MTHFR C677T mutation. Their findings did not support those of Bagley and Shelub, (1998) as they showed no difference between genotypes when plasma and urine samples were analysed. They suggest that there may be a

tissue difference in expression of the MTHFR TT genotype. More recently, Lathrop-Stern *et al.,* (2003) demonstrated in immortalised lymphocyte cell lines that although the concentration of intracellular folates did not differ with respect to the MTHFR C677CT genotype in folate adequate people, the intracellular folate co-factor distribution differed. Cells with the TT genotype had approximately half the concentration of 5-CH_3-THF polyglutamates and nearly double the concentration of 5-CHO-THF polyglutamates than the CC genotype cells. Yet despite the lower MTHFR enzyme activity, the mutant enzyme provided sufficient 5-CH_3-THF to meet the demands for methionine synthesis and homocysteine remethylation (Lathrop-Stern *et al.,* 2003).

1.7.3 Homocysteine levels associated with MTHFR C677T polymorphism

Homocysteine metabolism is at the intersection of two metabolic pathways; transulfuration and remethylation. During transulfuration, the enzyme cystathionine β-synthase (CBS) catalyses an irreversible condensation of homocysteine and serine to cystathionine, using vitamin B_6 as a co-factor. In methylation, the methyl donor for the conversion of homocysteine to methionine by the vitamin B_{12}-dependent methionine synthase enzyme, is provided by the reduction of 5,10-CH_2-THF to 5-CH_3-THF which is catalysed by the enzyme MTHFR. The transulfuration pathway contributes primarily to the maintenance of normal postprandial hcy concentrations, while the remethylation pathway maintains normal fasting hcy concentrations (Mudd *et al.,* 1989; Rosenblatt,1995). Homozygosity for the MTHFR C677T polymorphism is associated with mild hyperhomocysteinemia in humans with plasma folate concentrations below the median (Jacques *et al.,* 1996) and is the most common genetic cause of mild elevations in plasma hcy in humans (Schwahn and Rozen,

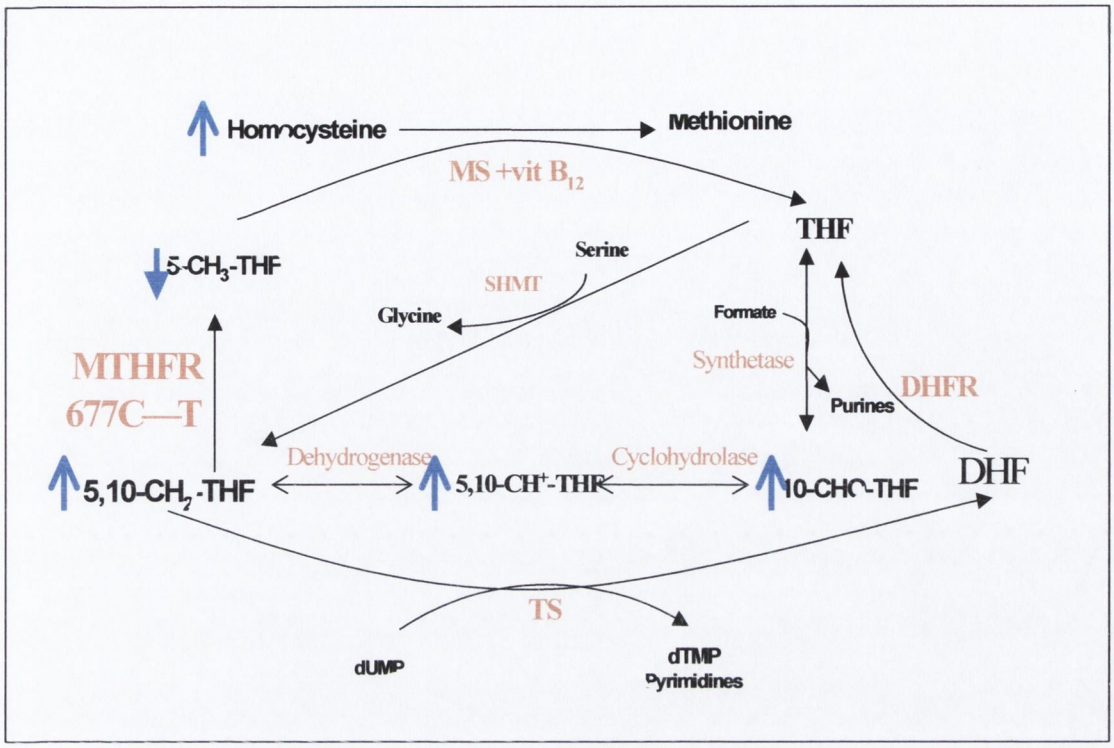


Figure 1.5: The effect of MTHFR C677T polymorphism on intracellular folate co-factor distribution. SHMT, serine hydroxymethyltransferase; THF, tetrahydrofolate; 10-CHO-THF, 10-formyltetrahydrofolate; 5,10CH^+-THF, 5,10-methenyltetrahydrofolate; 5,10-CH_2-THF, 5,10-metheylenetetrahydrofolate; 5-CH_3-THF, 5-methyltetrahydrofolate; 5-CHO-THF, 5-formyltetrahydrofolate; MS, methionine synthase; MTHFR, methylene tetrahydrofolate reductase; MTHFS, methenyltetrahydrofolate synthetase; TS, thymidylate synthase; THF, Tetrahydrofolate; dTMP, deoxythymidine monophosphate; dUMP, deoxyuridine monophosphate; DHF, Dihydrofolate; DHFR, dihydrofolate reductase.

2001). Mildly elevated plasma hcy levels have been associated with an increased risk of coronary artery disease (Boushey *et al.,* 1995; Graham and O'Callaghan, 1997). Mice homozygous for a knock-out mutation in MTHFR show 10-fold elevations in plasma hcy, and abnormal lipid deposition in the proximal portion of the aorta is observed in older heterozygotes and in homozygotes, but not in wild type animals (Chen *et al.,* 2001). Hyperhomocysteinemia is also associated with complications in pregnancy including miscarriage, pre-eclampsia and neural tube defects in the human fetus (Molloy *et al.,* 2002). Decreased levels of red cell folates have been reported in pregnant and non-pregnant women homozygous for the thermolabile MTHFR variant when compared to heterozygous or wild-type groups (Molloy *et al.,* 1997).

1.7.4 Effects of riboflavin and folate status in association with the MTHFR 677C→T mutation

Riboflavin is a water-soluble vitamin essential for normal cellular function and growth. It is a precursor of flavin mononucleotide (FMN) and FAD, which serve as cofactors for enzymes involved in the metabolism of vitamin B_6, folate and cobalamin (Guenther *et al.,* 1999). MTHFR requires riboflavin in the coenzyme form of FAD. Humans and other mammals have lost the ability to synthesise riboflavin and therefore obtain their vitamin requirements from both bacterially synthesised riboflavin in the large intestine or from the diet via intestinal absorption. Studies have shown that riboflavin uptake takes place mainly in the proximal part of the small intestine and involves specialised, carrier-mediated mechanisms (Akiyama *et al.,* 1982; Said *et al.,* 1994). The normal microflora of the large intestine have been shown to synthesise considerable amounts of riboflavin with a significant portion of

the vitamin in its free form ready for absorption. The absorption of luminally introduced riboflavin has been reported to occur in the large intestine of humans and rats (Kasper., 1970). The mechanism and regulation of riboflavin uptake in the large intestine was investigated by Said *et al.,* (2000) using an *in vitro* model system of the human-derived colonic epithelial cell line NCM460. These cells possessed similar characteristics to that of the native colonocytes, including similar uptake mechanisms. They identified the existence of a specialised carrier-mediated mechanism for riboflavin uptake using the NCM460 cell line. The mechanism appeared to be regulated by the extracellular substrate level and by intracellular Ca^{+2}/calmodulin-mediated pathway (Said *et al.,* 2000). The results suggest that the absorption of bacterially synthesised riboflavin could occur in the large intestine and may contribute toward riboflavin homeostasis.

In vitro studies have suggested that the reduced activity of the thermolabile MTHFR is due to the inappropriate loss of its riboflavin cofactor (Guenther *et al.,* 1999; Yamada *et al.,* 2001). As discussed above *in vitro* studies in *E.coli* have shown that thermolabile MTHFR is approximately eleven times as likely as the wild-type enzyme to dissociate from its FAD prosthetic group and thus become inactivated (Guenther *et al.,* 1999). The human recombinant thermolable MTHFR is only three times as likely as the wild-type enzyme to dissociate from its FAD prosthetic group (Yamanda *et al.,* 2001). Folate appeared to protect the mutant enzyme from destabilisation in studies of human lymphocytes extracts and purified bacterial and human enzymes (Guenther *et al.,* 1999; Yamada *et al.,* 2001). Several studies have been carried out to investigate the protective effects of folate on the MTHFR enzyme and to evaluate whether riboflavin status affects the activity of the thermolabile enzyme *in vivo*, and

consequently hcy concentrations (Hustad *et al.,* 2000; Jacques *et al.,* 2002; McNulty *et al.,* 2002). Three different populations were used in these studies; Irish (McNulty *et al.,* 2002), Norwegian (Hustad *et al.,* 2000) and American (Jacques *et al.,* 2002). Results from all three populations reported that riboflavin status may affect the hcy concentration in individuals homozygous TT. Differences were reported in respect to the influence of folate on hcy levels. Hustad *et al.,* (2000) demonstrated a riboflavin-homocysteine association in the CT and TT genotypes regardless of the folate status. Jacques *et al.,* (2002) demonstrated that TT individuals with low riboflavin intake had increased levels of hcy only when the plasma folate concentration was low also. They also showed a modest but non-significant association between plasma folate and plasma riboflavin levels. McNulty *et al.,* (2002) demonstrated that adequate riboflavin status renders the TT genotype neutral in respect to hcy concentrations as was the case with wild-type and heterozygous genotypes for thermolabile MTHFR. Elevated hcy concentrations was only reported in subjects with the TT genotype who had poor riboflavin status with the mean hcy concentration in this group being almost twice that of the other genotype groups. It would appear that riboflavin status is relatively unimportant in determining hcy concentrations in the absence of the thermolabile variant of MTHFR. This is in contrast to folate status which is a strong predictor of hcy concentrations in all genotypes. McNulty *et al.,* (2002) suggested that a riboflavin-dependent mechanism contributes to the interrelationship between folate and plasma hcy among persons with the TT genotype. Lathrop-Stern *et al.,* (2003) reported that under all conditions, immortalised lymphocyte cell lines with the TT genotype have significantly lower *in vitro* MTHFR activity than cells with the CC genotype. Compared with cells exposed to adequate nutrient conditions, the MTHFR activity in the TT genotype was significantly lower when exposed to either reduced

riboflavin or combined low folate and riboflavin. Methionine synthesis was reduced 70-80% in both cell genotypes when grown in low folate medium, with significantly lower methionine synthesis levels being reported in the TT genotype when compared with the CC genotype in cells grown in low folate and riboflavin media. Both cell genotypes reported significantly higher hcy accumulation when grown in folate deficient media and combined folate and riboflavin deficient medium, but differences between genotypes in medium hcy accumulation was not observed for any of the media conditions (Lathrop-Stern *et al.,* 2003). Further studies are required to determine if riboflavin is an independent modifier of hcy levels in persons with the MTHFR TT genotype. If this was the case MTHFR TT individuals may benefit from additional riboflavin supplementation as hcy may respond more rapidly than non TT individuals in reducing the hcy levels. This is especially relevant in today's society as the enrichment of grain products with folate in the US is showing altered hcy levels (Jacques *et al.,* 1999). The identification of polymorphisms or common mutations in vitamin metabolism may result in large percentages of the population requiring increased requirements for specific vitamins.

1.7.5 Effects of vitamin B_6 and folate status in association with the MTHFR 677C→T mutation

The steady state concentration of hcy in tissues and plasma is a function of the rate of homocysteine formation, remethylation, catabolism and renal excretion (Refsum *et al.,* 1998; Selhub *et al.,* 1999). The concentration of hcy is modulated by the vitamin nutritional status of folate, vitamin B_6 and vitamin B_{12} (Mansoor *et al.,* 1999; Selhub *et al.,* 1993). Plasma hcy concentration is influenced by the intake of these vitamins from dietary sources and nutritional supplements. Vitamin B_6 has been used

successfully to treat homocystinuria caused by CBS deficiency (Mudd *et al.*, 1985). Both folate and vitamin B_{12} play an essential functional role in hcy remethylation producing methionine which occurs primarily by the transfer of a methyl group from 5-CH_3-THF, this reaction being catalysed by methionine synthase. Remethylation can also occur, albeit to a lesser extent from betaine, catalysed by betaine-homocysteine methyltransferase.

One carbon units derived from the inter-conversion of serine and glycine, catalysed by SHMT are essential for normal cellular functions which include the synthesis of purines and pyrimidines and the conversion of homocysteine to methionine. Folate deficiency causes an increase in fasting homocysteine levels by reducing the concentration of cellular folate co-factors necessary for one-carbon metabolism and their enzymatic reduction to methyl groups (Selhub, 1999). Vitamin B_6 deficiency alters hcy metabolism by reducing the activity of SHMT and suppressing homocysteine catabolism (Selhub *et al.*, 1999; Ubbink *et al.*, 1996). Pyridoxal 5'-phosphate (PLP) is a form of vitamin B_6 which is required as a co-enzyme for both cytosolic and mitochondrial forms of SHMT and in the transulfuration pathway of homocysteine catabolism, as both CBS and γ-cystathionase require PLP as a co-enzyme. Martinez *et al.*, (2000) demonstrated that vitamin B_6 deficient rats demonstrated substantially lower hepatic SHMT activity and *in vivo* rates of hcy remethylation. The influence of one-carbon metabolism and transulfuration reactions on hcy levels was investigated by using stable isotope-labelled serine, methionine and leucine tracers (Cuskelly *et al.*, 2001). This was the first kinetic investigation to identify the metabolic effects of folate and vitamin B_6 deficiencies in humans on 1) the appearance of serine, methionine and leucine 2) the remethylation of hcy to form

methionine 3) the synthesis of hcy from methionine via transulfuration 4) the acquisition of one-carbon units from serine and their use in hcy remethylation in basal state and each nutritional perturbation. The major result from this study was that a large reduction in the rate of methionine synthesis occurred in folate deficiency. This indicated that during folate depletion a substantial impairment of folate-dependent remethylation of hcy occurs. The folate deficient group showed increases of 115% in plasma hcy levels when compared with controls The folate deficient group also showed a 37% difference in the overall remethylation rate. Cuskelly *et al.,* (2001) suggests that because the plasma hcy concentration increased to a greater extent than the hcy remethylation level reduced indicates that the remethylation rate is but one of several mechanisms by which plasma hcy is controlled. Vitamin B_6 deficiency showed no effect on the overall rate of hcy remethylation (Cuskelly *et al.,* 2001) which is consistent with the results published by Selhub (1999) who reported that vitamin B_6 deficiency had little effect on hcy metabolism unless the pathway is challenged by excess substrate, as in a methionine load. These findings were in contrast to findings in rat model studies, which reported major reductions in both the remethylation and transulfuration kinetics in vitamin B_6 deficiency (Martinez *et al.,* 2000). Cuskelly *et al.,* (2001) suggest that this discrepancy may be due to a more severe degree of vitamin B_6 deficiency in the rat model or to the trend amongst the vitamin B_6 deficient group toward an increased rate of homocysteine remethylation via cytosolic one-carbon metabolism. In humans both folate and riboflavin replete elderly subjects have been shown that a low dose vitamin B_6 supplement can effectively lower fasting plasma hcy concentrations (McKinley *et al.,* 2001).

1.7.6 DNA methylation and MTHFR C677T mutation

MTHFR catalyses the reduction of 5,10-CH_2-THF to 5-CH_3-THF, the methyl donor for the synthesis of methionine from homocysteine. Methionine is the immediate precursor of SAM, the universal methyl donor for a multitude of biological methylation reactions (Selhub and Miller, 1992). Included among these essential methylation reactions is DNA methylation, which occurs almost exclusively at CpG dinucleotides repeats in the human genome. DNA methylation is an important epigenetic feature of DNA that plays critical mechanistic roles in gene regulation (Robertson and Wolfe, 2000) and cellular differentiation. Aberrant genomic DNA methylation is widely recognised to be associated with different diseases, and is implicated in the genesis of cancer (Schmutte and Jones, 1998) and neurodevelopmental disorders (Robertson and Wolffe, 2000). DNA methylation patterns in the cell are established and maintained by DNA methyltransferases, which use s-adenosyl-L-methionine (SAM) as the donor of methyl groups. A major source of methyl groups used by SAM for methylation reactions is the folate-dependent *de novo* synthesis of one-carbon units (Selhub, 1999).

Folates play a major role in DNA metabolism. They are required for the synthesis of dTMP from dUMP. Under conditions of folate deficiency dUMP accumulates and as a result uracil is incorporated into DNA instead of thymine. Excessive uracil misincorporation leads to point mutations and also results in the generation of single and double stranded DNA breaks, chromosome breakage and micronucleus formation (Blount *et al.*,1997; Duthie and Hawdon, 1998) . Folate and vitamin B_{12} are required for the synthesis of methionine and SAM. SAM is a common methyl donor required for the maintenance of methylation patterns in DNA that determine gene expression

and DNA conformation. When methionine and vitamin B_{12} are deficient, SAM synthesis is reduced, methylation of DNA is reduced and the inhibition by SAM of MTHFR is minimised resulting in the irreversible conversion of 5,10-CH_2-THF to 5-CH_3-THF. This favours an increase in the dUMP pool and leading to increased uracil incorporation into DNA (Blount *et al.,* 1997).

The MTHFR C677T mutation is reported to significantly reduce the risk of colorectal cancer (Chen *et al.,* 1996; Slattery *et al.,* 1999) and acute lymphatic leukaemia (Blount *et al.,* 1997). Individuals who are heterozygous CT and homozygous TT for this MTHFR C677T polymorphism have an *in vitro* enzyme activity that is 65% and 30% of normal, respectively. MTHFR 677TT homozygosity has also been associated with mild elevations in hcy when folate status is inadequate (Jacques *et al.,* 1996; Stern *et al.,* 2003). This suggests that the *in vivo* activity of the MTHFR 677TT is protected from inactivation when folate status is adequate despite its *in vitro* phenotype of a lower enzyme activity. Studies have shown that DNA methylation was substantially lower with the MTHFR TT genotype only in the presence of low folate status (Lathrop-Stern *et al.,* 2000; Friso *et al.,* 2002). As the folate status improved, genomic DNA methylation was higher and not different from that seen in persons with the wild-type CC genotype (Lathrop-Stern *et al.,* 2003). It has been hypothesised that TT homozygotes are protected against some cancers because reduced MTHFR enzyme activity causes a diversion of 5,10-CH_2-THF towards thymidine synthesis. This minimises intracellular dUMP:dTMP ratios and, therefore, the chance of uracil being incorporated into DNA (Blount *et al.,* 1997). This requires further investigation as two studies carried out by Crott *et al.,* (2001), showed that the

677C→T polymorphism did not effect the ability of the cell to resist chromosome damage induced by folic acid deficiency *in vivo* (Crott *et al.*, 2001).

1.7.7 MTHFR C677T polymorphism in association with MTHFR A1298C polymorphism

A second polymorphism in the MTHFR gene was identified (Viel *et al.*, 1997; Van der Put *et al.*, 1998; Weisberg *et al.*, 1998). It consists of an A to C transition at the nucleotide A1298C which results in a glutamate to alanine substitution in the presumed regulatory domain of the MTHFR protein (Viel *et al.*, 1997). It was reported that the A1298C mutation resulted in a decrease in MTHFR activity that was more pronounced in 1298CC than in 1298AC or normal 1298AA states and did not result in a thermolabile protein (Friedman *et al.*, 1999). Freidman *et al.*, (1999) also reported that the loss of MTHFR activity in the C677T mutation was greater than the 1298A→C mutation (Friedman *et al.*, 1999) but this was disputed by Yamanda *et al.*, (2001) as their *in vitro* studies showed no such effect. The MTHFR A1298C and C677T mutations were reported to be in linkage disequilibrium in the general population and in neural tube defect (NTD) cases, but were found not to contribute as a risk factor to NTD (Stegmann *et al.*, 1999; Parle-McDermott, 2003). As a result of the linkage disequilibrium the effect of the A1298C mutation should always be examined against a wild type MTHFR 677CC background.

In combination with the MTHFR C677T polymorphism, in individuals with a wild type genotype MTHFR 677CC combined with a mutant homozygous MTHFR 1298CC or heterozygous 1298AC genotype showed decreased MTHFR enzyme activity, whereas the total plasma homocysteine and folate levels were not altered (Stegmann *et al.,* 1999). Combined heterozygosity 677CT/1298AC was found to be associated with reduced enzyme activity, elevated plasma homocysteine and decreased plasma folate (Van der Put *et al.,* 1998). Yamada *et al.,* (2001) evaluated the biochemical properties of recombinant human MTHFR and found MTHFR 1298CC variant had no effect on the catalytic function or regulation regardless of whether the mutation occurred alone or in association with MTHFR 677TT variant. The MTHFR 1298A→C varaint appeared thermostable and unaffected by changes in folate concentration *in vivo* (Yamada *et al.,* 2001). Friedman *et al.,* (1999) demonstrated that the A1298C mutation is highly prevalent and similar in frequency to the C677T mutation in a study using 377 Jewish Israeli subjects. In contrast to the C677T MTHFR mutation Friedman *et al.,* showed no evidence to suggest an association between A1298C MTHFR mutation and an increase in plasma hcy concentrations (Freidman *et al.,* 1999). Significantly lower levels of plasma hcy were reported in subjects MTHFR 677CC/1298CC when compared to MTHFR 677CC/1298AA and only individuals homozygous MTHFR 677TT showed increased levels of plasma hcy which is consistent with previous studies carried out (Frosst *et al.,* 1995; Harmon *et al.,* 1996; Jacques *et al.,* 1996). In contrast to results obtained from Van der Put *et al.,* (1998), Freidman *et al.,* (1999) showed no significant increase in plasma hcy concentrations in MTHFR 677CT/1298AC individuals. Weisberg *et al.,* (2001) reported increased fasting plasma hcy concentrations in

double heterozygotes when compared with single heterozygotes for the C677T mutation.

Bailey *et al.,* (2002) was the first to identify an inverse association between vitamin B_{12} status and plasma hcy in young women with the A1298C (vitamin B_{12} metabolism is discussed in section 1.9). They demonstrated that individuals with either MTHFR C677T or A1298C variant exhibited an inverse relationship between plasma hcy and vitamin B_{12} concentrations that were in marked contrast to individuals who possessed 677CC/1298AA genotype who were non-responsive. The inverse relationship was unexpected because the plasma vitamin B_{12} concentrations were well within the normal range. The mechanism by which vitamin B_{12} status modulates homocysteine levels with MTHFR polymorphisms is unknown. Vitamin B_{12} is not a required co-enzyme for the MTHFR catalysed reaction and therefore, MTHFR polymorphisms may co-exist with other common polymorphisms that are vitamin B_{12} dependent *i.e.* MS and methionine synthase reductase (MTRR). The results obtained from the Bailey *et al.,* (2002) study suggest that enhancing vitamin B_{12} status may significantly modulate plasma hcy concentrations in individuals doubly heterozygous or homozygous for either the MTHFR C677T or A1298C polymorphisms, even when vitamin B_{12} concentrations are within the normal range. It is worth noting that regression analysis results for serum folate indicated a genotype-independent response of predicted plasma hcy as serum folate concentrations increased, which is consistent with previous studies (Jacques *et al.,* 1996; Bailey and Gregory, 1999)

1.8 The effect of genetic variability on one-carbon metabolism and the risk of neural tube defects (NTDs)

Failure of the embryonic neural tube to close normally within the first four weeks following conception results in a group of major congenital malformations known as neural tube defects. These defects include spina bifida (51%), anencephalus (40%), encephalocele (8%), and iniencephaly (1%). Anencephalus and iniencephaly are universally lethal conditions with death occurring either in utero or shortly after birth. Genetic and environmental factors that produce alterations in folate metabolism are likely to play a major role in the development of NTDs. Evidence of an inherited component of this condition is in the observation that 1-3% of families with a previous NTD child will have a second affected child (Cowchock *et al.,* 1980; Pietrzyk, 1983; Papp *et al.,* 1997). This recurrence risk in siblings has been used to estimate the genetic contribution to complex disease phenotypes. For NTDs, the empirical increased risk to siblings is less than that in dieseases with a pure genetic etiology such as cystic fibrosis, but greater than that observed for other complex diseases such as Type 2 diabetes (Todd, 1995). The use of folic acid supplements taken preconceptionally can greatly reduce (up to 72%) a woman's risk of both NTD occurrence and recurrence in her offspring (Czeizel and Dubas, 1992). The mechanism through which the prevention occurs is unknown but alterations in folate, hcy, vitamin B_{12} and methionine metabolism have been associated with NTD (Brody *et al.,* 1999). On the strength of these results the US Food and Drug Adminstration (FDA) have mandated that all grain products are to be fortified with FA in order to reduce the risk of NTD births. Several studies have indicated that the folate status in

the US population has been significantly enhanced since 1997 when FA fortification was initiated (Caudill *et al.,* 2001; Jacques *et al.,* 1999).

Folic acid absorption and metabolism is a complex multistep process involving numerous genes. Upon investigation, the absorption of dietary folates in mothers of children with NTDs was found to be normal (Bower *et al.,* 1993; Wild *et al.,* 1994). Women who have had NTD children have shown to have elevated plasma hcy levels when measured during pregnancy (Mills and Conley, 1995), in response to methionine loading (Steegers-Theunissen *et al.,* 1994). Vitamin B_{12} levels in the low normal range have also been reported to be an idependent risk factor for NTDs (Kirke *et al.,* 1993). These observations suggest that inherited variations in genes regulating methionine, vitamin B_{12}, homocysteine and FA homeostasis may modify the risks of NTDs.

The thermolabile variant MTHFR is the first folate related variant to be associated with increased risk of NTDs (Ou *et al.,* 1996; Van der Put *et al.,* 1995; Whitehead *et al.,* 1995). Approximately 5-20% of the population are homozygous for the thermolabile TT variant (Pepe *et al.,* 1998), homozygosity for the TT variant has been reported to be more common in individuals with spina bifida, and accounts for approximately 13% of the population attributable risk (Whitehead *et al.,* 1995; Shields *et al.,* 1999). Several studies have established that women carrying affected embryos with NTDs (Kirke *et al.,* 1993; Mills *et al.,* 1995) and women who have given birth to affected infants (Steegers-Theunissen *et al.,* 1991, 1994) have significantly increased plasma hcy levels and significantly reduced plasma folate and vitamin B_{12} concentrations. However, several studies have found no association

between MTHFR C677T mutation and increased risk of NTDs (Papapetrou *et al.*, 1996; Mornet *et al.*, 1997; Speer *et al.*, 1997; Boduroglu *et al.*, 1999) therefore, the association of the MTHFR C677T mutation is still controversial. It seems likely that other genetic defects in folate related enzymes will be found that will explain NTDs. Candidate genes involved in folate metabolism have been screened in relation to NTDs. Some of these genes include CBS, methionine synthase (MS), methionine synthase reductase (MTRR), human trifunctional enzyme (MTHFD1) and several folate receptors (reviewed in Lucock,. 2000; Gelineau-van Waes and Finnell, 2001). Some of these candidate gene associations are discussed below:

1.8.1 Methionine Synthase (MS)

MS catalyses the vitamin B_{12} dependent conversion of hcy and 5-CH_3-THF to methionine and THF. Two of the four substrates homocysteine and vitamin B_{12} have been implicated in NTD formation which suggested that MS could be a candidate gene for NTDs. The role of genetic variation associated with MS was evaluated in families having children with NTDs (Brody *et al.*, 1999). The human MS gene had been cloned previously by Leclerc *et al.*, (1996) and Chen *et al.*, (1996). Brody *et al.*, (1999) used radiation hybrid and physical mapping techniques to localise the MS gene to a small region on chromosome one. They isolated YAC and BAC clones and previously identified polymorphic loci were mapped with respect to the MS gene. The segregation of these highly polymorphic short tandem repeat markers (simple-sequence repeat polymorphisms, SSRPs) physically linked to the MS gene were used to assess the role of variant MS alleles in the etiology of NTDs in an Irish population using a transmission disequilibrium test (TDT) with SSRP and coding polymorphisms (Brody *et al.*, 1999). From the results obtained from this study they concluded that if

specific MS alleles were associated with an increased risk of NTDs they are not common and do not show high prevalence (Brody *et al.,* 1999).

1.8.2 Methionine Synthase Reductase (MTRR)

MTRR is required to maintain the MS co-factor methylcobalamin in an active state. Therefore, it was suggested that variant alleles in MTRR may directly affect MS activity. An MTRR A66G variant was discovered and it was estimated to be present in approximately 25% of the population. Homozygous for the variant MTRR A66G was associated with significant increases in hcy levels when the vitamin B_{12} status was less than optimal (Wilson *et al.,* 1999; Leclerc *et al.,* 1999). Additional screening is needed to clarify this association. Leclerc *et al.,* (1999) cloned the MTRR gene and further studies are required to determine if this gene is associated with NTDs.

1.8.3 Human Trifunctional Enzyme

The enzymes 5,10-CH_2-THF dehydrogenase, 5,10-CH^+-THF cyclohydrolase and 10-CH0-THF synthetase catalyse three sequential reactions in the interconversion of one-carbon derivatives of THF which are substrates for methionine, thymidylate and *de novo* purine synthetases, see figure 1.4. In eukaryotes the three activities are properties of a single protein, a homodimer of polypeptides of approximately 100kDA (Barlowe *et al.,* 1989). The eukaryotic trifunctional enzyme (MTHFD1) consists of two major domains with the NADP-dependent dehydrogenase and cyclohydrolase sharing an overlapping active site on the NH_2-terminal and the COOH-terminal domain possessing the formyltetrahydrofolate synthetase activity which provides 10-CHO-THF for purine biosynthesis (Barlowe *et al.,* 1989). The formyltetrahydrofolate

synthetase is also thought to play a noncatalytic role in purine-synthesising multienzyme complex (Barlowe and Appling, 1990). Brody *et al.,* (2002) analysed several potential polymorphisms in MTHFD1 to determine if these polymorphisms were associated with NTDs in an Irish population. One such polymorphism the MTHFD1 R653Q lies within the 10-CHO-THF synthetase domain of MTHFD1 (Brody et a, 2002). This MTHFD1 R653Q single nucleotide polymorphism (SNPs) is polymorphic in an Irish population and the results of the MTHFD1 R653Q data show a clear excess of QQ homozygotes in mothers of children with NTD, compared with controls. This suggests that the variant plays a role in abnormal neural closure. No correlation was detected between MTHFD1 R653Q genotype and levels of plasma folate or red blood cell folate or between genotype and levels of homocysteine among mothers and patients with NTD or control individuals. Brody *et al.,* (2002) suggested that the MTHFD1 enzyme may play a key role in cell division and therefore may be an important influence on the maternal environment. The synthetase activity in the MTHFD1 enzyme provides the source of 10-CHO-THF for purine synthesis. MTHFD1 QQ homozygote mothers may have less efficient purine or pyrimidine synthesis, which is critically important during early embryo development because, during the neural tube closure at day 21-27 the embryo is entirely dependent upon maternally derived cells that protect and nurture its development. MTHFD1 is only the second folate-related gene to be identified as a risk factor for NTDs, and its identification.

1.8.4 Cystationine β-synthase (CBS)

CBS controls post-prandial levels of hcy and individuals carrying defective CBS alleles have reduced CBS activity and elevated hcy (Tsai, 1996; Mudd *et al.*,1989). Vitamin B_6 is a co-factor for CBS enzyme as previously discussed. Mildly elevated hcy levels have been associated with an increased risk NTD births (Mills and Conley, 1995) and therefore Ramsbottom *et al.*, (1997) investigated whether defects in the CBS enzyme increased the risk of NTD. In Ireland approximately 70% of defective CBS alleles in homocystinuria patients are G307S (Gallagher *et al.*, 1995), the underlying mutation being a G to A transition at nucleotide position 919 that changes a glycine amino acid to serine. Unlike other homocystinuria patients with other mutations in the CBS gene, G307S homozygotes are unresponsive to vitamin B_6 therapies. This would indicate that the G307S mutation is among the most severely dysfunctional. Another mutation in the CBS gene is the insertion/1278T haplotype in exon 8 (Sebastio *et al.*, 1995). This is a 68 base pair insertion and is located 11 base pairs downstream of 1278T. It encodes an additional 11 amino acids in frame followed by a premature termination codon (Sebastio *et al.*, 1995). It also specifies an extra downstream splice acceptor site, the use of which creates a normal sized product without the 1278T amino acid substitution. Ramsbottom *et al.*, (1997) did not identify any G307S alleles in either the NTD cases or mothers with a history of NTDs which they analysed. This suggested that heterozygotes carrying this defective CBS mutation did not constitute a significant portion of NTDs upon analysis. Therefore any risk associated with heterozygous G307S deficiency is likely to be insufficiently high to account for more than a tiny fraction of observed cases (Ramsbottom *et al.*, 1997). With regards to the insertion/1278T haplotype the frequency was similar in both cases and controls. When the CBS haplotype was analysed with the MTHFR TT genotype, it did not confer an increased risk of NTDs (Ramsbottom *et al.*, 1997). The

results obtained from this study suggested that inefficient transulphuration of hcy due to deficient CBS function did not contribute significantly to the aetiology of NTDs.

Sreening candidate genes for polymorphisms which may be identified as risk factors associated with NTD births is on-going. Other folate related genes may be identified as increased risk factors for NTD births in the future.

1.9 Vitamin B_{12} Metabolism

Vitamin B_{12} (cobalamin, cbl) is the generic name for a group of cobalt containing corrinoids with biological activity in mammals (Poston and Stadman, 1975). It is a large water soluble molecule that does not easily diffuse through cells. It is synthesised by bacteria, which is its only source, and is present in all forms of animal tissue, which acquire the vitamin indirectly from bacteria (Chanarin *et al.,* 1979). Vitamin B_{12} is not present in vegetables or fruit hence deficiency often presents itself in vegans. Dietary fish and meat are good sources of vitamin B_{12}. The core of vitamin B_{12} consists of a planar corrin-ring surrounding a central cobalt atom. The cobalt ion can be attached to various types of ligands *i.e.* methyl, hydroxy or adenosyl. These ligands determine the type of cobalamin molecule methylcobalamin (MeCbl), hyroxycobalamin (OH-Cbl), cyanocobalamin or adenosyl cobalamin (AdoCbl) respectively. AboCbl and MeCbl are naturally active forms of cobalamin, the MeCbl is a co-factor in the methionine synthase (MS) cataylsed reaction (Poston *et al.,* 1975) and the Ado-Cbl is a co-factor in the methylmalonyl CoA mutase catalysed reactions (Badior, 1975). Cobalamin in supplements often consists of

cyanocobalamin, which is converted via OH-Cbl to the biologically active forms MeCbl and AdoCbl.

On entering the stomach vitamin B_{12} is removed from dietary proteins by proteases and acid in the stomach (Cooper and Castle, 1960; Kittang and Schjonsby, 1987). Allen *et al.,* (1978) suggested that the vitamin B_{12} is bound to R-binders, a range of glycoproteins secreted in the saliva. This complex is digested by pancreatic enzymes in the small intestine, and Cbl is then free to bind to gastric intrinsic factors (IF). This IF-B_{12} complex passes down the ileal where it is absorbed by phagocytosis after attachment to specific membrane receptors. The vitamin B_{12} is freed probably by lysozomal digestion from the IF and is subsequently bound to transcobalamin II (TC-II). The TC II-Cbl complex is internalised by absorptive endocytosis using a specific high-affinity receptor (Fenton and Rosenberg, 1995). TC II is then degraded by lysosomal proteases, releasing the Cbl, which exits from the lysosome and is converted to either MeCbl in the cytosol which binds to MS or to AdoCbl in the mitochondria and binds with methylmalonyl-CoA (Weir and Scott, 1999). Plasma Cbl is mainly MeCbl (60-80%).

Cobalamin deficiency is rare in the general population due to the body's huge reserves and the high cobalamin content found in the normal diet. There are sub-groups within the population that are more at risk of cobalamin deficiency these include vegans and individuals with extreme protein deficiencies (Weir and Scott, 1999). Malabsorption of cobalamin in the intestine can lead to cobalamin deficiency. Pernicious anaemia is an auto-immune disease associated with cobalamin deficiency (Chanarin, 1979). This disease results in the production of auto-antibodies against

parietal cells causing atrophy. The cells cannot produce IF or secrete hydrochloric acid (HCl) and therefore cannot reabsorb the cobalamin that is secreted in the bile and a negative balance of the vitamin results. Following the establishment of the negative vitamin balance a megloblastic anaemia presents. An identical megaloblastic anaemia can be seen in folate deficiency. If the anaemia is not treated quickly by cobalamin replacement sub-acute degeneration of the spinal cord (SACD) occurs. The nerve damage is untreatable and irreversible after a certain point. The incidences of pernicious anaemia is markedly increased with age, the incidences at six months of age is 1/1million people whereas the incidences at seventy years of age is 1/200 people (Herbert, 1985).

Vitamin B_{12} deficiency produces a megaloblastic anaemia folate metabolism which inhibits cell division and therefore presents itself with identical metabolic effects as folate deficiency which also inhibits cells division. In vitamin B_{12} deficiency the 5-CH_3-THF cannot be converted to THF and homocysteine to methionine because of the diminished MS activity which requires vitamin B_{12} as a co-factor for the reaction. As a result the 5-CH_3-THF is trapped and therefore deprives the cell of folate co-factors required for purine and pyrimidine synthesis, this phenomen is referred to as "the methyl-trap hypothesis" (Scott and Weir, 1981). In addition reduced levels of methionine and SAM and increased levels of SAH occur since homocysteine the product of SAH cannot be re-methylated to methionine due to the diminished activity of MS caused by the vitamin B_{12} deficiency. A reduction in SAM releases the inhibition of MTHFR producing an increased diversion of folate co-factors into 5-CH_3-THF. An increase in SAH and reduction in SAM causes the ratio of SAM to SAH to be reduced resulting in the inhibition of methylation reactions. This

inhibition causes a state of hypomethylation in the brain, which in turn causes SACD (Weir *et al.,* 1988). In order for folates to be retained in the cells they need to be in their polyglutamate form, the enzyme FPGS is responsible for this polyglutamylation (Chen et al, 1995). 5-CH_3-THF which accumulates in the cell as a result of cobalamin deficiency is not a good substrate for FPGS and therefore the 5-CH_3-THF cannot be retained by cell (Savage and Lindenbaum, 1995) which results in folate depletion.

Vitamin B_{12} deficiency is treated with an intramuscular injection of cobalamin in the form of cyanocobalamin or hydroxycobalamin. This corrects the anaemia and treats the neuropathy as oral doses are not absorbed adequately. The anaemia associated with cobalamin can also be treated with folic acid but this will not treat the neuropathy as it cannot re-start the methylation cycle which is caused by the cobalamin deficiency. As a result the neuropathy can progress undetected due to the correction of the haematological manifestation. The folic acid enters the cell and is reduced to DHF and subsequently to THF by the enzyme DHFR. The THF is polyglutamated and retained by the cells where it is used in purine and pyrimidine synthesis and therefore correcting the anaemia but not the neuropathy (Weir *et al.,* 1988). This is referred to as a "masking effect" and is a serious problem in elderly people suffering from vitamin B_{12} deficiency megaloblastic anaemia as folate supplementation will mask the anaemia resulting in severe irreversible neuropathy if the deficiency goes undetected.

1.9.1 Vitamin B_{12} status and NTD risk

Boddie *et al.,* (2000) evaluated vitamin B_{12} in NTD case mothers compared with controls. NTD case mothers had significantly lower levels compared with controls.

The data suggests small differences in vitamin B_{12} within the normal range can have significant effects on NTD risk. The effect vitamin B_{12} status on plasma hcy in young women with combined MTHFR C677T polymorphism suggest that vit B_{12} requirements increase in individuals with certain polymorphisms affecting folate and vitamin B_{12} dependent pathways (D'Angelo *et al.,* 2000). Molloy *et al.,* (2002) investigated the influence folate, vitamin B_{12} and the MTHFR C677T variant on maternal and fetal plasma hcy levels at birth. They reported that the plasma folate and vitamin B_{12} levels were inversely related to plasma hcy levels in both mothers and fetus. After accounting for plasma folate and vitamin B_{12}, MTHFR C677T was not a predictor of maternal or fetal hcy levels (Molloy *et al.,* 2002). They further suggest that the potential impact of vitamin B_{12} on plasma hcy levels and NTD risk will emerge as an important additional factor but further studies are required to verify this.

1.10 Folate Catabolism

There are at least three ways that intracellular folate turnover occurs. Newly transported folate monoglutamates can escape polyglutamylation and efflux from the cell. Folate polyglutamates can undergo hydrolysis of the polyglutamate peptide by γ-glutamyl hydrolase (GGH), resulting in their conversion to folate monoglutamates, which can then efflux from the cell. Lastly, folates can be catabolised to yield inactive degradation products (Geoghegan *et al.,* 1995; McNulty *et al.,*1995). Folate catabolism is thought to be the predominant mechanism of folate turnover in both rat and human models (Caudill *et al.,* 2002) and directly affects intracellular folate concentrations (Suh *et al.,* 2001).

There may be advantages in maintaining low cellular folate concentrations through catabolism because it permits: 1) metabolic channelling of folate co-factors among folate-dependent enzymes, 2) competitive regulation of folate metabolic pathways and 3) translational control of TS expression (Anguera *et al.,* 2003). Folates and mRNA bind to TS competitively, this regulatory mechanism is operative only if TS is not saturated with folate co-factors. These biochemical advantages may be realised during physiological states of rapid cell division that are associated with increased rates of folate catabolism including cancer (Kim, 1999) and pregnancy (McPartlin *et al.,* 1993; Higgins *et al.,* 2000).

As discussed in section 1.1 reduced forms of folates are unstable *in vitro* and can readily undergo oxidative degradation (Blakley *et al.,* 1967). Oxidation has been proposed to occur by at least two distinct irreversible mechanisms, the first being the sequential oxidization of the pteridine ring resulting in DHF and then to folic acid through a quinoid DHF intermediate (Archer and Reed, 1980), the second resulting in the oxidative cleavage of the C9 and N10 bond of the dihydrofolates and tetrahydrofolates resulting in the formation of pterin derivative and *p*ABG (Archer and Reed, 1980). Pterin aldehydes generated by folate catabolism through cleavage at the carbon 9 and nitrogen 10 bonds are further metabolised, retained in the liver and slowly released (Suh *et al.,* 2001). Turnover rates of folates appear to be tissue specific with the heart, spleen, testes having a higher rate than the liver and kidneys.

The susceptibility to oxidative cleavage is even more pronounced with DHF, which is very readily cleaved. The oxidation of THF appears to take place almost exclusively

through DHF with cleavage albeit at a somewhat slower rate because it first forms DHF (Quinlivan *et al.,* 2000). Cellular accumulation of DHF in prokaryotes resulted in increased folate catabolism. Bacteria exposed to DHFR inhibitor TMP display rapid accumulation of both DHF followed by *p*ABG accumulation, with subsequent folate deficiency (Quinlivan *et al.,* 2000).

In vivo cleavage of folate results in the formation of primary aromatic, diazotizable amines in rat urine following administration of both 5-CHO-THF and FA. The formation of these catabolites was determined to be a major excretory process associated with folate turnover (Krumdieck *et al.,* 1978). These products were identified as *p*ABG and *ap*ABG and were shown to be present in the urine of human subjects (Krumdieck *et al.,* 1978). Following folate cleavage the export of *p*ABG polyglutamates was facilitated by the removal of the polyglutamate polypeptide by GGH, and the *p*ABG is likely to be acetylated in the cytosol by arylamine N-acetyltransferase to produce A*p*ABG (Minchin, 1995), which represents greater than 85% of the total *p*ABG excretion in humans (McPartlin *et al.,* 1992: Gregory *et al.,* 1998). A*p*ABG does not accumulate in cells but is rapidly excreted in the urine (Murphy and Scott, 1979). Methods were developed for the detection and quantitation of these catabolites in the urine of rats (McNulty *et al.,* 1993) and humans (Geoghegan *et al.,* 1995) by using a solid-phase extraction technique followed by reverse-phase HPLC analysis. Geoghegan *et al.,* (1995) developed a method which involved analysing urine samples using both an ion-pair reverse-phase HPLC system reverse-phase HPLC method. This enabled the detection of both the intact labile folates and the folate catabolic products in rat urine. The results suggested that the presence of free *p*ABG in urine was not due to incomplete acetylation *in vivo* but

rather to non-specific extracellular degradation of intact labile folates in the circulation or bladder or to the degradation of labile folates during urine collection (Geoghegan *et al.,* 1995).

The presence of folate catabolites in urine was considered a reliable indicator of folate status and turnover (Geoghegan *et al.,* 1995; Higgins *et al.,* 2000). It had been suggested that folate intake may influence the rate of folate turnover and catabolism in humans (Caudill *et al.,* 1998). In mammals, increased rates of folate catabolism are associated with increased rates of cell division, presumably resulting from robust thymidylate synthesis and therefore DHF formation. McNulty *et al.,* (1995) investigated the relationship between growth rates and folate catabolism in weanling rats. Over a twelve day period weanling rats were put on a restricted feed diet which provided 50% folate intake required for normal growth. The a*p*ABG excretion was significantly higher in normally growing rats compared with growth-restricted rats. Following the restriction diet a*p*ABG levels increased, tending to exceed those of normally growing rats of comparable age. These results demonstrated that growth retardation resulted in decreased rates of folate catabolism and accelerated growth rates following dietary restriction was associated with increased levels of folate catabolism (McNulty *et al.,* 1995).

Gregory *et al.,* (1998) designed a mathematical kinetic model of folate metabolism in non-pregnant women to investigate the relationship among rates of intake, turnover, masses of *in vivo* folate pools and relative significance of excretory processes. Over a ten week period non-pregnant women were fed a diet which contained both naturally occurring folate in food and [$^2H_2$] folic acid present in orange juice. The

isotopic enrichment of total urinary folate and the primary catabolite a*p*ABG was determined. The model predicted that; 1) varying the level folate intake from 200-400μg/day had little effect on the mass of the large saturable folate pool, 2) the fast turnover of nonsaturable folate pools increased proportional to folate intake and 3) the slow nonsaturable folate pools also tended to increase (Gregory *et al.,* 1998). The results also demonstrated that the mean residence time of folates decreases with increasing folate intake, which suggested that the newly absorbed folate molecules may compete for binding sites in tissues and therefore accelerate turnover. Wolfe *et al.,* (2003) reported a significant reduction in urinary folate catabolites in response to a low-folate diet and the different responses to dietary folate intakes of 200-400 g/day which supported the assumption that total urinary folate catabolite excretion is sensitive to changes in folate intake (McNulty *et al.,* 1995; Gregory *et al.,* 1998). Folate catabolite excretion responded to changes in dietary folate intake in a similar manner in both young and old (Wolfe *et al.,* 2003). The results supported the measurement of folate catabolites as a folate status indicator in both the young and elderly population (Wolfe *et al.,* 2003).

Accelerated rates of folate catabolism may explain folate deficiencies arising from certain clinical conditions or physical states including cancer (Kelly *et al.,* 1983), pregnancy (McPartlin *et al.,* 1993; Higgins *et al.,* 2000), alcohol abuse (Shaw *et al.,* 1989), Crohns disease (Hoffbrand *et al.,* 1969) and anticonvulsant therapies (Kelly *et al.,* 1979). The relationship between increased rate of folate catabolism and both pregnancy and cancer is discussed in section 1.8.2 and 1.8.3 respectively.

Recently, a protein was purified from rat liver that appeared to catalyse the oxidative catabolism of 5-CHO-THF to *p*ABG and a pterin derivative (Suh *et al.,* 2000). The purified protein was identified as ferritin, the major iron-storage protein in the body. Ferritins are 24mers that form a hollow protein shell and can store up to 4500 atoms of Fe^{+3} (Harrison and Arosio, 1996). In vertebrates, ferritin is primarily localized to the cytoplasm and is comprised of varying proportions of heavy chain ferritin (HCF) and light-chain ferritin (LCF) subunits (Harrison and Arosio, 1996). Both cell type and iron status determines the relative proportions of HCF and LCF. Ferritin-mediated folate catabolism exhibits less than a single turnover per ferritin monomeric subunit and therefore is not a catalytic event *in vivo* (Suh *et al.,* 2000). The K_m of 5-CHO-THF for ferritin is in the millimolar range however, the affinity for other forms of folates was not determined in the study (Suh *et al.,* 2000). If the more oxidatively labile folates are the best substrates for ferritin-mediated catabolism, their accumulation may result in low cellular folate concentrations. Previous attempts to purify an enzyme from mammalian tissue that catalysed the oxidative cleavage of folates to *p*ABG had not been successful. Commercial rat ferritin did not display catabolic activity, but activity could be acquired with iron loading. The ferritin-catalysed reaction displayed burst kinetics, and the enzyme catalysed only a single turnover *in vitro.* Increased expression of rat heavy chain ferritin cDNA resulted in increased rates of folate turnover and reduced intracellular folate concentrations in Chinese hamster ovary cells and human mammary carcinoma cells (Suh *et al.,* 2000). Folates were shown to be less susceptible to ferritin-mediated catabolism with increased residency time in the cell, indicating that ferritin preferentially scavenges non-protein-bound folates that lack glutamate polypeptides (Suh *et al.,* 2000). These results indicated that ferritin catalyses folate turnover *in vitro* and *in vivo* and may be

an important novel pathway in regulating intracellular folate concentrations (Suh *et al.*, 2000). Further work is required before this hypothesis can be accepted or rejected.

1.10.1 Folate catabolism and Methenyltetrahydrofolate synthetase (MTHFS) 5-CHO-THF is thought to be a chemically stable form of folate (Kruschwitz *et al.*, 1994). It is not known to accumulate in the mammalian cells because of the enzymatic activity of methenyltetrahydrofolate synthetase (MTHFS) (Holmes and Appling, 2002). MTHFS catalyses 5-CHO-THF to 5,10-CH^{+}-THF in the cytoplasm and depletes 5-CHO-THF levels in the cells (Girgis *et al.*, 1997). MTHFS is expressed in many organisms ranging from bacteria to humans, which suggests that it plays an important role in one-carbon metabolism (Holmes and Appling, 2002; Roje *et al.*, 2002). Girgis *et al.*, (1997) isolated human MTHFS cDNA and expressed it in MCF-7 and SH-SY5Y cells. The transfected cells displayed increased MTHFS activity (Girgis *et al.*, 1997). Anguera *et al.*, (2003) determined the effect the increased MTHFS activity had on intracellular folate concentrations and folate turnover in these transfected cells when compared with normal human MCF-7 mammary adenocarcinoma cells and human neuroblastoma SH-SYSY cells. Overexpressing MTHFS activity resulted in; 1) increased rates of folate turnover 2) elevated levels of *p*ABG in the culture media 3) reduced concentrations of cellular folate independent of the folic acid concentration of the media and 4) increased average polyglutamate chain lengths of folate co-factors (Anguera *et al.*, 2003). MTHFS activity appears to regulate intracellular folate concentrations by accelerating rates of catabolism. Newly imported folate, which is in the monoglutamate form and therefore not protein-bound, is vulnerable to turnover resulting from increased

MTHFS activity (Anguera *et al.,* 2003). Furthermore, the correlation between MTHFS message levels and rates of folate catabolism in tissues indicates that MTHFS may play a key role in determining cellular folate concentrations in a tissue specific manner (Anguera *et al.,* 2003).

There are two potential mechanisms whereby MTHFS accelerates folate catabolism. First, MTHFS may have a second catalytic activity and directly catalyses the oxidation of reduced folate co-factors. MTHFS has been shown to have high affinity for several folate derivatives including 5-CHO-THF. 5-CH_3-THF, THF and DHF (Bertrand *et al.,* 1987), and therefore these folate derivatives may be potential candidate substrates for MTHFS-mediated catabolism. In view of the existence of several multifunctional enzymes of folate metabolism, the possibility that MTHFS catalyses an oxidative reaction in addition to the synthesis of 5,10-CH^+-THF is worth consideration (Anguera *et al.,* 2003). Alternatively, MTHFS may accelerate folate catabolism by altering the distribution of folate co-factors, resulting in the accumulation of more labile folate co-factors (Anguera *et al.,* 2003). Therefore, MTHFS-mediated catabolism may modulate tissue or cellular folate concentrations independent of whole-body folate status, and thereby regulate folate metabolic pathways.

1.10.2 Evidence of increased folate catabolism rates associated with pregnancy

Increased folate is required in pregnancy due to the growth of the fetus, placenta and maternal tissue. It is also required for uterine enlargement and expansion of blood

volume, which necessitates increased red cell production (McNulty *et al.,* 1993). Newborns have significantly higher plasma and red blood cell folate levels compared with maternal values so the increased folate need is not at the expense of the fetus (Caudill *et al.,* 1998). Inadequate folate status in pregnancy is common and is associated with increased incidence of maternal megaloblastic anaemia, lower birth weight babies and pre-term delivery. In pregnant women, the need for folate exceeds that of the calculated total and placental folate content at term, indicating that increased folate turnover may occur during pregnancy (McPartlin *et al.,* 1993). McPartlin *et al.,* (1993), showed increased urinary A*p*ABG during pregnancy, following an initial pilot study of six healthy nonpregnant women and six age-matched pregnant women. The results showed that urinary *p*ABG and A*p*ABG increased during the second and third trimesters in pregnant women compared with controls (McPartlin *et al.,* 1993). A larger study of 25 non-pregnant and 25 pregnant women were examined and the results supported the previous study results (Higgins *et al.,* 2000). The maximum rate of folate catabolism occurred during the third trimester. From this study it was estimated 200-300μg/day of folic acid is required to offset losses from catabolism (Higgins at al, 2000). In contrast Caudill *et al.,* (1998) reported no increase in catabolism in the second trimester for pregnant women in week 14-16 of gestation. Excretion of A*p*ABG at twenty-six weeks of gestation was significantly lower than in non-pregnant controls when women had been given 450μg of folate per day. No reduction was reported when women were supplemented with 850μg per day. (Caudil *et al.,* 1998). These disparate results between the two studies were attributed to the different analytical procedures used to measure A*p*ABG and various aspects of the patient treatment protocol, including duration of controlled dietary intake (Suh *et al.,* 2001).

Studies of rat models have demonstrated not only an increased need for folate co-factors and increased one-carbon metabolism during pregnancy but also elevated rates of folate catabolism. In rat models the urinary A*p*ABG production is increased in pregnant dams relative to nonpregnant dams when free-fed or pair-fed on a purified folate free diet (McNulty *et al.,* 1993). Some of the increase was accounted for by weight gain and therefore was independent of pregnancy, but there was also an authentic effect of pregnancy on folate turnover. Between day 2 and day 18 of gestation urinary A*p*ABG excretion increased up to 3 times that of nonpregnant rats but fell off significantly despite increased weight gain until day 21. Elevated A*p*ABG excretion may represent the increasing needs of folate for DNA synthesis by fetal and placental tissues, whereas the decrease in excretion corresponds to reduced needs as the placental tissue switches to hypertrophic growth. Elevated rates of folate catabolism continued during the growth of weanling rats (McNulty *et al.,* 1995). This suggests the rate of folate catabolism is related to folate utilisation in cell division, presumably resulting from increased generation of oxidatively labile DHF synthesis (McNulty *et al.,* 1995).

Pregnancy is associated with elevated ferritin expression (Suh *et al.,* 2001). Heavy chain ferritin (HCF) message levels were found to be markedly elevated during the implantation stage of pregnancy in rats (Zhu *et al.,* 1995), compared with minimal levels of HCF mRNA normally found in the uteri of non-pregnant rats. HCF mRNA is increased 8-10 fold in pregnant rats (Zhu *et al.,* 1995). Fetal iron requirements are greatest in the third trimester of pregnancy. Maternal serum ferritin levels declines

with gestation as a result of haemodilution occurring during early pregnancy and as a result of heavy demands for iron (Murphy and Scott, 1979). Maternal serum ferritin concentrations are lowest during the third trimester of pregnancy, with mean levels approximately those of iron-deficiency anemia, and they decline despite the use of supplements (Murphy and Scott, 1979). Failure of serum ferritin levels to decline in the third trimester is associated with iron-deficiency anaemia, anaemia, and lower levels of serum and RBC folate during pregnancy. High maternal serum ferritin is also a marker for maternal infection (Tamura *et al.,* 1996). Furthermore, in the third trimester, both high ferritin levels and low red blood cell (RBC) folate are associated with pre-term and very pre-term delivery. High ferritin levels in the second trimester are also a predictor for pre-term birth (Goldenberg and Tamura, 1996). Suh *et al.,* (2001) suggest that it may be possible that low maternal ferritin levels during periods of high fetal iron requirements may occur in order to spare folate by decreasing catabolism (Suh *et al.,* 2001).

1.10.3 Evidence of increased folate catabolism rates associated with cancer

Rapidly proliferating cancer cells have accelerated DNA synthesis and therefore an increased requirement for folate (Kim, 1999). Kelly *et al.,* (1983) demonstrated that mice with ascitic tumors displayed a 50% increase in folate catabolism, as measured by the presence of urinary catabolites [^{3}H]*p*ABG and [^{3}H] a*p*ABG (Kelly *et al.,* 1983). Cancer patients with active, untreated, or metastic malignancies exhibit folate deficiency without any evidence of malnourishment, malabsorption, or increased intact folate excretion (Gruner and Weitman, 1999). Patients suffering with colorectal adenoma exhibit localised tissue deficiency in the absence of low serum or RBC folate levels (Kim *et al.,*1998). The folate content of neoplastic cells is significantly

lower than the adjacent normal cells, and folate levels in normal cells from patients and controls are not significantly different (Meenan *et al.,* 1997). Tumour cells have been shown to have increased rates of folate catabolism (Kelly *et al.,* 1983). Increased folate catabolism in cancerous tissue may be at least partly due to altered ferritin levels. Increased generation of intracellular ferritin has been detected in leukaemic cells, kidney cancer and mammary carcinomas relative to normal tissue (Suh *et al.,* 2001). The levels may be influenced by the expression of the oncogene c-*myc.* Both HCF and c-*myc* were identified as mRNAs that are overexpressed in chemically induced hepatocellular carcinoma in rats. In the same model, the expression of HCF increased ten fold as the tumour progressed (Wu *et al.,* 1996), although, there have been no studies relating ferritin levels and folate concentrations in cancer cells.

1.11 Antifolates

The critical function of DHFR in restoring THF concentrations following thymidylate biosynthesis established the basis for antifolate drug therapy not only in infection but also in cancer, inflammatory and autoimmune conditions (Brumfitt *et al.,* 1980). The first folate antagonists to be developed were the large molecule analogues of folic acid such as methotrexate (MTX) and aminopterin. They are potent inhibitors of all types of DHFR. Aminopterin was used chemotherapeutically as early as 1948 when temporary remissions were produced in cases of leukaemia (Pochedly *et al.,* 1984), although, these folic acid antagonists showed little selective toxicity as they were equally active against the DHFR of both host and parasite. Small molecule inhibitors of DHFR are more selective. One such molecule is trimethoprim (TMP), a member of

the 2,4-diaminopyrimidine family. It is a folic acid antagonist, active against bacteria and protozoans.

It had been postulated that the diaminopyrimidines were inhibitors of DHFR (Burchall and Hitchings, 1965) and the elucidation of the primary and secondary structure of DHFR was further proof that this was in fact the case. Their selectivity had been attributed to the fact that they are structural analogs of only part of the folic acid molecule and not all of it, as is the case with methotrexate and aminopterins (Brumfitt *et al.,* 1980). TMP is a selective inhibitor of bacterial DHFR with no notable toxicity against mammalian forms of the enzyme (Brumfitt *et al.,* 1980). Quinlivan *et al.,* (2000) proposed that the commonly observed depletion of the folate pool upon anti-folate treatment could be explained by catabolism of folate. They showed that although DHF accumulated acutely upon inhibition of DHFR with TMP, it diminished to a very low level with time as the concentration of *P*ABG increased. Quinlivan *et al.,* (2000) also suggested that the parallel effects of antifolates *i.e.* disruption of the cell redox state and the decrease in intracellular pH contributed to spontaneous cleavage of labile folates such as DHF. The relative degree of catabolism or oxidation to folic acid might vary depending on such factors as species of bacteria, the redox state of the cell or on the therapeutic regime of antifolates however, further investigation is required to prove or disprove this point.

1.11.1 The mechanism of action of MTX

MTX is a folic acid analogue anti-metabolic drug. It was developed in the 1940's and along with its close analogue aminopterin were the first drugs to induce remission in acute leukaemia (Chu *et al.,* 1996) (review). It has since been used in low doses to treat two chronic inflammatory disorders; rheumatoid arthritis (RA) and psoriasis. Due to its efficacy in treating inflammatory disorders in the 1980's it was used later to treat inflammatory bowel disease.

The principle mechanism of action of MTX to which its cytotoxic activity has been ascribed is its inhibition of DHFR (Jolivet *et al.,* 1993). This resulted in the reduction of THF production, leading to the accumulation of DHF polyglutamates (DHFPG) and oxidized folates. Current evidence suggests that in RA, MTX does not simply act as a cytotoxic agent, but has other mechanisms of action that ameliorate the inflammatory response. A brief summary of the mechanism of action of MTX in treating RA is discussed below with a more detailed explanation in section 1.11.3 MTX is a substrate for the enzyme folypolyglutamate syntethase (FPGS) which enable glutamic acid residues to be added. Polyglutamated MTX (MTXPG) are retained longer in the cells than the parent drug and exhibit extended biological activity (Chabner *et al.,* 1985). Parent MTX, MTXPG and another major metabolite, 7-hydroxymethotrexate are all folic acid analogues, which confer inhibitory effects on many of the enzymes in the metabolic pathway (Matherly *et al.,* 1986; Skibula *et al.,* 1999). These enzymes include thymidylate synthetase (TS), 10formyl tetrahydrofolate synethase, cyclohydrolase, dehydrogenase, AICAR transformylase (Baggott *et al.,* 1986). Dihydrofolate polyglutamates are also potent inhibitors of folate dependent enzymes, including TS, AICAR transformylase and methionine synthase (MS).

As previously discussed in section 1.6, AICAR transformylase is a bifunctional protein which also contains the activity for IMP cyclohydrolase in a single polypeptide chain. These two enzymes catalyse the last two steps in *de novo* purine ring biosynthesis (Rayl *et al.,* 1996). Both MTXPG and DHFPG act as inhibitors of AICAR transformylase (Allegra *et al.,* 1985). Inhibition of AICAR transformylase leads to the accumulation of AICAR and it had been shown that patients with malignancies treated with high dose MTX excreted elevated urinary levels of AICAR (Luhby and Cooperman, 1962). AICAR like MTXPG and DHFPG leads to the accumulation of adenosine partly due to the inhibition of adenosine deaminase (ADA) (Baggott *et al.,* 1986). The anti-inflammatory activity of adenosine is well recognised (Cronstein *et al*, 1994), but a mechanism to explain its elevated secretion from the cells following MTX treatment has not been fully elucidated. Another consequence of inhibiting AICAR transformylase is the inhibition of *de novo* purine biosynthesis. This mechanism of action was demonstrated in both normal T cells and a T cell line (Fairbanks *et al.,* 1995; Budzik *et al.,* 2000). Activated T cells were used because they depend solely on *de novo* purine and pyrimidine biosynthesis as compared to salvage pathways (Fairbanks *et al.,* 1995), whereas most cells are capable of a combination of both the *de novo* and salvage pathways to provide the required nucleotide precursors for RNA and DNA synthesis (Yamaoka *et al.,* 1997).

Numerous mechanisms of actions have been investigated to attempt to explain the effectiveness of MTX in RA. It had been suggested that the inhibition of DHFR would lead to downstream inhibition of transmethylation reactions, thereby yielding immunosuppression (Nesher and Moore, 1990). It has also been demonstrated that MTX can suppress the production of several pro-inflammatory cytokines including

TNF-α (Smith-Oliver *et al.,* 1993), IL-1 (Connolly *et al.,* 1988), IL-6 (Barrera, 1995) and IL-10 (Seitz *et al.,* 1992). However, none of these mechanisms can explain fully the pharmacological activity of MTX *in vivo.* In summary the net effects of MTX include inhibition of folate dependent reactions involving the transfer of one-carbon fragments such as the production of thymidylate and purines, inhibition of production of methionine and local accumulation of adenosine.

Several lines of evidence suggest that the cellular action of low-dose MTX, which underlies its anti-inflammatory/immune modifier effect, may not be related to the inhibition of DHFR. Firstly, overt inhibition of DHFR is not required for efficacy but rather is a sign of toxicity in low-dose MTX. Co-administration with folinic acid which by-passes the blockage of DHF ameliorates many of the side effects of MTX but if given in excess can retard the drugs efficacy (Shiroky *et al.,* 1993; Weinblatt *et al.,* 1993). However, folic acid given in a high dose has been reported to reduce the side effects of the drug without reducing its efficacy (Morgan *et al.,* 1994; Duhra, 1993). Lastly trimetrexate, which is an unpolyglutamated analogue of folic acid and which inhibits DHFR strongly but is inactive against other folate enzymes, is ineffective in a rat model of chronic inflammation. These results suggest that inhibition of folate dependent enzymes distal to DHFR in the metabolic pathway of folic acid by MTX and its metabolites may be the events responsible for this drugs anti-inflammatory activity.

1.11.2 Results of high dose MTX treatment

MTX causes folate deficiency and the folate nutrition declines precipitously resulting in a significant increase in plasma homocysteine levels. Clinical studies have shown

that plasma and urinary levels of hcy respond to MTX therapy in cancer and psoriasis patients (Refsum *et al.,* 1986), indicating that hcy in extracellular fluids may be an indicator of an antifolate effect. Clinical data indicate that the burden of proliferating cells, cytotoxicity and the folate status are also determinants of extracellular hcy. Intracellular hcy is either converted to cysteine or is salvaged to methionine through remethylation. Remethylation is catalysed by betaine- hcy transmethylase in liver and methionine synthase, which is widely distributed in mammalian cells. An alternate fate of hcy is export into the extracellular medium. This process occurs at a rate reflecting the balance between hcy production and utilisation (Refsum *et al.,* 1991). Extracellular hcy may be an indicator of the function of enzymic processes or availability of co-factors or substrates involved in hcy metabolism (Ueland *et al.,* 1986). The effect MTX treatment has been studied both *in vitro* and *in vivo*. It appears to enhance the hcy egress from confluent, quiescent fibroblasts in culture (Ueland *et al.,* 1986) and also increases the plasma hcy in patients treated with a wide range of doses (Refsum *et al.,* 1991).

The primary mechanism of MTX relies upon interfering with the activation of folic acid and the degree of folate depletion during MTX therapy depends primarily upon the weekly administration dose. As a chemotherapeutic agent doses of MTX are high (up to 30μg/m^2), interference with folic acid is intentional and so individuals prescribed this drug for cancer treatment should limit their supplementation of folic acid to a maximum of 400μg per day. The hcy level in patients with acute lymphoblastic leukaemia or solid tumors exposed to MTX was reduced upon administration of 5-CHO-THF (Refsum *et al.,* 1986, Van Ede *et al.,* 2001). Related observations have been made in folate-deficient patients with elevated plasma hcy.

Increased levels of FA reduces plasma hcy concentrations which indicates plasma Hcy responds rapidly to changes in intracellular folate status. Moreover, the effect on plasma hcy from low doses of MTX in psoriasis patients suggest that hcy is a particularly sensitive indicator of an antifolate effect (Refsum, 1991). Hcy export from cultured cells increases during proliferation and during MTX exposure and reduces upon exposure to 5-CHO-THF. MTX-dependent enhancement of hcy export was not related to cytotoxicity or inhibition of cell growth, but probably reflects cellular depletion of 5-CH_3-THF. An investigation of time course and concentration dependence suggests that increased hcy export is an early and sensitive measurement of the antifolate effect of MTX, but the increased rate was antagonised and the export decreased when cytotoxicity and inhibition of cell growth ensued (Refsum, 1991).

1.11.3 Results of low dose treatment of MTX

MTX in low doses has been found to be a useful in the treatment of a wide variety of nonmalignant disorders, including psoriasis (Hanno *et al.,* 1980), polymyositis (Arnett *et al.,* 1973), dermatomyositis and RA (Morgan *et al.,* 1987). In autoimmune diseases MTX is effective in a low dose 7-15mg/week (Herman *et al.,* 2003). Early diagnosis and treatment is critical for rheumatoid arthritis (RA) patients. Moreover, an early plateau of clinical response to MTX is reached only after six months of treatment therefore it is very important to get treatment early. Over the past decade many RA patients failed to respond to MTX drug treatment therefore it is necessary to predict the immunosuppressive effects of MTX as early as possible (Herman *et al.,* 2003).

Lymphocyte activation and cell apoptosis can be monitored and measured by fluorescence polarisation (FP) of fluorescein diacetate labelled cells using an

Individual Cell Scanner (IC-S) (Zurgil *et al.,* 2000; Herman *et al.,* 2003). Using this technique the effects of MTX on activated lymphocyte cells was investigated and the most suitable suppression effect of MTX on these activated lymphocytes was identified (Herman *et al.,* 2003). The IC-S was used to monitor the development of the apoptotic process, following MTX administration, in an effort to establish an early event that demonstrates the MTX suppression effect. This model may help RA patients to receive appropriate treatment as well as to predict dosage and timing for better disease therapy (Herman *et al.,* 2003). Future work using this method includes focusing on utilising and adjusting this model for other disease modifying anti-rheumatic drug (DMARD) options to improve treatment and provide optimal care for these patients. The data from Herman *et al.,* (2003) demonstrated that MTX inhibits cell proliferation and induces effective cell death of highly activated lymphocytes. The progressive increase in apoptotic cells after the prolonged incubation period (72hr) was noticeable mostly in MTX administration ranging from 0.1 to 1μM MTX. This model provides evidence for the immunosuppressive activity of low-dose MTX.

Evidence suggests that the cell subpopulation T-helper 1 (Th1) plays a predominant role in RA pathogenesis. Singh *et al.,* (1999) demonstrated that lymphocytes obtained from RA patients produce Th1 type cytokines. The cytokines produced by Th1 cells are thought to be of critical importance in disease development or subsiding. The decrease in the TNF-α production and the increase in IL-10 production demonstrated by Rudwaleit *et al.,* (2000) suggests that MTX is involved in a cellular regulatory mechanism which is relevant to the disease remission. Schuerwegh *et al.,* (2001) evaluated the influence of long-term MTX treatment on the general normalisation of the cytokine balance (Th1/Th2) in both $CD4^+$ and $CD8^+$ lymphocytes (Schuerwegh *et*

al., 2001). Expanded $CD4^+$ clones were present in the blood and infiltrate into the joints of RA patients, persisting for years (Waase *et al.,* 1996).

1.11.4 MTX rescue agents

Initially researchers assumed that MTX effects on folic acid were the source of its presumed benefits in cases of RA, as in chemotherapeutic uses. However practice shifted to support supplementation with folates to counter the adverse effects of MTX in the cases for several reasons.

The administration of folinic acid after high-dose MTX, usually 24h is used to prevent otherwise lethal effects of the drug (Gorlick *et al.,* 1999). These protocols have been developed to promote penetration of MTX into pharmacological sanctuary sites and drug resistant tumours and to selectively rescue proliferating tissues from the toxic effects of the drug folinic acid is commonly used later in patients on standard dose therapy who experience unexpected or protracted toxic response. A number of mechanisms, probably acting in concert, have been suggested to explain the folinic acid rescue of MTX cytotoxicity at the cellular level (Matherly and Angeles, 1994). These include: (a) circumvention of DHFR inhibition by replenishment of the folate pool; (b) displacement, by DHF polyglutamates, of MTX from DHFR; and (c) in the continued presence of extracellular MTX, prevention of further drug uptake and polyglutamation. However it should be emphasised that although folinic acid has a place in the rescue of toxicity, its use should not be advocated routinely because it is likely to induce similar effects in tumours (Farrugia *et al.,* 2000). The British Medical

Association are introducing practical measures to reduce the risk of errors in oral MTX in response to deaths and serious events in patients taking the MTX drug incorrectly. A range of factors had contributed to these adverse events including patients not given sufficient information on how often to take the drug and variations in patient monitoring .

1.11.5 MTX and genetic variability in MTHFR and MTHFD1 enzyme

In addition to MTX inhibiting DHFR, resulting in reduced availability of reduced folates, MTX may also inhibit the conversion of 5,10-CH_2-THF to 5-CH_3-THF (Goldman and Matherly, 1985). Homozygosity of the MTHFR C677T mutation gene also results in reduced activity of the MTHFR enzyme. The resulting decrease in the level of 5-CH_3-THF causes impeded remethylation of hcy to methionine and consequently, hyperhomocysteinaemia (Frosst *et al.,* 1995).

Many adverse effects of hyperhomocysteinemia have been reported, especially increased cardiovascular risks (Boushey *et al.,* 1995) and NTD Czeizel and Dudas, 1992). Van Ede at al (2002), investigated the effects of MTX treatment in RA patients on folate and homocysteine metabolism, the role of folinic acid and folic acid supplementation and the influence of the MTHFR C677T mutation. They also investigated the relationship of homocysteine metabolism with the efficacy and toxicity of MTX treatment (Van Ede *et al.,* 2002). They reported low-dose MTX treatment in RA patients leads to an increased plasma homocysteine level. Concomitant folate supplementation with either folinic acid or folic acid caused a decrease in the level of plasma homocysteine. Theoretically, the presence of C677T mutation in the MTHFR gene would lead to additional or synergistic alterations in

folate and homocysteine metabolism when MTX is administered. However, no relationship was found between the presence or absence of the MTHFR C677T mutation and the homocysteine level in patients (van Ede *et al.,* 2002). The number of patients with the TT genotype may have been to small for such an interaction to be observed in this study. Furthermore, homocysteine metabolism was reported not to be associated with efficacy or toxicity of MTX treatment (van Ede *et al.,* 2002).

In contrast a study carried out by Ulrich *et al.,* which investigated the extent to which the MTHFR 677C→T mutation modified the response to MTX in patients undergoing bone marrow transplantation showed a relationship between the MTHFR genotype and MTX (Ulrich *et al.,* 2001). MTX is administered to patients undergoing marrow transplantation to prevent graft-versus-host disease. Ulrich *et al.,* hypothesised that MTX sensitivity would vary with genetic variability in folate-metabolising enzymes. They reported patients who were MTHFR TT experiencing higher toxicity which was evidenced by increased oral mucositis and a trend toward delayed platelet recovery. A low folate status (dietary or MTX induced) may further decrease availability of folate for nucleotide synthesis (Ulrich *et al.,* 1999).

Taniguchi *et al.,* (2002) also investigated the link between the MTHFR genotype and the efficacy or toxicity of MTX in the treatment of RA. Two common polymorphisms were investigated MTHFR C677T mutation and the A1298C mutation. Examining each locus separately showed that patients with 1298C were receiving significantly lower doses of MTX compared to patients without, while a higher rate of overall MTX toxicity was observed in patients with 677T than those without. Patients with the 677T-1298C haplotype were receiving lower doses of

MTX than those without, while subjects 677T-1298A had a higher frequency of side effects from MTX. These results suggest that the polymorphisms within the MTHFR gene are associated with both efficacy and toxicity of MTX in RA patients (Taniguchi *et al.,* 2002). Further work is required to prove such an association.

Since MTHFR and MTHFD1 are key players in folate metabolism, the differences in their activity due to their respective gene variants, could not only modify susceptibility to different diseases (Schwahn and Rozen, 2001) but might modulate therapeutic response to antifolate chemotherapeutic agents. Krajinovic *et al.,* (2004) investigated the association of MTHFR and MTHFD1 polymorphisms and MTX sensitivity in the outcome of childhood acute lymphoblastic leukemia (ALL). They did not observe any association between MTHFR or MTHFD1 polymorphisms and MTX toxicity in childhood ALL patients but further studies are needed to confirm this result as small numbers were used in this study (Krajinovic *et al.,* 2004). The association of both genes polymorphisms appear more obvious when combined with other variants within the same pathway. As discussed MTX-induced depletion of folate co-factors such as 10-CHO-THF and 5,10-CH_2-THF which contribute to the inhibition of folate-dependent enzymes of *de novo* purine and pyrimidine syynthesis (Jolivet *et al.,* 1982) along with MTXPG which directly inhibits folate-dependent enzymes (Allegra *et al.,* 1985). TS requires 5,10-CH_2-THF for its activity and therefore it is possible that increased levels of this folate co-factor, produced by altered MTHFR and/or MTHFD1 action might facilitate thymidine synthesis therefore interfering with MTX action. This would be particularly noticeable in ALL patients homozygous TS 3R (triple repeat 3R at the 5'UTR TS polymorphism) who have increased TS levels (Horie *et al.,* 1995; Krajinovic *et al.,* 2002). A study was

undertaken to assess the association of MTHFR and MTHFD1 polymorphisms in the context of TS 3R homozygosity (Krajinovic *et al.,* 2004). It was suggested that lower levels of TS and 5,10-CH_2-THF would facilitate MTX action while in contrast, high levels would mediate MTX resistance. The latter would be expected for homozygous TS 3R individuals with the MTHFR 677T/1298A haplotype or possibly with the MTHFD1 R653Q variant, thus explaining the highly significant reduction in the relapse rate for individuals with those genotype combinations. Higher 5,10-CH_2-THF and TS levels associated with these variants were suggested to limit DNA damage by reducing uracil incorporation into DNA, thus explaining the protective role of these variants against leukaemia (Skibola *et al.,* 1999). On the other hand, this might favour the proliferation of residual malignant clone. In order to understand fully the role of gene polymorphisms underlying the enzymatic variants in the folate pathway, and their gene-gene interactions in ALL outcomes, it will be necessary to carry out studies with larger numbers than the present study.

1.11.6 MTX resistance

MTX plays an important role in the treatment of a variety of human malignancy and inflammatory disorders as previously discussed. However, the emergence of MTX drug resistance has limited its efficacy (Gorlick *et al.,* 1996). Numerous studies investigating intrinsic and acquired resistance to MTX using *in vitro* and animal model systems have been carried out (Schweitzer *et al.,* 1990: Allegra and Boarman, 1990; Dicker *et al.,* 1993; Mauritz *et al.,* 2002). The four main causes of MTX resistance are thought to be; 1) qualitative and quantitative alterations in DHFR activity (Carman *et al.,* 1984; Dicker *et al.,* 1993) 2) impaired membrane transport of MTX into the cell via the RFC or increased efflux via members of the multidrug

resistance protein family (Hooijberg *et al.,* 1999) 3) decreased polyglutamylation either due to decreased activity of FPGS (McCloskey *et al.,* 1991) or decreased activity of folypolyglutamate hydrolase (FPGH) (Rots *et al.,* 1999) and 4) more recently MTX resistance was associated with the loss of functional retinoblastoma protein which leads to increased levels of E2-F, which is involved in the transcriptional regulation of DHFR and TS genes (Li *et al.,* 1995).

1.12 Folate Analysis

1.12.1 Separation and analysis of folate cofactors

The main problems associated with developing a method a method to identify and quantitate folate co-factors is the relative instability of reduced folates and their interconversion during extraction and analysis. High sensitivity is required in analytical methods because of low concentrations of the folates in biological materials. Conventional assays by microbiological methods provide high sensitivity but are slow and do not readily differentiate between folate co-factors (Molloy *et al.,* 1997). Competitive binding radiometric methods exhibit sensitivity comparable to that of the microbiological assay, although differences in responses of various folates represent major limitations for many analyses (Shane *et al,* 1980). The development of HPLC systems enabled the folate co-factors to be separated and identified individually and in some instances quantified. A brief summary of these methods is discussed below.

The basic principle behind the microbiological assay is that naturally occurring folates can promote the growth of three bacterial strains *L.casei*, *S. faecalis*, *P. cerevisiae*

(Blakley and Benkovic, 1984). *L.casei* responds to the majority of folate derivatives including those with up to three glutamate moieties (Tamura *et al.,* 1972). *S.faecalis* does not respond to 5-CH_3-THF or to folates with polyglutamate chain length of two or more. *P. cerevisiae* is effective in assays of THF derivatives but does not respond to 5-CH_3-THF (Blakley and Benkovic, 1984). Originally the microbiological assay was considered time-consuming and difficult to maintain but modifications made to the method throughout the years have meant the microbiological folate assay has become a reliable method, easily maintained and considerably less costly than the radioassay (Molloy *et al.,* 1997). The modifications included the development of a chloramphenicol resistant strain of *L.casei* (NCIB 10463), which saved time by avoiding aseptic precautions (O'Broin and Kelleher, 1992). Cryopreserving the inoculum of *L.casei* in multiple individual vials resulted in standardised growth curves that are reproducible for hundreds of assays (Molloy and Scott, 1997). The microbiological assay remains one of the most popular methods for the measurement of total folates.

The principle behind the radioassay of folates is that labelled and unlabeled folates (ligands) compete for a limited number of binding sites on a folate-binding protein. There are two types of radioassays competitive and non-competitive ligand binding. In the competitive system, the amount of binding is such that only about 50% of the folates added (labelled and unlabeled) can be bound. The binding of the labelled folate is monitored and is reduced by increases in the concentration of unlabeled folates due to competition for binding sites on the binding protein (Blakley and Benkovic, 1984). The competitive binding radiometric methods exhibit sensitivity comparable to that of the microbiological assay, although differences in responses of

various folates represent major limitations for many analyses (Shane *et al*, 1980). In the non-competitive ligand-binding radioassay, unlabeled folates are incubated with the binder protein, and then labelled folate is added to titrate the unoccupied sites on the binding protein (Blakley and Benkovic, 1984).

High performance liquid chromatography (HPLC) is a fast, efficient and sensitive method used to separate a wide variety of molecules, from ionised to non-ionised and from high molecular weight proteins to very small compounds. The most common HPLC method used is reverse phase chromatography, which uses a non-polar stationary phase with a polar mobile phase. The principle of the method is that the less polar (*i.e.* less water-soluble) a sample is the better it is retained by the column. The main advantage of reverse phase chromatography is that it is easy to use and gives reproducible retention times. The method chosen to separate the folate co-factors in this study is an ion-pair reverse phase chromatography method developed by Quinlivan *et al.,* (2000). This method involves the addition of an ion-pair reagent to the mobile phase, this forms an ion pair with sample components of the opposite charge. The ion-pair reagent used to separate folate co-factors is tetrabutylammonium phosphate or PicA, which binds to the hydrocarbon side chain on the column and forms a complex with a charged sample component due to electrostatic interaction. The ion-pairing agent is normally a rather bulky molecule, and the ion-ion bond formed will be more or less shielded by the rest of the molecule. The complex of the ion pair will be considerably more hydrophobic than the separate parts. The pI of folates is between 3-4 therefore at the pH of the mobile phase (pH 6.0) all the folate co-factors with the exception of 5,10-CH^+-THF are anions (negatively charged). As a result 5,10-CH^+-THF will elute in the void volume. The column will then retain the

negatively charged folates with varying degrees depending on the strength of their charge. Figure 1.6 shows a diagrammatic representation of a HPLC unit.

1.12.2 A brief summary of method developed to analyse folates

McMartin *et al.,* (1981) developed a coupled HPLC and microbiological assay to separate and determine folate co-factors in rat liver. Liver folates were extracted by boiling in 1.5M 2-mercaptoethanol and enzymatically hydrolysed at pH 6.0 using hog kidney polyglutamate hydrolase. The folate co-factors were separated using ion-pair reverse phase HPLC and the folate co-factors were collected as fractions and a microbiological assay was then performed on these fractions. During the extraction process they observed that 50% of 10-CHO-THF was converted to 5-CHO-THF. These results showed that there was equal amounts of 10-CHO-THF and 5-CHO-THF so they concluded from these measurements that no 5-CHO-THF was present in the liver. This process although time consuming appeared to be very sensitive to folate levels. They verified their results by using [2-^{14}C] folic acid, which was injected into the rats 24hrs before their livers were removed.

Eto and Krumineck (1982) developed a method which relied on the differential susceptibility to oxidative cleavage at the C9 and N10 bond of various folate derivatives to estimate the level of three hepatic folate pools. Phase-bonded octodecylsilane columns were developed isocratically, and the effluent was monitored at 560nm. Using this method three determinations had to be performed and strict adherence to various conditions were required which lead to the possibility of errors in assaying.

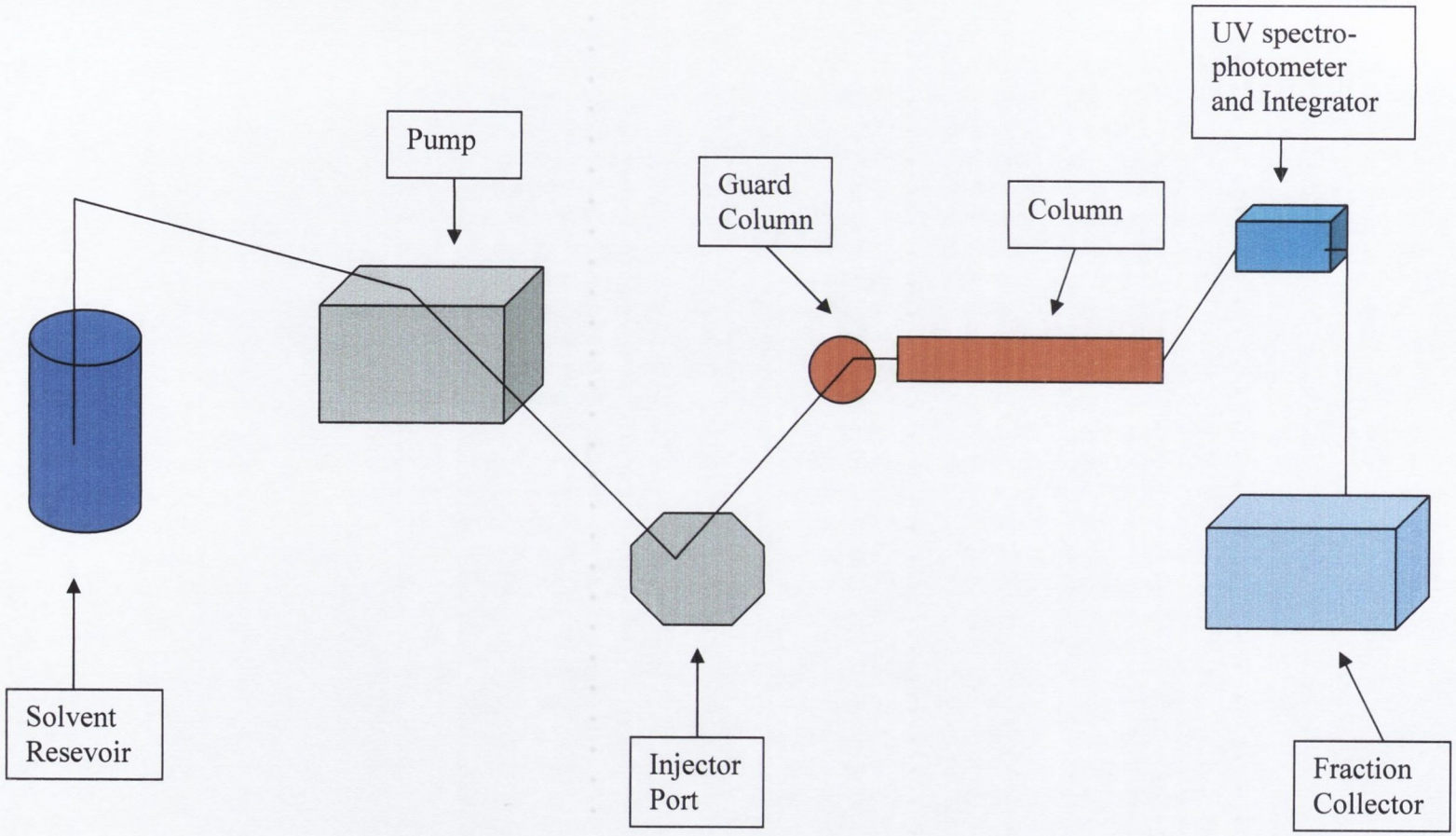


Figure 1.6 : Schematic diagram of HPLC

Duch *et al.,* (1983) developed an ion-pair reverse phase chromatography method for the analysis of folate co-factors in tissue. The sample was purified using Dowex 50 chromatography and concentrated 20-fold by overnight lyophilization before HPLC. This method was the first to allow quantitation of tissue folates following conversion of polyglutamates to monoglutamates and using HPLC in conjunction with UV detection. The problem with this method is that sample preparation is cumbersome, long and leads to degradation of the more labile folates.

Gregory *et al.,* (1984), developed a method for separating folates which consisted of a DEAE-sephadex stage to partially purify the folates and subsequent HPLC separation at pH 2.3 using a flurometric detector. The interconversion of 10-CHO-THF to 5-CHO-THF was a problem in this procedure which meant neither could be quantified directly. Two spectrophotoflourimeters were required and a postcolumn derivatisation system to determine the amount of folate present. This method was cumbersome and expensive when the equipment required was taken into account (Gregory *et al.,* 1984).

Wilson and Horne, (1984) developed a method for the separation of pteroylmonoglutamtes using a method which had been previously devised by Horne *et al.,* (1981) and had since been modified by Wilson and Horne (1983). The changes eliminated the problem of interconversion of 10-CHO-THF to 5-CHO-THF by using an extraction buffer which consisted of 2% (w/v) sodium ascorbate, 0.2M 2-mercaptoethanol, 50mm HEPES, 50mM CHES, pH 7.85. Rat plasma was used as the source of conjugase to hydrolyse the folate polyglutamates. They treated folate standards using the same conditions as the sample would be subjected to and when the

HPLC-*L.casei* assay was performed no interconversion was detected between folate cofactors. It was thought that the presence of 2-mercaptoethanol in the extraction buffer which formed a hemithioacetal with formaldehyde (which can be generated from ascorbate solutions at elevated temperatures) and this prevented the conversion of 5,10-CH_2-THF to THF. The microbiological assay was reported to have a detection limit of 0.025ng (0.053pmol) and because it was very specific for folates once they had been deconjugated unlike UV detection and fluorescence emission no prior purification was necessary before the assay was carried out. The results were verified by injecting rats with [^{3}H] folic acid and comparing the spread of radiolabelled folates following separation. Only four of the folate co-factors were identified using this method 10-CHO-THF , THF, 5-CHO-THF and 5-CH_3-THF. They detected a small amount of 5-CHO-THF present in rat liver which sheds doubt on the assumption that had been previously made by McMartin *et al.,* (1981).

Geoghegan *et al.,* (1995) developed a HPLC method combining both ion-pair reverse phase chromatography similar to Duch *et al.,* (1983) and reverse phase chromatography. Although, primarily he was investigating folate catabolism in rats it was necessary for him to identify and separate all the folate co-factors. This method was sensitive and the main advantage was that it enabled both in-tact folate co-factors and degradation products in the sample being identified. The disadvantage of this method is that two HPLC runs are required and therefore the method becomes time-consuming.

Kelly *et al.,* (1996) designed a HPLC-microbiological assay for serum folic acid. Using this reverse phase HPLC method he was able to separate folic acid from 5-CH_3-

THF in serum and then quantify it by *L.casei* microbiological assay. This enabled the identification of unmetabolised folic acid in the serum so the effect of intervention studies on a population can be monitored. This assay has major implications on food fortification programmes because of the risk of unaltered folic acid in circulation masking the effect of pernicious anaemia. As a method of identifying all the folate co-factors in tissues and cells it would fall short as only two folate co-factors identified. Subsequently Sweeney *et al.,* (2003) modified this method in order to improve the sensitivity by concentrating the fractions recovered from the HPLC system by running the fractions through a C_{18} Sep-Pak cartridges prior to the microbiological assay. Unfortunately only 2 folate cofactors are identified using this improved method namely folic acid and 5-CH_3-THF.

Belz and Nau (1998), determined folate patterns in mouse plasma, erythrocytes, and embryos by ion-pair reverse-phase HPLC coupled with a microbiological assay. They were able to detect all 8 folate monoglutamtes co-factors but some folate cofactor interconversion was reported hence, the level of these interconversion are taken into account when the final folate cofactor concentrations were calculated. The advantage of this method was that the fractions were collected directly into the mircotitre plates and then lyophilized to remove the methanol concentration in the mobile phase which can interfere with the growth of *L.casei*. This procedure meant that there was no loss of sample during HPLC analysis and fraction collecting.

Selhub *et al* (1980), developed an efficient method for the purification and concentration of naturally occurring folates as intact molecules using affinity chromatography which utilised immobilised milk folate binding proteins.

Subsequently this affinity-column chromatography method was used in combination with HPLC-diode array UV detection system for the isolation and quantification of folates in biological matter including foods and red blood cells (Bagley and Selhub, 1997 and 1998). The advantage of this method was the folate cofactor distribution could be identified and quantified intact, including oxidation states, 1C substitutions and glutamyl-chain lengths of a complex mixture of naturally occurring folates using this method. This method has since been used to identify folate co-factors in immortalised lymphocyte cell lines (Lathrop-Stern *et al.*, 2003).

Quinlivan *et al.*, (2000) developed a method for separating folate co-factors in bacteria using ion-pair reverse phase chromatography. The main differences with this method from the previously described methods (Geoghegan *et al.*, 1995: Duch *et al.*, 1983) were that a phenomenex kingsorb C18 column instead of a waters C18 column. The mobile phase not only contained picA reagent and methanol but also sodium acetate and was at pH 6.0. Following sonication and centrifugation of the bacterial cells the intracellular folates were incubated with rat serum for 45min to allow full deconjugation to occur before injection onto the column. The sample preparation was quick and very little degradation of the labile folates occurred. It had been reported that the only folate co-factor to be degraded was DHF during extraction with a loss of 13% (Quinlivan *et al.*, 2000). The bacteria were fed with [^{3}H] pABA, which became incorporated into the cell to become labelled folate co-factors. Following HPLC separation the radiolabelled folates they were collected as timed fractions and counted on a scintillation counter. The folate co-factors present in each peak was expressed as a percentage of total radiolabel injected onto the column. This method was modified and used for the folate cofactor analysis in this thesis.

Nelson *et al.,* (2001) developed a method to identify 5-CH_3-THF in human plasma using liquid chromatography-electrospray-ionization mass spectrometry. Liquid chromatography (LC) in direct combination with mass spectrometry (MS) has been shown to be a good analytical technique for the selective separation and detection of labile folate monoglutamates. In this method 5-CH_3-THF present in human plasma is extracted using a C18 solid-phase extraction cartridge and quantitatively detected using mild MS conditions. The LC-MS sensitivity of 5-CH_3-THF is excellent with a calculated detection limit of 0.39ng/ml based on isotope-dilution internal standard calibration (Nelson *et al.,* 2001). This method can baseline separate in <15min 5-CH_3-THF, 5-CHO-THF, THF, DHF, FA standards. The main advantage of this method over the HPLC methods discussed above is the speed and sensitivity of this LC-MS.

1.12.3. Folate extraction and deconjugation methods

Naturally occurring folates are present in tissues and cells of humans and plants in polyglutamated forms. In order to analyse these folates most methods require these polyglutamated folates to be deconjugated into their monoglutamated forms. Deconjugation of folates is achieved by using γ-glutamyl hydrolase (also referred to as conjugase), which is found in rat serum, rat liver, human plasma, chicken pancreas, hog kidney (Wang *et al.,* 1985). Chicken pancreas and rat serum are the most commonly used conjugase treatments. The majority of the end product following deconjugation using rat serum is monoglutamyl folates (Horne *et al.,* 1981). In HPLC complete deconjugation is required to eliminate the possibility of ghost peaks or unidentified peaks being obtained. The endogenous folates present in rat serum can be removed easily by dialysis in buffer containing acid washed charcoal (McMartin *et*

al., 1981; Duch *et al.,* 1983; McKillop *et al.,* 2002) and therefore small concentrations of folates can be analysed in samples using the microbiological assays.

Chicken pancreas deconjugates folates into mono-, di-, and tri-glutamate forms. Contrary to the general belief that *L.casei* responded equally to mono- and di-glutamates it was reported that in fact the *L.casei* response to di- and triglutamates was approximately 90% and 60% respectively when compared with the response to monoglutamates. This could lead to results reading falsely high when the *L.casei* microbiological assays is used to determine the folate content in samples. Therefore, it is essential to determine the growth response for *L.casei* for di- and tri-glutamate standards prior to sample analysis.

In 1990 a tri-enzyme treatment method was developed which, in addition to the traditional treatment with conjugase, proteases and α-amylase were also used (Eitenmiller *et al.,* 1990: Martin *et al.,* 1990). In 1997, it was reported that the trienzyme treatment was necessary to obtain proper folate content in certain foods (Tamura *et al.,* 1997). Shrestha *et al.,* (2000) compared the traditional single conjugase treatment with the tri-enzyme treatment on spinach, fortified bread and two types of breakfast cereals using the *L.casei* microbiological assay to determine the folate content recovered. They concluded that the tri-enzyme treatment was not necessarily the best method for folate extraction for all foods. In spinach the tri-enzyme (unautoclaved) and single enzyme treated samples yielded significantly higher folate values than tri-enzyme (autoclaved) treatment with chicken pancreas as

the source of conjugase. With human plasma as the source of conjugase, there was no significant difference in folate values between single and tri-enzyme (autoclaved) treated spinach samples but both were significantly lower than autoclaved tri-enzyme treated sample. In bread, the apparent folate value obtained from tri-enzyme autoclaved sample was significantly higher than single enzyme treated sample with both sources of conjugase. In breakfast cereals there was a significant decrease in folate, in all samples extracted with the tri-enzyme method in all, except the unautoclaved samples deconjugated with chicken pancreas where the apparent decrease was not significant. These results obtained by Sherestha *et al.,* (2000) concluded that it is not necessary to use the tri-enzyme method for every single food and that variations in the source of conjugase significantly affected the apparent folate content of foods; chicken pancreas yielded more folate than human plasma. Tamura *et al.,* (1998) also reported that the tri-enzyme method may not always be necessary and the choice of methods depended on the food type.

1.13 Aims of this project:

1) To confirm the work of Quinlivan *et al.,* (2000) in demonstrating an effect of the antifolate drug trimethoprim has on the folate co-factor distribution in bacterial cells could be duplicated (Quinlivan *et al.,* 2000). It would be expected that dihydrofolate would accumulate acutely upon inhibition of dihydrofolate reductase with TMP and it would diminish to a very low level with time as the concentration of *p*-aminobenzoylglutamate increased. The appearance of folic acid should also be observed which might explain the bacteriostatic effect of trimethoprim has on bacteria.

2) To extend the work of Quinlivan *et al.*, (2000) to mammalian cells in primary cultures and cell lines both adherrant and in suspension to demonstrate that the technology could be used in mammalian cell models to investigate the intracellular folate co-factor distribution in relation to the methylenetetrahydrofolate reductase C677T mutation.

3) To investigate the effect the antifolate drug methotrexate has on the folate co-factor distribution in wild type and mutant methylenetetrahydrofolate reductase C677T immortalised lymphocyte cell lines.

Chapter 2: Materials and Methods

2.1 Equipment

2.1.1 HPLC Assay

- Waters HPLC:

Pump (model number 510)	**Waters Corporation, Milford, USA**
Rheodyne Injector	**Phenomenex, Chesire, England**
UV Spectrophotmeter	
Fraction collector (frac 100)	**Pharmacia LKB**
Hamillton syringe	**Hamillton Co., Reno, Nevada**
Column Heater (model 7955)	**Jones Chromatography, UK**

- Shimazdu HPLC: **Shimazdu**

  Pump A CL-6A

  Pump B CL-6A

  UV Spectrophotometer SPD-6A

  Integrator C-R3A

- HPLC Columns:

Kingsorb 3μ C18 column, (150 x 4.6mm)	**Phenomenex, Chesire, England**
Kingsorb 3μ C8 column, (150 x 4.6mm)	**Phenomenex, Chesire, England**
Waters μbondapak C18 column (3.9 x 300mm)	**Waters Corporation, Milford, USA**

- Multiskan Plus plate-reader **Lab systems, Helsinki, Finland**

- Orbital Shaking Incubator (model G-25) **New Brunswick Scientific, New Jersey, USA**

- Sonicator 450 **Branson Ultrasonic**

- Scintillation Counter — **Tricab 1200, Packard, Berks, UK**
- Sep-Pak C18 column — **Waters Corporation, Milford, USA**

2.1.2 Cell Tissue Culture:

- Microscope (Eclipse TS100) — **Nikon**
- Laminar flow hood (Class II Microbiogical Cabinet) — **Atlas Clean Air LTD**
- Incubator (CO_2 Water-Jacketed Incubator) — **NuAire, US**
- Centrifuge (Super T21) — **Sorvall Products, Newtown, CT06470**
- Haemocytometer — **Biosciences**

2.1.3 DNA Extraction and Genotyping

- Gel Box (Horizon 11.14) — **Life Technologies GibcoBRL, Paisley, Scotland**
- Battery , Model 400L — **Life Technologies GibcoBRL, Paisley, Scotland**
- UV Transilluminator — **Life Technologies GibcoBRL, Paisley, Scotland**

- PCR Machine, PTC-100 **MJ Research Inc.**

2.1.4 Equipment for Microbiological Assay

- Spectrophotometer 35 UV/Vis **Beckman Ltd.**
- Titerek Mutliscan Plus **Flow Laboratories, Scotland**

  (Microtitre Plate Reader)
- Microtitre Plus (Flat Bottom) 96 well **Sarstedt, Wexford, Ireland**
- Triteck Plate Sealer **ICN Biomedical, Ayreshire, Scotland**

2.2 Materials

2.2.1 HPLC Assay

- PIC A Reagent (tetrabutyl-ammonium phosphate) **Waters Corporation, Milford, USA**
- Methanol: HPLC Grade **Scharlau Chemie, Barcelona, Spain**
- Di-Sodium hydrogen phosphate **Sigma-Aldrich, Steinheim, Germany**
  Citric Acid
- Folate Standards **Sigma-Aldrich, Steinheim, Germany**
  Folic acid (Pteroyl-L-glutamic acid)
  Folinic acid (N^5-Formyl-5,6,7,8-tetrahydropteroyl-L-glutamic acid) or (5-CHO-THF)
- Folate Standards **Schircks Laboratories, Jona, Switzerland**
  (6R,S)-5,10-methylene-5,6,7,8 THF magnesium salt
  (6R,S)-5-methyl-5,6-THF calcium salt
  (6R,S)- 5,10-methenyl-5,6,7,8-THF hydrochloride
  7,8-Dihydrofolic acid
  (6R,S)-5,6,7,8-tetrahydrofolic acid trihydrochloride
  (6R,S)-5-methyl-5,6,7,8 tetrahydrofolic acid calcium salt
  p-Aminobenzoyl-L-glutamic acid
  (6R,S)-5-Methyl-5-6-dihydrofolic acid, ammonium salt
  10-formylfolic acid
  Pteroyldi-γ-L-glutamic acid
  Pteroyltri-γ-L-glutamic acid
  Methotrexate

2.2.2 *E.coli* Assay

- Di-Potassium hydrogen phosphate trihydrate — **Merck, Dormstadt, Germany**
- Magnesium Sulphate — **BDH Laboratory Suppliers, England**
  Potassium dihydrogen orthophosphate
  Mercaptoethanol

- Ammonium Chloride — **Sigma-Aldrich, Steinheim, Germany**
  Sodium Chloride
  Calcium Chloride dehydrate
  Glucose (Grade H)
  Sodium Acetate trihydrate
  Sodium ascorbate

- Glacial Acetic Acid — **Labscan, Stillorgan, Dublin**
  Tetrahydrofuran
  Acetonitrile

- (2-Hydroxyethyl)piperazine-1-ethanesulfonic acid,N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonicacid) (HEPES) — **GibcoBRL, Life Technologies, Paisley, Scotland**

- [3,5-^{3}H] –*p*-Aminobenzoic acid (MT-599, 40.9Ci/mmol) — **Moravek Biochemicals, Brea, CA, USA**

- *Esherichia coli* NCIMB 8879 — **NCIMB, Aberdeen, Scotland**

- Horse Blood Columbia blood agar **Microbiology Dept, SPD, St James Hospital**

- Monotrim® (Trimethoprim) **Solvay Healthcare, Southampton**

- Ecoscint (scintillation fluid) **Kimberly Research, Georgia, USA**

- Rat Plasma **BioResources Unit, TCD, Dublin 2**

2.2.3 Cell Culture Consumables

- Foetal Bovine Serum (FCS) **Gibco, Invitrogen Corporation**
  Antibiotic-Antimycotic(100X)
  Phosphate Buffered Saline (PBS) 10X without Ca^{+2} and Mg^{+2}
  Medium 199 with glutamine and sodium bicarbonate
  Medium 199 without glutamine, methionine and folic acid
  Medium 1640
  Medium 1640 without folic acid
  Trypsin/EDTA (100X)
  Glutamine
  Methionine

- Virkon **Antec, Lennox, Naas Rd, Dublin 12**

- Gelatin Powder **Sigma-Aldrich, Steinheim, Germany**
  Endothelial Cell Growth Supplement

Heparin
Collagenase Type 2s
Trypan Blue Stain — **Sigma-Aldrich, Steinheim, Germany**
Gentamicin solution

- ((6S)-Folinic acid, diammonium salt, [3',5',7,9-3H(N)]-) *[^{3}H] –folinic ac*id (MT-521, 16.6Ci/mmol) — **Moravek Biochemical, Brea, CA, USA**

2.2.4 DNA Extraction and Genotyping Reagents

- Qiagen DNA Extraction Kit (QIAamp DNA blood mini kit) — **Qiagen Ltd, Boundary , West Sussex**
- *Hin F1* restriction enzyme — **New England Biosciences**
- Taq Polymerase — **Sigma-Aldrich, Steinheim, Germany**
  Bromophenol Blue
  Xylene Cyanol
  Glycerol
  PCR Buffer 10X without Mg^{+2}
  Stock Magnesium Chloride (25mM)
  100bp Molecular Weight Marker
- DNTP's (PCR Nucleotide Mix 10mM) — **Boehringer Mannheim, Germany**
- Primer 1: MW=7608µg/µmole — **Gibco, Biosciences, Tallaght**

(Sequence TGAAGGAGAAGGTGTCTGGGGGA)

Primer 2 : MW=6626µg/µmole,
(Sequence AGGACGGTGCGGTGAGAGTG)

Agarose

- HPLC Grade water — **Labscan, Stillorgan, Dublin**

2.2.5 Microbiological Assay

- Ascorbic acid (Sodium Salt)
  Potassium Dichromate
  Ascorbic Acid
  Tween 80 — **Sigma-Aldrich, Steinheim, Germany**

- Vitamin Folic Acid Assay Base Broth (Ref No. 882470) — **Merck, Darmstadt, Germany**
- Chloramphenicol — **Parke Daves & Co. Pontypool, Wales**
- *Lactobacillus rhamnosi* (NCIB 19463) — **Torrey Research, Aberdeen, Scotland**
- Sulphuric Acid — **Merck, Darmstadt, Germany**

2.2.6 Immortalised Lymphocyte Cell lines

- Immortalised lymphocyte cell lines — **NIGMS Human Genetic Cell Repository, Coriell Institute for Medical Research, New Jersey, USA.**

15041	MTHFR CC
15070	MTHFR CC
15931	MTHFR CC
17180	MTHFR CC
17242	MTHFR CC
17219	MTHFR CC
17259	MTHFR TT
17265	MTHFR TT
15083	MTHFR TT
17219	MTHFR TT

17274 MTHFR TT

17234 MTHFR TT

2.2.7 Consumables

- 50ml and 15ml sterile centrifuge tubes **Gibcobrl, Biosciences, Tallaght,**
  6-well Sterile Tissue Culture Plates **Dublin**
  25cm^2, 75cm^2, 175cm^2 sterile cell tissue culture flasks

- Sterile double grip umbilical cord clamps **Hollister ULC, Ballina, Ireland**

- Gauze **Smith and Nephew, Dun Laoghaire**
  Swabs
- Sterile Needles **Sterican, B. Braun, Germany**
  Sterile Syringes 20ml
- Needle Cutter **Destruclip, Hypodermic safety device**

- Sterile T extension sets **Intra Veno, Tallaght, Dublin**

- Sterile Pasteur Pipettes **Sparks, Tallaght, Dublin**
  250ml Sterile Collection Jars

2.3 HPLC method for separating folate co-factors

2.3.1 HPLC Buffer Preparation

Pic A reagent Mobile Phase

0.1M sodium acetate was dissolved in 850ml of HPLC grade water and 1 bottle of 5mM Pic A was added. The pH was adjusted to pH 6.0 using glacial acetic acid. The volume was made up to 900ml with water and then 100ml of methanol was added. The buffer was filtered, degassed and stored at room temperature. When necessary the volume of methanol was adjusted to give better separation of peaks.

Citrate Phosphate Mobile Phase

0.1M citric acid buffer was prepared by dissolving 14.4g citric acid in 750ml of HPLC grade water. 12.16g disodium hydrogen phosphate was dissolved in 250ml HPLC grade water and added to the 750ml of 0.1M citric acid. If necessary the pH was adjusted to pH 4.0 by adding a few sodium hydroxide crystals. 430ml of 0.1M citric acid buffer pH 4.0 was added to 445ml of 0.1% acetic acid and 125ml of methanol. The buffer was filtered, degassed and stored at room temperature.

2.3.2 Folate Standard Preparation

2mg of each commercially available folate standard was weighed on a fine balance and transferred to a 20ml grade A volumetric flask. The solution was brought up to the mark with 1% sodium ascorbate. A few drops of 1M sodium hydroxide was added to the folic acid standard in order to dissolve the standard completely. Nitrogen gas was bubbled through each standard before being aliquoted into 0.5ml amounts and frozen at –40°C.

Preparation of 5,10-CH^+-THF and 10-CHO-THF

2mg of 5,10-CH^+-THF was dissolved in 20ml of 1% ascorbic acid. 10ml of the standard was removed and the pH adjusted to 8.5 with 2N potassium hydroxide to

yield 95-100% 10-CHO-THF (Stover and Schirch, 1992). Nitrogen gas was bubbled through each standard before being aliquoted into 0.5ml amounts and frozen at -40°C.

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2.3.3 Standard Runs on HPLC

The pump flow rate was set at 1ml/min, the column was heated to 37°C in a column heater and the spectrophotometer was blanked with the mobile phase at 280nm. The standards (prepared as in 2.3.2 above) were injected onto the HPLC and their elution profiles and times were determined.

2.3.4 Column Cleaning Protocol

The column was reversed and the following solutions were run through it:
200ml 95% water/5% acetonitrile (for buffer removal)
200ml Tetrahydrofuran
200ml 95% acetonitrile/5% water
200ml mobile phase
The column was returned to its original direction of flow and was ready to be re-used. Standards were run to ensure the column was working satisfactorily

2.4 Calibration of Scintillation Counter

When quench is present in a sample the number of scintillations is reduced. The degree of quench can be obtained from the degree of reduction in scintillations, using a correction curve prepared with quenched standards. In the case of the Packard counter, the instrument records each pulse arising from a compton electron in a channel appropriate to its energy. It then uses the data to calculate a Transformed Spectral Index of the External Standard (tSIE) for the sample. A standard curve relating tSIE to efficiency of counting can be used to give the efficiency of counting for each sample. Modern liquid scintillation counters will correct for quench

automatically and provide disintegrations per minute (dpm) results. An example of a quench curve can be seen in figure 2.1.

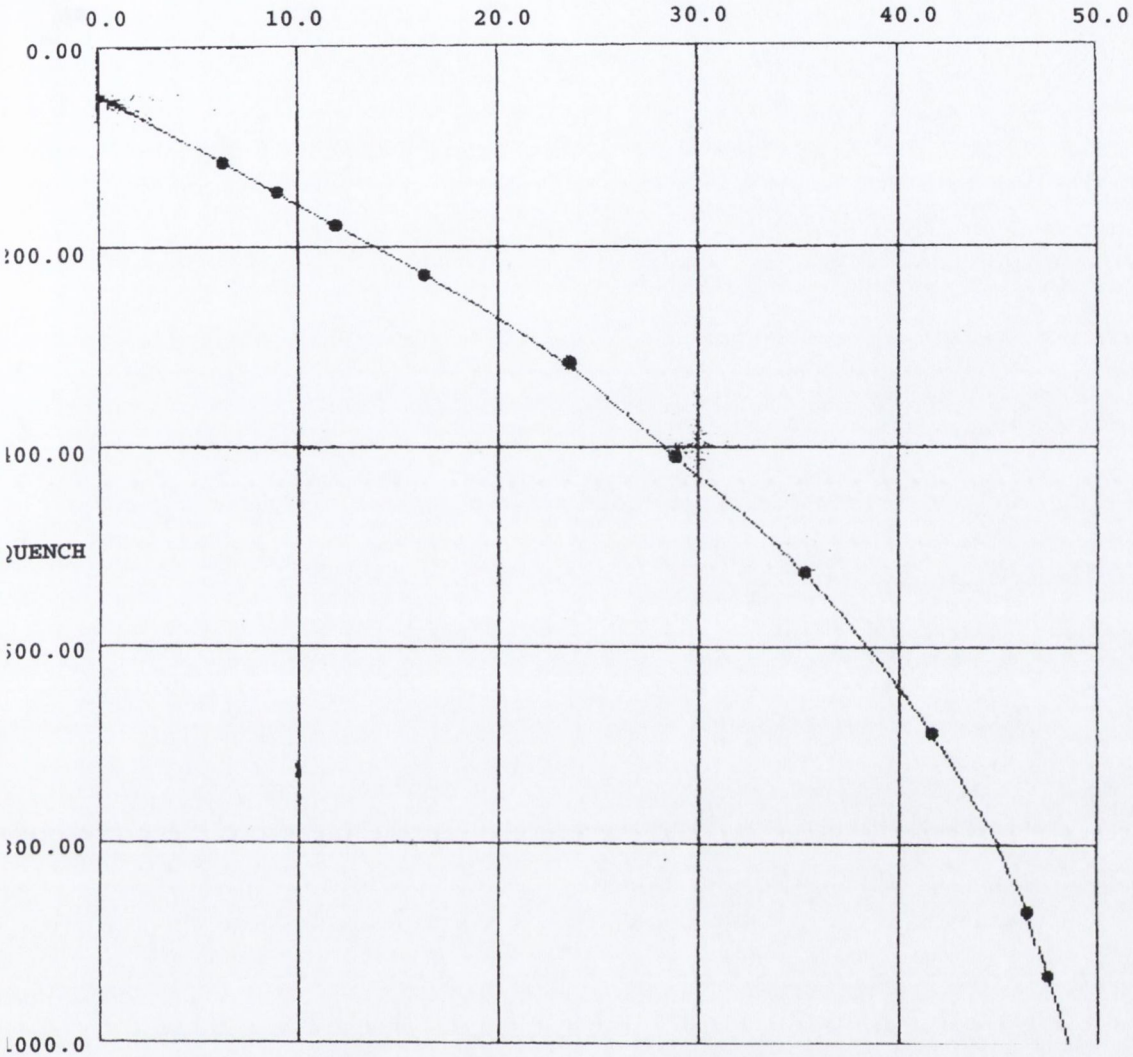
2.5 Preparation of *E.coli* 8879 for HPLC Assay

2.5.1 Dialysis of Rat Serum

Blood (10ml) was obtained from a rat by cardiac puncture and allowed to clot. The sample was centrifuged at 3,200rpm for 10min and the serum was removed. The rat serum was dialysed overnight in PBS(1X) buffer with 2g of acid washed activated charcoal per litre in a cold room (Wilson and Horne, 1982) to remove endogenase folate. Following dialysis the rat serum was tested for conjugase activity using folate polyglutamate standards, as described below.

2.5.2 Determination of Conjugase Activity in Freshly Dialysed Rat Serum

A standard solution of 0.143mM pteroyltri-γ-L-glutamic acid was prepared as per method 2.3.2. 100µl of the standard solution was mixed with 20µl of dialysed rat serum and incubated at 37°C for varying timepoints from 0 to 2hr and 100µl of the standard was run on the HPLC. Full deconjugation had occurred when all the pteroyltri-γ-L-glutamic acid peak was converted to a mono glutamic acid standard peak which was identified by the difference in elution times of the standards. An incubation time of 1hr allowed for the full deconjugation of 14.3nmoles pteroyltri-γ-L-glutamic acid into its monoglutamate form. 14.3nmoles is higher than the intracellular folate concentration in cells and therefore there was sufficient evidence to suggest this procedure would deconjugate all the intracellular folate polyglutamates.



QUENCH	ELA	QUENCH	ELA	QUENCH	ELA
115.96	6.36	145.36	9.07	177.70	11.94
228.09	16.36	318.30	23.65	408.51	28.94
526.02	35.33	687.65	41.74	864.61	48.57
929.78	47.61				

Figure 2.1 : An example of a quench standard curve. A standard curve relating tSIE to efficiency of counting is used to give the efficiency of counting for each sample. %Eficency is plotted on the x-axis and quench is plotted on the y-axis.

2.5.3 Preparation of Glucose Growth Media

The glucose growth media was prepared by dissolving the following in sterile distilled water:

NaCl 0.05M

NH_4Cl 0.05M

KH_2PO_4 0.25M

$CaCl_2$ 0.067M

$MgSO_4$ 2mM

Glucose 5.55mM

The pH was adjusted to pH 7.2 using saturated K_2HPO_4. The solution was stored at 2-4°C for one week.

2.5.4 Preparation of stock *E.coli* 8879

A plate of horse blood columbia agar was streaked with *E.coli* 8879 and incubated for 24 hours at 37°C. One colony was used to inoculate 5ml glucose growth media which was incubated in an orbital shaker at 37°C, 270 rpm for 10 hours (*E.coli* will have reached log phase of growth after 10 hours incubation). A columbia blood agar plate was lightly streaked with this bacterial suspension and incubated at 37°C for 24 hours. One colony was again inoculated into 5ml of growth media and incubated at 37°C, 270rpm for 9 hours. This bacterial suspension was diluted 1/2 with a sterile glycerol solution (50% (w/v) with distilled water and autoclaved). This suspension was aliquoted over dry ice into cryotubes and frozen at –70°C until required.

2.5.5 Optimisation of Growth Periods for *E.coli* 8879

A horse blood columbia agar plate was streaked with *E.coli* and incubated at 37°C for 24 hours. Approximately 20 colonies were resuspended in 1ml of glucose growth media. The absorbance reading of 300µl of the suspension was read using a Multiscan Plus plate-reader at 590nm. The sample was diluted to give a final reading of 1.0A.U. 100µl of this sample was used to inoculate 5ml of glucose growth media and was

incubated at 37°C in an orbital incubator at 270rpm for 10 hours. An incubation time of 10 hours was required for the bacteria to have reached log phase of the first growth. 250µl of this suspension was used to inoculate 5ml of glucose growth media containing 300pmol [^{3}H]-PABA and was incubated at 37°C, 270rpm for 6.5 hours. Following the 6.5 hour incubation the bacteria had reached log phase of the second growth. The suspension was centrifuged at 3,500rpm for 8min. The pellet was resuspended in 2ml of glucose growth media and washed. The washed pellet was resuspended in 1ml of glucose growth media. 200µl was inoculated into 5ml of glucose growth media containing the required concentrations of TMP and incubated at 37°C, 270rpm for 4 hours, which allowed the bacteria to reach the log phase of the third bacterial growth.

2.5.6 Determination of the Minimum TMP Concentration for Maximum Inhibition

5ml aliquots of growth media containing varying concentrations of TMP ranging from 0-200µg/ml were prepared and inoculated with 200µl of the *E.coli* suspension after it had reached log phase in the second growth. The suspension was incubated at 37°C, 270rpm for 4 hours. Absorbance readings were recorded and from these results the minimum amount of drug required to kill the maximum amount of bacteria in 4 hours was calculated.

2.5.7 Preparation of *E.coli* samples for HPLC

The *E.coli* samples were prepared as described in method 2.6.5. After varying incubation times from 0-24hr with glucose growth media containing the required amount of TMP the bacterial suspensions were centrifuged at 3,500rpm for 10min. The bacteria pellet was resuspended in 500µl of lysate buffer containing 100mM HEPES, 100mM β-mercaptoethanol and 2% (w/v) sodium ascorbate. This suspension was transferred to a microfuge tube and placed on ice. The suspension was sonicated for 3x30sec with 30secs breaks in between each sonication. This was then centrifuged

at 13,000rpm for 2-3mins to pellet the cell debris. The supernatant was added to 100µl of freshly thawed rat serum and incubated at 37°C for 45mins. Quinlivan *et al.*, (2000) reported full deconjugation of folates following an incubation of 45min with rat serum. In order to compare results a 45mins incubation time was used to deconjugate the bacterial folates. In later studies using mammalian cells a 1hr incubation time was used as per method 2.5.2. The sample was then centrifuged at 13,000rpm for 3mins. The supernatant was filtered using a 0.45µ filter. The sample was spiked with folate standards and 50µl of this sample was mixed with 3ml of ecoscint and counted using a scintillation counter. From the remaining sample 100µl was injected onto the column. Fractions were collected at 0.25ml/min intervals. 3ml of ecoscint was added to each fraction and the dpm counts were obtained using a scintillation counter. Figure 2.2 shows a schematic diagram of the this method. The following equation was used to determine the percentage of radiolabelled folate per fraction:

$$\% \text{ folate recovery/fraction} = \frac{\text{dpm counts/fraction}}{\text{total dpm counts injected}} \times 100$$

2.6 Cell Culture Methods

2.6.1 Maintenance, Storage and Subculture of Immortalised Lymphocyte Cell Lines

Immortalised human lymphocyte cell lines were obtained from the Coriell Institute for Medical Research, New Jersey in a frozen stock. Each cell line had an assigned repository number from the Coriell Institute and the MTHFR 677C→T genotype information was obtained from Dr Larry Brody (Genome Technology Branch, National Human Genome Research Institute, National Institutes of Health). The cells were revived (see method 2.6.3) and seeded at a concentration of $2x10^5$cells/ml media. They were fed every 2-3 days with RPMI Medium 1640 containing 10% FCS and gentamicin. The flasks were incubated at 37°C in a humidified atmosphere and 5% CO_2. When the cells had reached confluency the media and the cells were aseptically poured into a sterile universal tube and centrifuged at 1,100rpm for 10min.

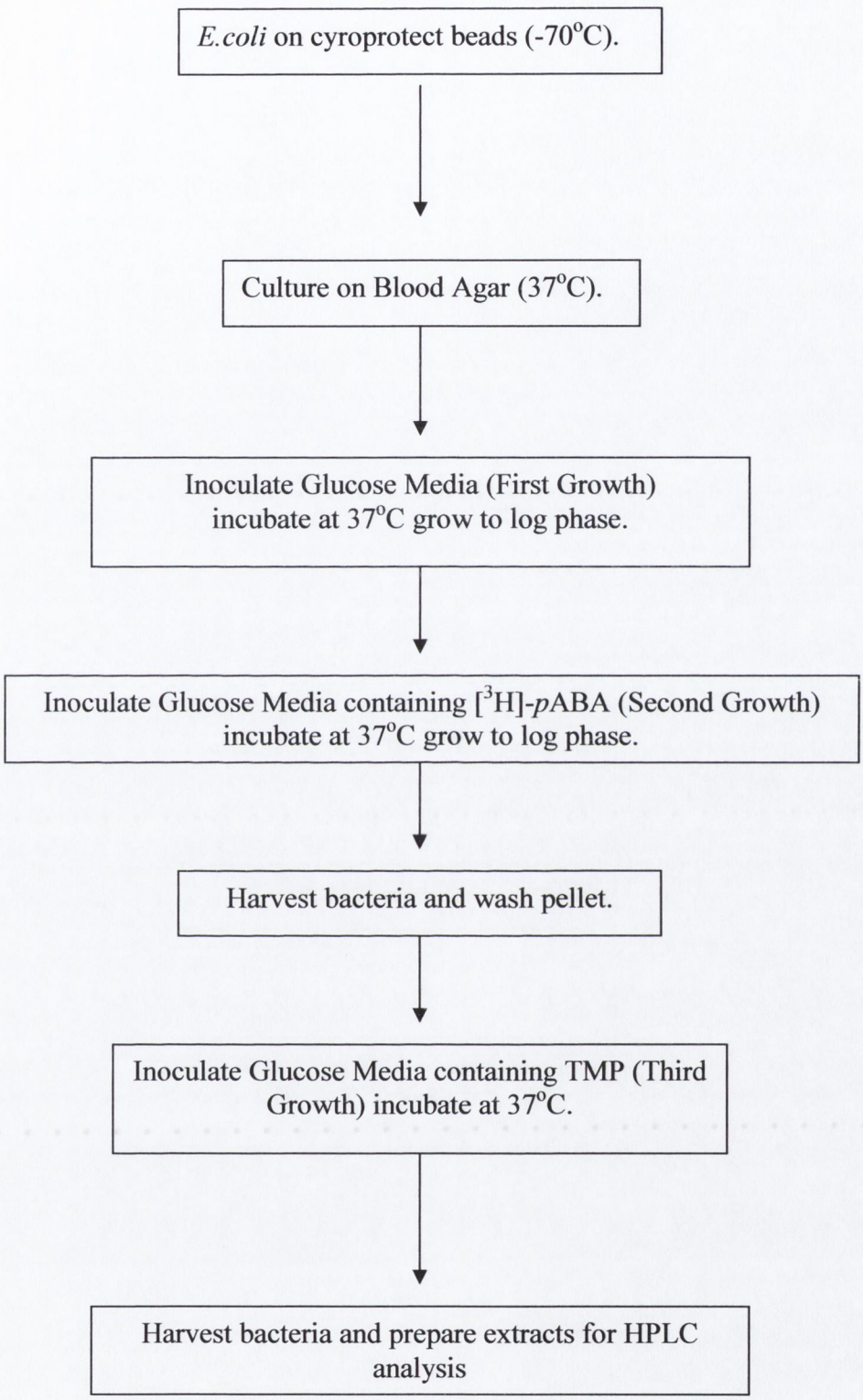


Figure 2.2 : Schematic chart of *E.coli* experimental procedure

The supernatant was removed and the cell pellet resuspended in 1ml fresh media. This was then ready to be split 1:3 ratio into fresh RPMI 1640 medium.

2.6.2 Counting Suspenson Cells using Trypan Blue Viability Stain

A 5µl aliquot of cells, which had been resuspended in 1ml of media, was mixed with 45µl of trypan blue stain. The cell suspension was then counted using a haemocytometer. Dead cells appeared blue using this stain and viable cells were clear; viable cells do not permit the stain to penetrate through the cell wall. Ideally the number of cells should be between 100-300/mm^2 to provide a reasonable degree of accuracy in counting. The greater the cell number the more accurate the counts become. The number of cells/ml could then be determined by the following equation:
(Cell count x 10) **x** 10^4 = Number of cells/ml

2.6.3 Freezing and Reviving Immortalised Lymphocyte Cell Lines

Immortalised lymphocyte cells have been reported to freeze best at 3 x 10^6cells/ml or higher. A pellet containing these numbers or higher was washed twice with PBS(1X) without Ca^{+2} and Mg^{+2} PBS(1X). A 7% DMSO/ RPM1 medium 1640 plus 10% FCS solution was prepared and the cells were resuspended in 1ml of this solution. The suspension was then transferred to a cryostat tube and placed in a cryostore box to be frozen for approximately 1hour at –20°C and then frozen overnight at –80°C. The cryostat tube was then transferred into a liquid nitrogen storage vessel.

To revive a cell suspension from liquid nitrogen, the cryostat tube was removed and thawed in the hand. One ml of RPMI media 1640 supplemented with 10% FCS was very slowly added and mixed to the cell suspension. The suspension was then transferred to a centrifuge tube containing approx 10ml of media. The cells were spun at 1,100rpm for 10min and the pellet was washed twice more with the media to ensure all the DMSO had been removed. The pellet was resuspended in 1ml of media and transferred to a 25cm^2 flask which contained 9 ml of media and incubated at 37°C in a humidified atmosphere and 5% CO_2. Once the cells reached confluency they were passaged as per method 2.6.1

2.6.4 Growth Curves for Immortalised Lymphocyte Cell Lines

The cells were seeded into 10ml of RPMI medium 1640 at a density of 2 X 10^5 cells/ml. The cells were counted at 24hr intervals for 72hr. Three flasks were counted at each time-point.

2.7 Preparation of immortalised lymphocyte cell samples for HPLC

Immortalised lymphocyte cells were seeded at a cell density of 2 x 10^5cells/ml in folate free RPMI 1640 medium and incubated at 37°C, 5% CO_2 grown for 96hr. The cells were then re-seeded at 2 x 10^5cells/ml into 10ml RPMI 1640 medium supplemented with 1.19nmol [^{3}H] 5-CHO-THF (0.5μCi [^{3}H]5-CHO-THF) and incubated for 72hr at 37°C, 5% CO_2. The cells were washed 3 times in PBS(1X) without Ca^{++} and Mg^{++} and resuspended in 100μl 1% sodium ascorbate. The cells were sonicated on ice, 10sec x 3times and centrifuged at 14,000rpm for 5min. The supernatant was removed and 25μl dialysed rat serum was added and incubated for 1hr at 37°C in order to deconjugate the intracellular folates. The sample was spiked with 10μl of a standard mix containing PABG, 5-CHO-THF, 5,10-CH_2-THF, FA and 5-CH_3-THF. 10μl of the sample was counted to determine the radioactivity present in the sample. 100ul of the sample was injected onto HPLC and fractions were collected at 0.33ml/min intervals and counted on the scintillation counter. The radioactivity in the aliquots were compared to the HPLC standard trace obtained and the radioactive peaks were identified. Figure 2.3 shows a schematic chart for this method. The following equation was used to determine the percentage of radiolabelled folate per fraction:

$$\% \text{ folate recovery/fraction} = \frac{\text{dpm counts/fraction}}{\text{total dpm counts injected}} \times 100$$

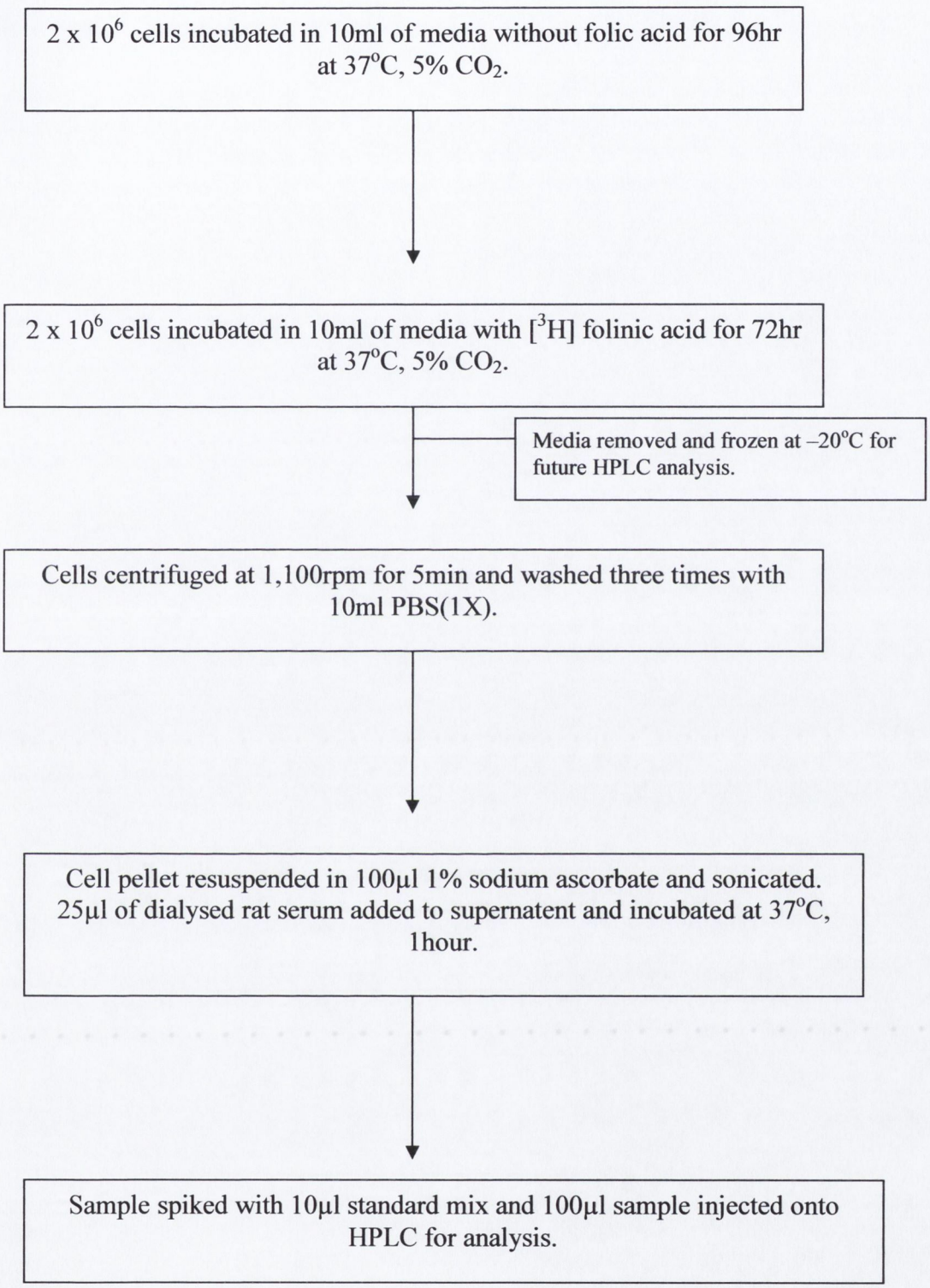


Figure 2.3 : Schematic chart of lymphocyte experimental procedure

2.8 Preparation of retained lymphocyte medium for HPLC analysis following folate radiolabelling

A Sep-Pak C18 column was conditioned by injecting 10ml 100% methanol followed by 10ml HPLC mobile phase. Slowly 10ml of media from radiolabelled cells (see figure 2.3) was injected through the column. The folates bind to the column because of the presence of the ion-pair reagent (PicA) in the mobile phase. The folates were then flushed from the column by injecting 1ml methanol slowly through the column and collecting the eluted methanol. This procedure was repeated and the two fractions were combined. A stream of nitrogen gas was passed over the combined fraction to allow the methanol to evaporate and the folates were resuspened in 100µl of mobile phase, spiked with a standard mix containing pABG, 5-CHO-THF and 5-CH_3-THF and analysed on the HPLC. Fractions were collected at 1ml/min intervals.

2.9 The Isolation and Culture of Human Umbilical Vein Endothelial Cells (HUVEC)

Umbilical cords were obtained from normal vaginal deliveries by consenting women of Irish nationality. The cords were placed in sterile jars containing cord buffer (sterile PBS 1X without Ca^{++} or Mg^{++}). The cords were stored at 4°C until use. Any cords stored for any longer than 48hr after delivery were discarded as cell viability upon isolation was low.

Using an aseptic technique under the laminar flow hood the cord was removed from the specimen jar and wiped clean with sterile gauze. It was then inspected for clamp marks. These areas were cut off giving a cord length of approx. 15-20cm. The umbilical vein was located using a sterile mounted needle and gently expanded. A hubless 23 gauge needle shaft 1 1/4 inch in length was blunted and the vein was canulated with the needle, which was secured in place with a cord clamp. A sterile "T" adaptor was connected to the needle. A sterile 20ml syringe filled with warm cord buffer was connected to the "T" extension and the buffer was run through until the buffer at the distal end of the vein was clear. This removed any remaining blood

and blood clots. The distal end of the vein was then canulated as described above. A sterile 20ml syringe containing a collagenase type 2s (10mg/ml) solution was attached to the proximal end and the solution was slowly flushed through and drained into the distal syringe whilst drawing back on the latter. The proximal extension was clamped shut using the attached haemostat. The cord was expanded with the collagenase solution (4-8ml depending on the size). The distal extension was also clamped with the haemostat. The cord was wrapped in tin foil and left in a 37°C incubator for 7minutes. Upon removal of the cord it was massaged between the thumb and index finger to help dislodge the remaining cells. The collagenase was flushed into the distal syringe upon opening of the haemostat by injecting approximately 20ml of medium 199 containing 10% FCS. This solution was then placed in a sterile 50ml centrifuge tube, which already contained about 20ml of media. The tube was centrifuged at 1,100rpm for 10mins. The supernatant was discarded and the pellet, which contained the cells, was resuspended in 1ml of growth media. This was then added to a $25cm^2$ flask, which had been gelatinised with a sterile 0.2% gelatin solution and contained 3ml of growth media. The flask was incubated overnight at 37°C and 5% CO2. The flask was examined for endothelial cells, which had adhered. The media was replaced with 4mls of fresh media and 133µl of endothelial cell growth supplement (ECGS). The flasks were incubated until a confluent monolayer of cells had grown. Endothelial cells should appear as a cobble-stoned monolayer, which is what identifies them from smooth muscle cells, and fibroblasts, which can sometimes outgrow the endothelial cells. Figure 2.4 shows a schematic chart of this method.

2.9.1 Maintenance, Storage and Subculture of Endothelial Cells

The cells were fed every 2-3 days with medium 199 containing 10% FCS, antibiotic/antimycotic agent and 100µg ECGS/10µg heparin/ml media. The flasks were incubated at 37°C in a humidified atmosphere and 5% CO_2. When the cells had reached a confluent monolayer they were passaged and split in a 1:3 ratio. The media in the flask was poured off aseptically and the monlayer of cells were washed with PBS(1X) without Ca^{+2} and Mg^{+2}. A 1% Trypsin/ EDTA solution was prepared by thawing a 1ml (10X) solution and adding 9ml of PBS (1X) without Ca^{+2} and Mg^{+2}. One ml of this solution was used to wet the cells and was poured off immediately. An

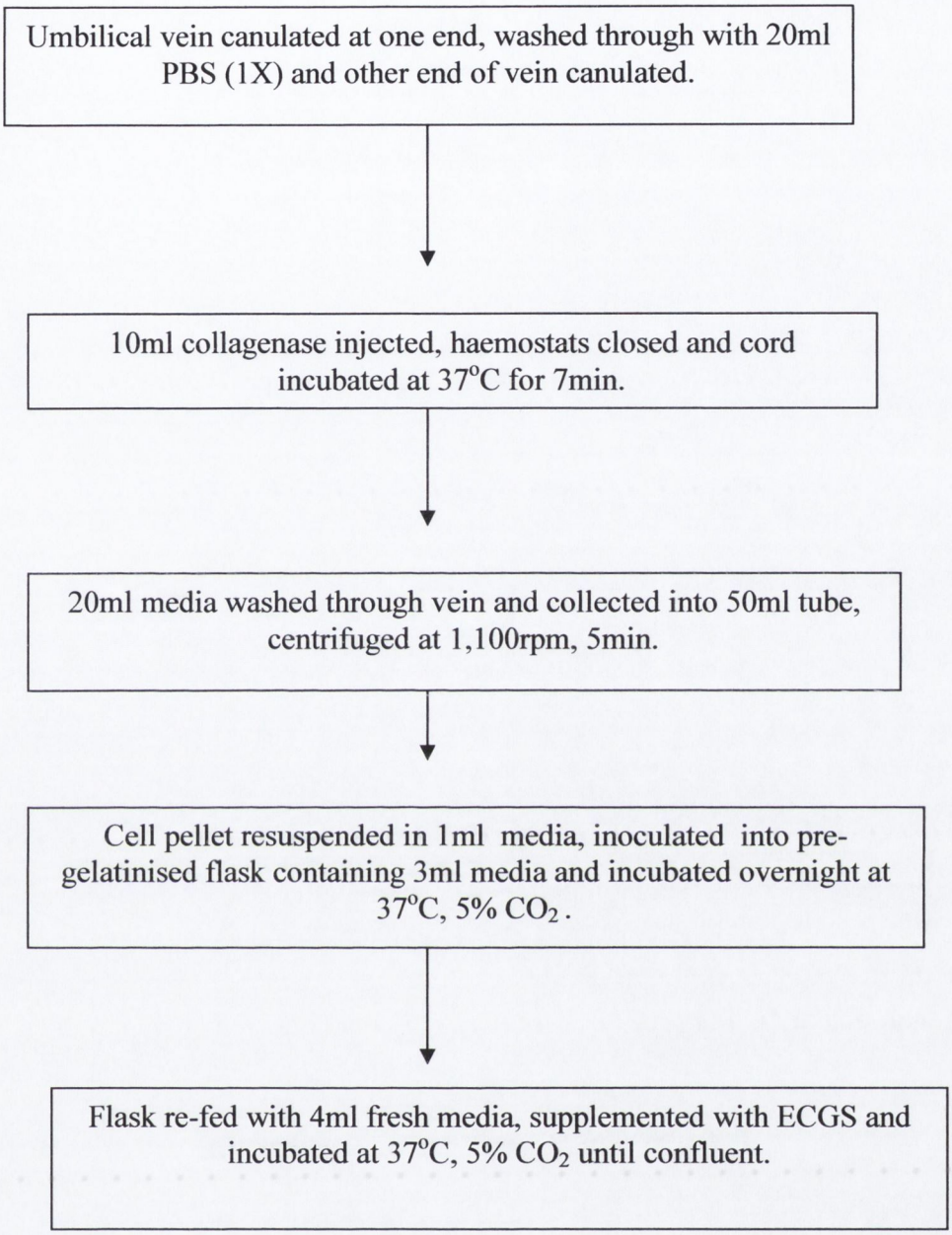


Figure 2.4 : Schematic chart of the isolation of HUVEC cells from an umbilical cord.

appropriate amount of Trypsin/EDTA was then added to each flask (2ml/25cm^2 flask, 5ml/75cm^2 flask, 7ml/ 175cm^2 flask) and incubated at 37°C, 5% CO_2 for approx. 5-10mins. The exact time was determined by examining the flask under the microscope and observing when all the cells had detached and were floating in solution. This process could be aided by tapping the flasks gently to dislodge the cells. Three times the volume of medium 199 containing 10% FCS was added. This inhibited the action of the trypsin/EDTA solution had. The cell suspension was transferred to a centrifuge tube and centrifuged at 1,100rpm for 10min. The supernatant was removed and the cell pellet resuspended. This was then ready to be split 1:3 ratio in pre-gelatinised flasks or counted or to be washed further for freezing.

2.9.2 Counting Endothelial Cells using Trypan Blue Viability Stain

A 10µl aliquot of freshly trypsinised cells, which had been resuspended in an appropriate amount of medium, depending on the size of the cell pellet, was mixed with 10µl of trypan blue stain. The cell suspension was then counted using a haemocytometer. Dead cells appeared blue using this stain and live cells did not permit the stain to penetrate. The number of cells/ml could then be determined by the following equation:

(Cell count x 2) **x** 10^4 = Number of cells/ml.

2.9.3 Freezing and Reviving Endothelial cells

Endothelial cells have been reported to freeze best at 1 X 10^6cells/ml or higher. A pellet containing these numbers or higher was washed twice with PBS(1X) without Ca^{++} and Mg^{++} PBS(1X). A 7.5% DMSO/medium 199 plus 10% FCS was prepared and the cells were resuspended in 1ml of this solution. The suspension was then transferred to a cryostat tube and placed in a cryostore box to be frozen for approx. 1hour at –20°C and then frozen overnight at –80°C. The cryostat tube was then transferred into a liquid nitrogen storage vessel.

To revive a cell suspension from liquid nitrogen, the cryostat tube was removed and thawed in the hand. One ml of Medium 199 with 10% FCS which had been

previously warmed to approx 37°C was very slowly added and mixed to the cell suspension. The suspension was then transferred to a centrifuge tube containing approx 10ml of media. The cells were spun at 1,100rpm for 10mins and the pellet was washed twice more with the media to ensure all the DMSO had been removed. The pellet was resuspended in 1ml of media and transferred to a pre-gelatinised $25cm^2$ flask that contained 3ml of medium containing 10% FCS and ECGS. The cells were fed for a few times with this 10% FCS media until the cells appeared to look healthy and began to divide.

2.9.4 Growth Curves for Endothelial Cells

6 well multiwell plates were gelatinised. The cells were seeded at a density of 1 X 10^5 cells/well. They were synchronised by incubating them for 24hrs at 37°C, 5% CO_2 in 2ml of the appropriate media minus FCS. The wells were then checked under a microscope to determine if the cells had attached. Two wells were counted at this stage to determine the number of cells that had attached (approximately 70%). The media was changed to 4ml of media containing 10% FCS and ECGS. The cells were refed every 2-3 days and after the appropriate time intervals the wells were trypsinised and counted.

2.10 Preparation of endothelial cell samples for HPLC

The endothelial cells were seeded at a cell density of 3 x 10^5cells/$25cm^2$flask and grown for one passage in 4ml of folate free medium 199 and ECGS. The cells were then re-seeded at 3 x 10^5cells/$25cm^2$flask into 4ml of folate free medium 199 and ECGS, supplemented with 3.0nmol [^{3}H] 5-CHO-THF (0.5μCi [^{3}H] 5-CHO-THF) and incubated for 96hr at 37°C, 5% CO_2. The cells were harvested and the samples prepared using the method described in 2.7.

2.11 DNA Extraction Procedure

At least 2 x 10^4 cells were resuspended in 200µl of PBS (1X) and the DNA was extracted using a Qiagen kit (QIAamp DNA blood mini kit). 20µl of QIAGEN protease and 200µl of lysis buffer was added to the cell suspension in a microcentrifuge tube and pulse-vortexed for 15secs. The sample was incubated for 10mins at 56°C. After a brief centrifugation, 200µl of (96-100%) ethanol was added and it was again pulse-vortexed for 15seconds and briefly spun. The mixture was applied to a QIAamp spin column without wetting the rim. The cap was closed and centrifuged at 8,000rpm for 1minute. The filtrate was discarded, the spin column was placed in a clean 2ml collection tube. 500µl of buffer AW1 was added and the tube was centrifuged at 8,000rpm for 1minute. The filtrate is discarded the spin column was placed in a clean 2ml collection tube. 500µl of buffer AW2 was added and the column centrifuged at 14,000rpm for 3minute. The last spin was repeated to ensure there was no carryover of buffer. The QIAamp spin column was placed in a clean 1.5ml microcentrifuge tube and 200µl Buffer AE was added, this was incubated for 5min at room temp, and centrifuged at 8,000rpm for 1minute. This last buffer wash was repeated to ensure all the DNA has been collected. For samples which contained low cell numbers the sample was frozen at –20°C and then freeze-dried to concentrate the DNA. This step ensured that sufficient DNA was present for PCR analysis. The freeze-dried sample was resuspended in 25µl of PBS(1X). 2µl of the sample was mixed with 2µl of loading dye and run on a 1.5% agarose gel with ethidium bromide.

2.12 The Preparation of a Gel and Loading Samples

To make 50ml of loading dye:

125mgs Bromophenol blue

125mgs Xylene cyanol

15nls of Glycerol

35nls of Water

1.5g of electrophoresis grade agarose was added to 100ml of 1X TBE buffer in a 500ml Pyrex bottle. The solution was boiled either in a microwave, 1.5µl of Ethidium Bromide (10mg/ml) was added and mixed by swirling. A 20 tooth 1mm comb was put

in place and the gel was poured and allowed to solidify which takes about 20-30minute. The samples were loaded with the loading dye. The gel was run at 100-150V for 2.5-3 hours, removed and placed on an UV transilluminator to view the fluorescent bands of DNA.

2.13 Preparation of Samples For PCR

The extracted DNA samples were thawed to room temperature. To each labelled, autoclaved eppendorf tube, 1.0 µl of DNA was added to the PCR mix, containing:

4 µl	10X PCR Buffer
5 µl	Stock Magnesium
8 µl	dNTPs (deoxyNucleotide Tri Phosphates)
1 µl	Primer 1
1 µl	Primer 2
0.2 µl	Taq
30.8µl	HPLC Grade Water

This brought the total volume to 50µl.

An overlay of approximately 3 drops of mineral oil was added to the tube to prevent evaporation of the sample during the PCR cycles. A negative control, with only the P.C.R. mix, was also included to ensure only the cell sample DNA was amplified. A positive control with a known heterozygote for the mutation of interest was also included. The samples were placed in the PCR cycler, ensuring that each tube was positioned tightly into each well. The cycler was programmed for MTHFR PCR in the following way:

1. 94 °C for a 5 minute ramp
2. 94 °C for 1 minute
3. 62 °C for 1 minute
4. 72 °C for 1 minute

Steps 2.-> 4. were repeated 30 times, *i.e.* 30 cycles. The programme took approximately 3 hours to complete. After completion, a sample 10 µl of the PCR product was run out on a 1.5% agarose gel to ensure that amplification of our target DNA had occured.

2.14 Restriction Enzyme Digest of MTHFR PCR Product

If the reaction was successful, 17µl of the PCR product was digested with the enzyme *HinF 1*, (for MTHFR PCR)

The digestion mix was as follows:

1. 17 µl of PCR product
2. 2µl of reaction buffer
3. 1µl of enzyme, *HinF 1*

The samples were allowed to digest overnight in a water bath, at 37 °C. After digestion, the samples were run on a 1.5% agarose gel in 1 X TBE Buffer. Figure 2.5 shows a typical gel containing MTHFR CC, CT and TT genotypes. The wild type genotypes are represented by an uncut band of 198 base pairs. The heterozygote samples are represented by two bands of 198 base pairs and 175 base pairs. Homozygous for the mutation is represented by a 175 base pairs.

2.15 Microbiological Assay of Standards

2.15.1 Microbiological Assay for Folate

Preparation of assay medium (to make 100ml)

5.7g folic acid assay broth

3.0mg chloramphenical

30µl Tween

75mg ascorbic acid

100ml distilled water

The folic acid broth, chloramphenicol, and water were mixed together and heated. The tween was added and the mixture was heated to boiling point. After cooling 75mg of ascorbic acid was added. The cryopreserved *L.casei* was made up by the laboratory technicians as described by Molloy and Scott, (1997). This was added to the media at the concentration of 200µl/100ml and was mixed thoroughly using a magnetic stirrer.

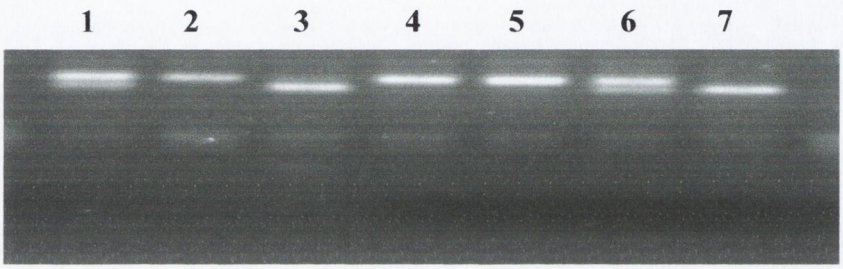


Figure 2.5: A typical example of a gel containing DNA samples genotyped for the MTHFR C677T mutation. DNA was extracted from cell samples and amplified using MTHFR PCR. The PCR product was digested with the enzyme *HinF* 1 overnight at 37˚Cand run on a 1.5% agarose gel in 1XTBE buffer. Lanes 1,6 are MTHFR 677CT genotypes represented by two bands of 198 base pairs and 175 base pairs, lanes 2,4,5 are MTHFR 677CC genotypes represented by an uncut band of 198 base pairs. and lanes 3,7 are MTHFR 677TT genotypes represented by a band of 175 base pairs.

2.15.2 Preparation of standard curve

A working standard of each folate co-factor (0.2265μM) was prepared by the addition of 500μl of a stock standard of 45.3μM to 0.5% sodium ascorbate buffer, which was brought to a final volume of 100ml. A standard curve was constructed in the region 0-113.3fmol/well which is the equivalent of 0-50pg/well (0-100μl) using a 96 well microtitre plate. Twelve columns with 8 replicates for each standard were prepared containing each folate co-factor standard fmol/well and pg/well as shown below:

fmol/ well	0	0	5.7	11.3	17.0	22.7	28.3	34.0	45.3	68.0	90.6	113.3
pg/ well	0	0	2.5	5	7.5	10	12.5	15	20	30	40	50

All wells were brought to a volume of 100μl by the addition of 0.5% sodium ascorbate buffer.

2.15.3 Addition of media, incubation and reading of results

Media (200μl) was added to all wells of the microtire plate using a multi-channel pipette. The plates were sealed using a plate sealer. The plates were incubated at 37°C for 42 hours in a non-humidified warm air incubator. The plates were then mixed thoroughly by inversion, the plate sealers were removed and the plates were read by densitometry at 590nm. The test results were calculated from the standard curve by a computer package Genesis Lite version 3.0 (from Labsystems and Life Sciences International) to give results in ng/ml, which is then converted to pmol/ml. Figure 2.6 shows an example of a standard curve using folic acid as the standard.

2.16 Statistical Analysis

The descriptive statistics in this thesis were calculated using an Excel program, which gave the mean, standard deviation (SD), standard error of the mean (SEM) and percentage coefficient of variation (%CV) results for the data. A student t-test was

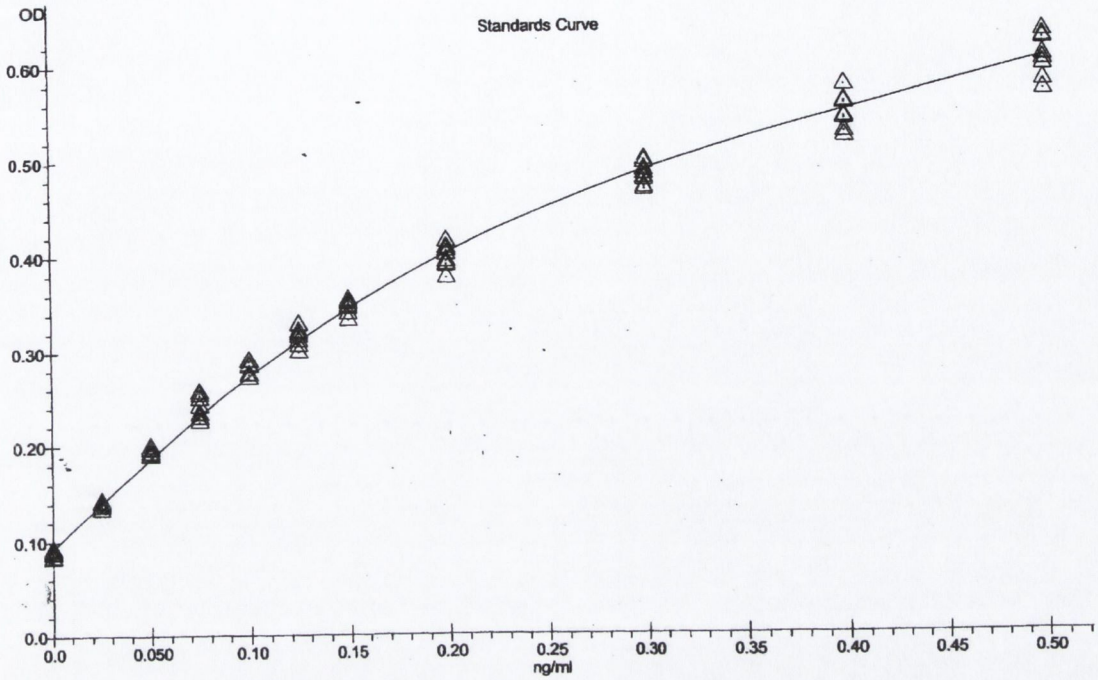


Figure 2.6: An example of a microbiolgical assay standard curve using Folic Acid as a standard. A folic acid standard was prepared at concentrations of 0.2265µm in 0.5%(w/v) sodium ascorbate and a standard curve was constructed in the region 0-113.3fmol/well which is the equivalent of 0-50pg/well using a 96 well microtitre plate. All wells were brought to a volume of 100µl by the addition of 0.5% sodium ascorbate buffer. 200µl of folate media inoculated with *L.casei* was added to the wells. The plates were incubated at 37°C for 42hr in a non-humidified warm air incubator. The plates were then mixed thoroughly by inversion and read by densitometry at 590nm. The test results were calculated from the standard curve by a computer package Genesis Lite version 3.0 (from Labsystems and Life Sciences International) to give results in ng/ml, which is then converted to pmol/ml. The x-axis represents the concentration of the folic acid standard (ng/ml) and the y-axis the absorbance readings at each concentration.

used in chapter 5 to assess the level of significance between MTHFR C677T genotypes for both the radiolabelled [^{3}H]5-CHO-THF incorporation and the percentage distribution of the radiolabelled intracellular folate co-factors. The level of significance p(value) was given for comparisons between MTHFR CC and TT immortalised human lymphocyte cell lines. The student t-test was carried out using a Datadesk computer program. A two-way ANOVA was performed in chapter 6 using a Graphpad Prism program, version 2.0. An ANOVA tests for differences between group means and a two-way ANOVA determines how a response is affected by two different categorical variables. The test simultaneously asks three questions; 1) Does the first variable systematically affect the results. 2) Does the second variable systematically affect the results. 3) Is there an interaction between the two variables.

Chapter 3:

Development and validation of a method to measure the intracellular folate co-factors in mammalian cell lines.

3.1 Introduction

Within a cell there is a complex mixture of folates which differ only by the state of oxidation of the pteridine ring, the substitution of a one-carbon residue on nitrogen position 5 or 10 and the number of glutamate residues attached. Folates are essential for *de novo* purine and pyrimidine synthesis as well as for the supply of methyl groups to remethylate homocysteine and methionine and thereby supply s-adenosylmethionine (SAM) for biological methylation reactions. Disturbances in folate metabolism may also be related to an increased risk of cardiovascular disease (Graham *et al.,* 1997; Quinlivan *et al.,* 2002), carcinogenesis and also may lead to congenital malformations such as neural tube defects (Molloy *et al.,* 2002). As a result, the development of a reliable method for quantifying the various folate co-factors within tissues and cells would enable further investigation into the effects of various metabolic states *e.g.* folate and Vitamin B_{12} deficiency on folate metabolism and catabolism.

The main problem associated with developing a method that can identify and quantify folate co-factors is the relative instability of reduced folates and their inter-conversion during extraction and analysis. Furthermore, high sensitivity is required in analytical methods because of low folate concentrations in biological materials. Conventional assays by microbiological methods provide high sensitivity but are slow and do not differentiate between folate co-factors (Molloy *et al.,* 1997). Competitive binding radiometric methods exhibit considerably lower sensitivity compared to that of the microbiological assay and differences in responses of various folates represent major limitations for many analyses (Shane, 1980). Various methods have been developed for the separation of folates by HPLC, combined HPLC/microbiological assays and HPLC mass spectrophotometric methods (Gregory *et al.,* 1984: Wilson and Horne,

1984: Duch *et al.,* 1983: Belz and Nau, 1998: Bagley and Selhub, 1998: Quinlivan *et al.,* 2000, Pawlosky *et al.,* 2003) but numerous problems have been associated with these methods, including inadequate extract purification and insufficient sensitivity or specificity of detection methods. Below is a brief summary of some of the methods used to extract and analyse folate co-factors in tissues and cells.

Duch *et al.,* (1983) developed an ion-pair reverse phase chromatography method for the analysis of folate co-factors in tissue. The sample was purified using Dowex 50 chromatography and concentrated 20-fold by overnight lyophilization before HPLC. This method was the first to allow quantitation of tissue folates following the conversion of the polyglutamates to monoglutamates and subsequently HPLC analysis was used in conjunction with UV detection. The problem with this method was that the sample preparation was cumbersome and long, which resulted in the degradation of the more labile folates. Geoghegan *et al.,* (1995) developed a HPLC method combining both ion-pair reverse phase chromatography similar to Duch *et al.,* (1984) and reverse phase chromatography. Although primarily they were investigating folate catabolism in rats by analysing catabolic products present in their urine, it was also necessary for them to identify and separate all the folate co-factors. The two HPLC methods in combination enabled them to identify both the breakdown products of folates and the reduced folate co-factor forms .

Bagley and Selhub, (1998) developed a method using affinity chromatography combined with HPLC, the sensitivity of the method was increased by the use of an electrochemical (coulometric) detection system instead of UV detection. The folates were separated on the basis of their pteridine ring structure and number of glutamate

residues. For folates with the same pteridine ring structure, retention time of the folate co-factors increased with increasing glutamate content. Using this method the presence of formylated and methylated folates were identified in red blood cells from individuals homozygous for the MTHFR 677TT mutation while MTHFR 677CC individuals contained only methylated folates (Bagley and Selhub, 1998). Similarly in immortalised lymphocytes using the same method the proportion of 5-CH_3-THF in cells with the MTHFR TT genotype was approximately half that of the MTHFR CC genotype under adequate nutrient conditions (Stern *et al.,* 2003). However, this method required the use of equipment that is not usually widely available in laboratories and therefore it is not widely used.

Quinlivan *et al.,* (2000) developed a method for separating folate co-factors in bacteria using ion-pair reverse phase chromatography. The main differences of this method from the previously described methods (Geoghegan *et al.,* 1995: Duch *et al.,* 1983) were that a Phenomenex Kingsorb C_{18} column was used as opposed to a waters μbondapak C_{18} column. The mobile phase not only contained picA reagent and methanol but also sodium acetate and was at a pH 6.0. Following sonication and centrifugation of the bacterial cells, the folates were incubated with rat serum at 37°C for 45min to allow full deconjugation of all the folate co-factors before injection onto the column. The main advantage of this method was that sample preparation was quick with very little degradation of the labile folates occurred. It had been reported that the only folate co-factor to be degraded during extraction was DHF, with a loss of 13% (Quinlivan *et al.,* 2000). The principle of the method involved the bacteria being fed with [^{3}H] PABA, which became incorporated into the cell and eventually became labelled folate co-factors. Following HPLC separation the radiolabelled folates were

collected as timed fractions and counted on a scintillation counter. The radiolabelled folate co-factors present in each peak was expressed as a percentage of total intracellular radiolabelled folates. This method allowed the relative distribution of co-factors to be assessed but was not quantitative.

The reason Quinlivan *et al.,* (2000) developed this HPLC method was to investigate the metabolic consequence of TMP inhibition on folate co-factor distribution in bacterial cells. As discussed in chapter 1 TMP is a structural analogue of the pteridine portion of DHF and acts as a competitive inhibitor of bacterial DHFR. It has no notable toxicity against mammalian forms of the DHFR enzyme (Brumtiff *et al.,* 1980). It is used on its own or in combination with sulfamethoxazole (SMX) as the first line of defence against urinary tract infections (Nicolle, 2003 review) and acute respiratory tract infections (Mahmud *et al.,* 2003). SMX is a structural analogue of p-ABA and inhibits the synthesis of DHF, a precursor of THF. The drug combination blocks two consecutive steps in the bacterial biosynthesis of essential nucleic acids and proteins (Wormser *et al.,* 1982). With or without SMX the common features of TMP action are; the disruption of folate metabolism and the cessation of nucleotide biosynthesis due to the depletion of the folate co-factor pools.

Quinlivan *et al.,* (2000) demonstrated that the commonly observed depletion in the folate co-factor pool after anti-folate treatment could be explained by folate catabolism. In their study they pre-labelled *E.coli* 8879 with [^{3}H]-*p*ABA, which allowed them to distinguish the metabolic effects of TMP from the folate biosynthetic effects, since both processes lead to the formation of DHF. They reported that DHF accumulated acutely upon inhibition of DHFR with TMP but, diminished to a very low level with time while

the level of *p*ABG and FA increased overtime. Their results suggested that the DHF which initially accumulated in the TMP treated *E.coli* 8879 had two fates, it could be either catabolised to *p*ABG or re-oxidised to FA. The removal of DHF through the scission to inactive catabolites would further enhance the effect of TMP through decreased competition for DHFR binding and would results in TMP having a bactericidal effect on the bacteria. However, the accumulated FA could be converted back into reduced folate co-factors upon removal of TMP resulting in TMP exerting only a bacteristatic effect on the bacteria as opposed to the desired bactericidal effect. (Quinlivan *et al.,* 2000). The duel effect of TMP may have physiological consequences for bacterial survival and decolonisation on premature termination of treatment of infection. Quinlivan *et al.,* (2000) suggest that the degree of DHF catabolism or oxidation to FA may vary with the species of bacteria, the redox state of the cell or the therapeutic regime of antifolate. If this proved to be the case these factors may determine the bateriostatic or bactericidal effect of TMP. Bacterial resistance to TMP and TMP/SMX treatment has become a growing problem especially in the treatment of urinary tract infections (Gupta *et al.,* 2003) and acute respiratory tract infections (Mahmud et at, 2003) and therefore it is crucial to understand how the mechanism of action of TMP and TMP/SMX affects the intracellular folate co-factors.

The first aim of this chapter was to repeat the study of Quinlivan *et al.,* (2000) in order to establish if their findings could be reproduced. Following this the method was to be transferred to mammalian cell model in order to investigate the effect the antifolates drug MTX would have on the intracellular folate co-factor distribution in mammalian cells. MTX is an inhibitor of the mammalian form of DHFR and its primary mechanism of action is thought to occur through its inhibition of DHFR and result in

the depletion of intracellular folate co-factors. 5,10-CH_2-THF and 10-CHO-THF are essential folate co-factors required for the biosynthesis of thymidylate and purines, repectively, and as a result of their depletion DNA synthesis cannot occur. However, MTX is a substrate for the intracellular FPGS enzyme and once inside the cell FPGS adds glutamate residues. MTX and its polyglutamate forms have been shown to inhibit other folate metabolising enzymes including AICAR transformylase, TS and MTHFR (Baggott *et al.,* 1986). Therefore, MTX inhibition of DNA biosynthesis is multifactorial, including both partial depletion of reduced folate co-factors and direct inhibition of folate dependent enzymes. Quinlivan *et al.,* (2000) demonstrated that the commonly observed depletion in the folate co-factor pool in bacterial cells following TMP treatment could be explained by folate catabolism. The question remains as to whether the depletion in the folate co-factors in mammalian cells following MTX treatment could in part be explained by folate catabolism as both TMP and MTX inhibit DHFR and would result in DHF accumulation.

It has been hypothesised that MTX sensitivity would vary with genetic variability in folate metabolising enzymes. Studies have shown that toxicity to MTX is aggravated among individuals homozygous for the common MTHFR C677T polymorphism (Ulrich *et al.,* 2001). The ultimate aims of this thesis which are addressed mainly in chapter 6 was firstly, to determine if folate catabolism could in part explain the depletion in folate co-factors following MTX treatment in mammalian cells and secondly, to determine if the MTHFR C677T polymorphism would effect the mechanism of action of MTX in mammalian cells.

The order to achieve the aims in this thesis the following experiments were carried out;

1) The method developed by Quinlivan *et al.,* (2000) was set up and his study repeated to ensure their results were reproducible. Once the reproducibility of these results was established, the two sets of results were compared. The next step was to determine if this method could be transferred from a bacterial cell model to a mammalian cell model. Modifications to the method were required to achieve optimum folate co-factor separation conditions. These modifications were validated to ensure the repeatability and reproducibility of the results obtained using the newly modified method.

2) It had been previously established in our laboratory that the mammalian cell line ECV304 incorporated [^{3}H]FA upon proliferation. Allegra *et al.,* (1986) and Baram *et al.,* (1987) had also shown this to be the case for the MCF-7 breast cancer cell line and for human myeloid precursor cells (MPCs). Baram *et al.,* (1987) had reported that a higher percentage of radiolabelled folate was incorporated into MPC cells using [^{3}H]5-CHO-THF as the radiolabelled folate source instead of [^{3}H] FA. In a system where [^{3}H]5-CHO-THF is the source of exogenous folate, it is rapidly converted to 5,10-CH$^+$-THF by the enzyme MTHFS in the cytoplasm. Monoglutamate folates can be either converted to folate polyglutamates in the cytoplasm by the enzyme FPGS, or the folate monoglutamates can enter the mitochondria where they are converted to polyglutamates and sequestered in that compartment (Anguera *et al.,* 2003). 5-CHO-THF is not an effective substrate for FPGS and therefore these newly imported folate derivatives are unlikely to receive a polyglutamate tail prior to equilibration in the folate pool (Garrow and Shane, 1993). In this study [^{3}H]5-CHO-THF was to be used as the exogenous source of folate for the mammalian cells.

As discussed the ultimate aim in developing this method was to determine if a change in folate co-factor distribution could be identified for the common MTHFR C677T polymorphisms and to examine the effect MTX would have on the folate co-factor distribution in mammalian cells of both MTHFR 677CC and MTHFR 677TT genotypes before and after MTX treatment. Initially the robust and rapidly proliferating ECV304 cell line was used to develop and validate the methodology in mammalian cells.

3.2 RESULTS

3.2.1 Verification of Folate Standards

In order to verify each folate co-factor standard received from Shircks Laboratory, the absorption spectra for each was determined. The λ_{max} for each folate co-factor standard was identical to that reported in Blakley and Benkovic (1984) and therefore each of the folate co-factor standards could be verified.

An attempt was made to use the combined HPLC/microbiological method developed by Belz and Nau (1998) to identify and quantify the set of folate co-factor standards injected onto the HPLC columns. Microbiological assay results are acceptable only if the background absorbance readings at 590nm are less than 0.1. Initially, a background 96 well plate was set-up, which contained 9 drops of eluted mobile phase. The plate was freeze-dried, resuspended in 100 µl of 1%(w/v) sodium ascorbate and assayed using the *L.casei* microbiological assay as per Belz and Nau (1998). The purpose of the background plate was to determine if the mobile phase eluting from the column contained any folate contamination that could interfere with microbiological assay results. The background readings for the eluted mobile phase varied from 0.1 to 0.4, which indicated that following elution from the HPLC column the mobile phase was contaminated. The microbiological assay is very sensitive to background folate contamination and as a result the eluted folate co-factor standards could not be quantified using the microbiological assay as per Belz and Nau (1998).

Instead a microbiological assay was carried out on each the folate co-factor standards in order to establish that each of the folate standards were, as expected biologically active folate co-factors. For the microbiological assay a set of standards was prepared at concentrations of 0.2265µm in 0.5% sodium ascorbate and the assay was performed on each as described in method 2.15. A comparison was made between the folate co-factors and a folic acid standard (in-house standard) see figure 3.1. A set of low, medium and high folate quality control samples were included in each assay to ensure method validity. All the folate co-factor standards were biologically active as they all supported the growth of *L.casei.* The *L.casei* growth response to each of the folate co-factors varied slightly however, these assays were not carried out on the same day and therefore the variation could be due to intra-assay variation. The *p*ABG standard did not support the growth of *L.casei*, which is what would be expected, as *p*ABG is a degradation product of folate and is biologically inactive.

3.2.2 Identification of Folate Standards on the HPLC

A set of folate co-factor standards were prepared as described in section 2.3.2 and separated using the HPLC method developed by Quinlivan *et al.,* (2000). This ion-pair reverse phase chromatography method used a picA reagent as the ion-pair molecule in the mobile phase. PicA is a quaternary amine and is bound to the hydrocarbon side-chain of the C_{18} Phenomenex Kingsorb column and the folate co-factors were bound to this complex in varying degrees depending on their relative charge. The eight folate co-factor standards eluted in the following order

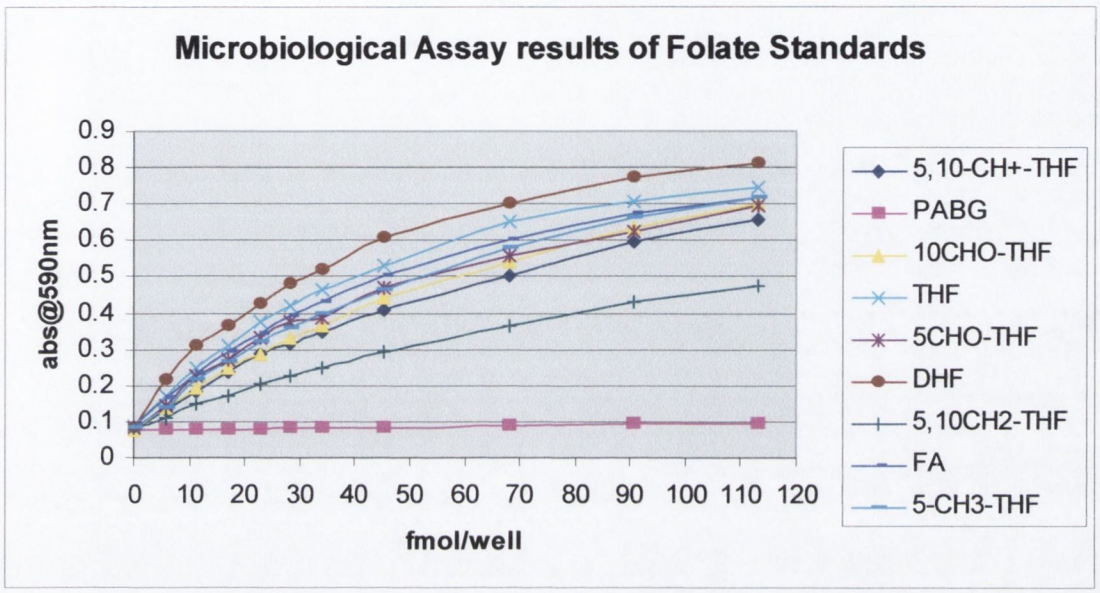


Figure 3.1: The effect of folate co-factor standards on the growth of *L.Casei* compared with an "in-house" folic acid standard. A set of folate co-factor standards were prepared at concentrations of 0.2265µm in 0.5%(w/v) sodium ascorbate and a standard curve was constructed in the region 0-113.3fmol/well which is the equivalent of 0-50pg/well using a 96 well microtitre plate. All wells were brought to a volume of 100µl by the addition of 0.5% sodium ascorbate buffer. 200µl of folate media inoculated with *L.casei* was added to all wells. The plates were incubated at 37°C for 42hr in a non-humidified warm air incubator. The plates were then mixed thoroughly by inversion and read by densitometry at 590nm. The test results were calculated from the standard curve by a computer package Genesis Lite version 3.0 (from Labsystems and Life Sciences International) to give results in ng/ml, which is then converted to pmol/ml.

and gave retention times as follows; 5,10-CH^{+}-THF (2.5min), *p*ABG (5.3 min), 10-CHO-THF (6.2 min), THF (7.2 min), 5-CHO-THF (10.1 min), 5,10-CH_2-THF (12.8 min), DHF (11.9 min), folic acid (16.5 min) and 5-CH_3-THF (23.5 min). Each standard was injected in triplicate and this was carried out on three consecutive days. The mean peak elution times for each triplicate standard run was recorded on three consecutive days, the standard deviation and percentage coefficient of variation was calculated for each folate co-factor to ensure the reproducibility and repeatability of the method, see table 3.1. The intra-assay variation for each folate co-factor standard was less than 5%, which confirmed that the method gave reliable and reproducible results. Each cell extract sample to be analysed using this HPLC was spiked with a standard mix and the elution times of the folate co-factors in the standard spike were identical to those recorded previously without the cell extracts.

3.2.3 Determination of Growth Conditions for *E.coli* 8879

Prior to determining the effect TMP had on folate co-factors within *E.coli*_8879 the optimum growth conditions for the bacteria had to be determined. This was performed in triplicate as per method 2.5.5. From the recorded absorbance readings at 590nm for each time-point, the beginning and end of log phase growth could be established and hence the incubation time required for the bacteria to have reached logarithmic growth before harvesting, see table 3.2. Establishing the correct growth conditions was necessary to ensure the maximum uptake of [^{3}H]PABA prior to treatment with TMP. The first logarithmic growth phase was reached following a 10hr incubation. The second logarithmic growth phase was

	Peak Elution Times (min)								
Result no.	5,10-CH^+-THF	pABG	10-CHO-THF	THF	5-CHO-THF	DHF	5,10-CH_2-THF	FA	5-CH_3-THF
1	2.4	5.3	6.3	7.2	10.1	11.7	12.7	16.4	23.5
2	2.4	5.3	6.2	7.2	10.1	11.7	13	16.6	23.1
3	2.6	5.4	6.2	7.2	10.1	11.7	12.5	16.5	23.8
Mean	**2.5**	**5.3**	**6.2**	**7.2**	**10.1**	**11.7**	**12.7**	**16.5**	**23.5**
SD	**0.1**	**0.1**	**0.1**	**0.0**	**0.0**	**0.0**	**0.3**	**0.1**	**0.4**
%CV	**4.7**	**1.1**	**0.9**	**0.0**	**0.0**	**0.0**	**2.0**	**0.6**	**1.5**

Table 3.1: **The mean peak elution times of folate co-factor standards.** The mean peak elution time of triplicate runs for each of the eight folate co-factor standards was recorded on hree consecutive days, using freshly made mobile pahse each day. The mean, standard deviation and percentage coefficient of variation of the mean elution times was calculated in order to determine the inter-assay variation for each folate co-factor standard.

	Growth Curve Results for *E.coli* 8879 in Glucose Media		
	Start of Log Growth (hrs)	End of Log Growth (hrs)	Bacterial Harvest Time (hrs)
Growth Phase 1	8	12	10
Growth Phase 2	5	9	6.5
Growth Phase 3	2	4	3

Table 3.2: The growth response of *E.coli* 8879 in glucose growth media. The logarithmic growth was determined for each growth phase from absorbance readings recorded hourly at 590nm.

reached following a 6.5hr incubation and the third following a 3hr incubation time. All *E.coli* 8879 experiments were performed on cells in log phase growth.

3.2.4 Establishing the Minimum Concentration of TMP to cause a Maximum Inhibitory effect on *E.coli* 8879

The next step was to determine the minimum concentration of TMP required to cause a maximum inhibitory effect using the method described in section 2.5.6. Based on the previous experiments, untreated *E.coli* 8879 had reached the end of logarithmic growth following a 4hr incubation period therefore varying concentrations of TMP from 0-200µg/ml in media were prepared and the cells were incubated for 4hr in 5mls of the media. From the recorded absorbance readings a TMP concentration of 2µg/ml was shown to cause a maximum inhibitory effect on *E.coli* 8879 following a 4hr incubation, see figure 3.2.

3.2.5 Effect of TMP on folate co-factors in *E.coli* 8879

*E.coli*_8879 was labelled with 12.27µCi[^{3}H]-PABA, which is the equivalent of 300pm[^{3}H]-PABA, during the second growth phase and harvested. The bacteria were re-suspended in 5ml of media containing 2µg/ml of TMP and incubated for varying time points between 0-24hr as per method 2.5.6. The *E.coli* samples were harvested and the folate co-factors were extracted as per method 2.5.7 and separated by HPLC as described by Quinlivan *et al.*, (2000). The mobile phase contained 5mM picA in 0.1M sodium acetate (pH 6.0) and methanol at (16%) (v/v). The column was eluted isocratically at 1ml/min and fractions were

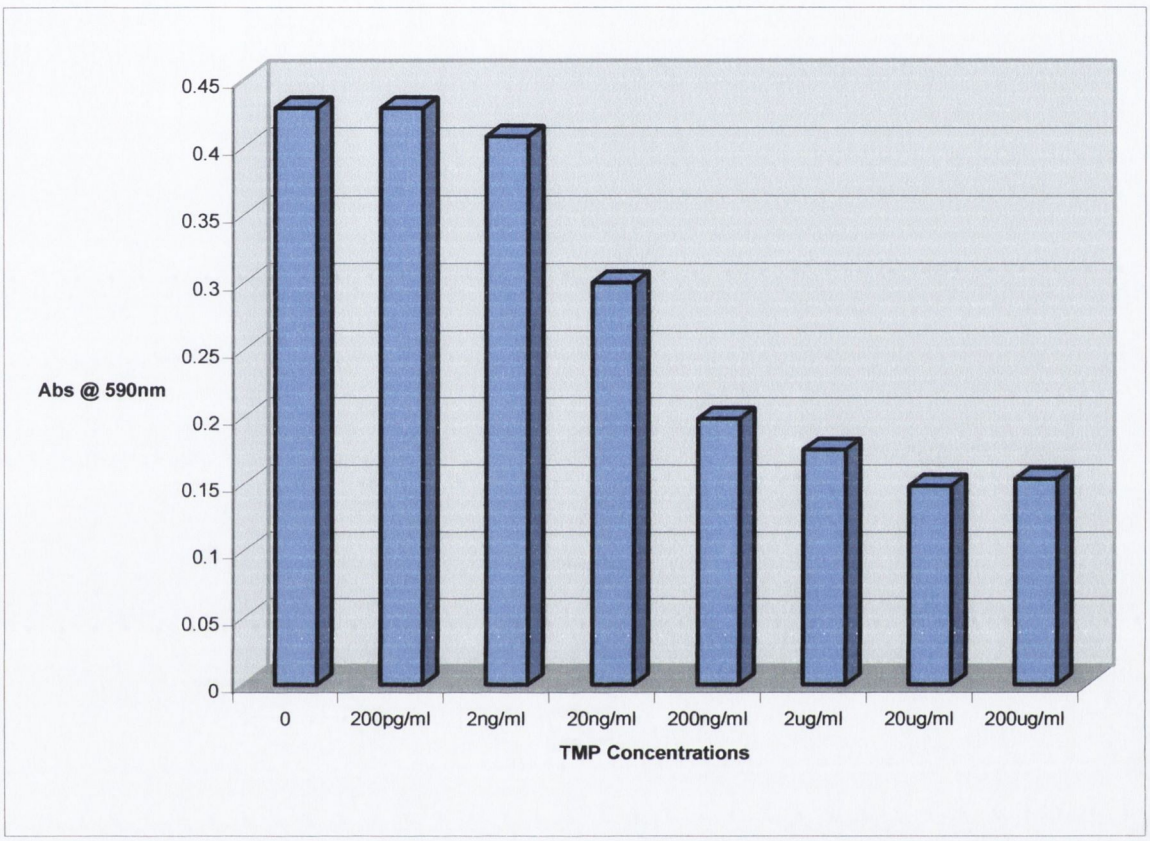


Fig 3.2: Effect of TMP concentration on the growth of *E.coli* 8879. *E.coli* 8879 was incubated in glucose growth media containing varying concentrations of TMP ranging from 0-200µg/ml for 4 hours. Absorbance readings were recorded and the minimum TMP concentration which caused the maximum growth inhibitory effect on the *E.coli* 8879 was calculated to be 2µg/ml. This was the concentration of drug used to treat the *E.coli* prior to HPLC analysis.

collected at 0.5ml/min intervals. The results were graphed in figure 3.3. The original method reported that a 16% (v/v) MeOH mobile phase separated all the folate co-factors but, as shown in figure 3.4, this could not be repeated using the HPLC system available. As shown, the 10-CHO-THF peak and the THF peaks co-eluted as did the 5,10-CH_2-THF and the DHF peaks. In order to improve the separation it was decided to lower the percentage MeOH concentration in the mobile phase to 14% (v/v) and to collect fractions at 0.25ml/min instead of 0.5ml/min.

To examine the effectiveness of these changes, the TMP treated *E.coli* samples were re-analysed using the modified method. It was expected that due to the instability of some of the folate co-factors, especially DHF, THF and 10-CHO-THF, some breakdown would occur during storage, but the lysate buffer contained mercaptoethanol and sodium ascorbate which can be protective against oxidation of the labile folates, and the problem of poor separation could still be addressed using these samples. Fresh mobile phase was prepared using methanol at (14%) v/v and fractions were collected at 0.25ml/min. As can be seen from figure 3.4 using this method the folate co-factors were better separated and this was the method used in further studies to verify the findings of Quinlivan *et al.,* (2000).

3.2.6 Reproducibility of Assay Results

Using the improved method, duplicate *E.coli* 8879 samples were labelled with 300pmol[^{3}H]-PABA and assayed as per method 2.5.7. A set of folate co-factor standards had been run on the column prior to the *E.coli* sample to establish their

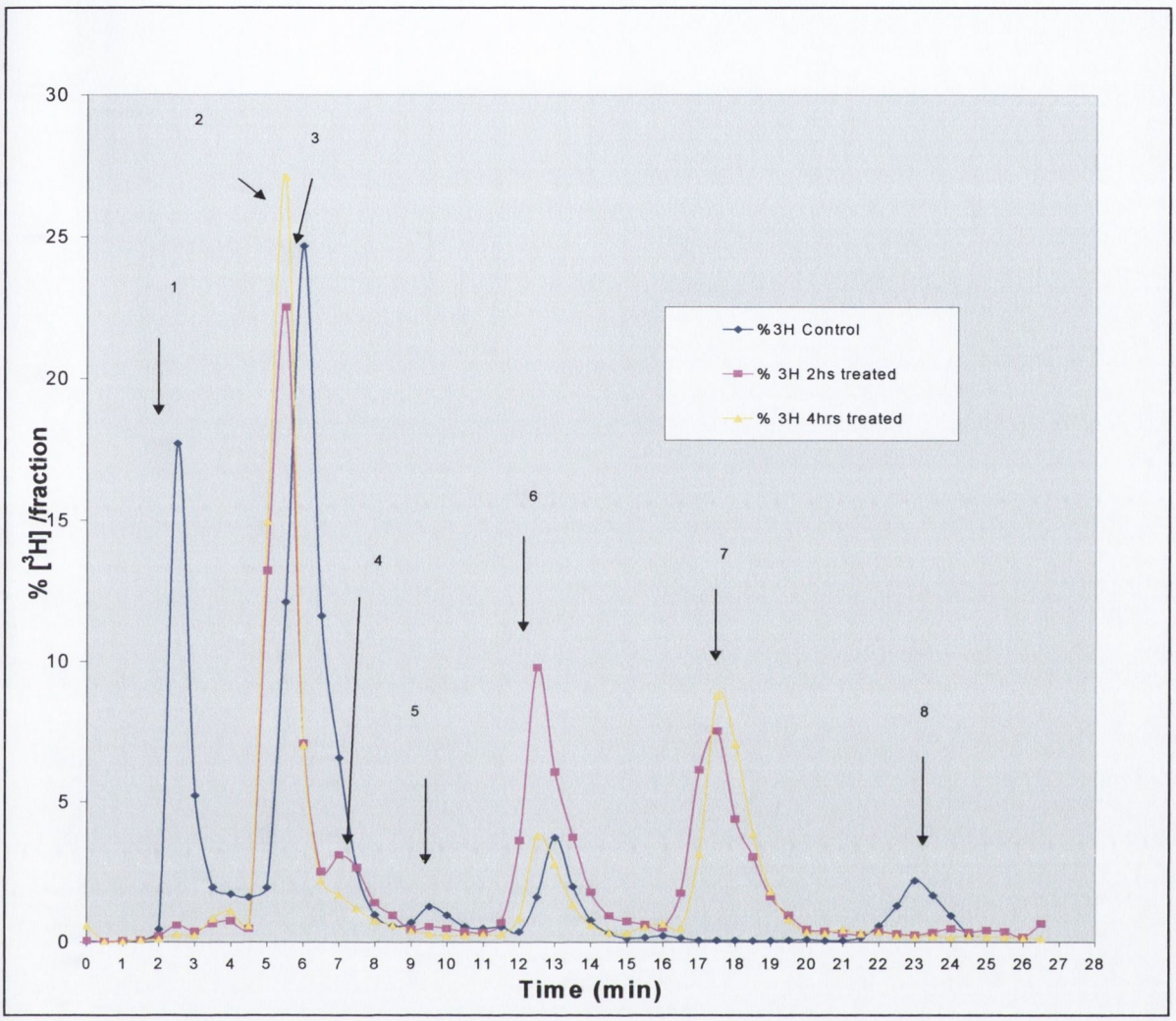


Figure 3.3: Effect of TMP on the intracellular folate co-factor distribution in *E.coli* 8879 using 16% (v/v) methanol mobile phase. *E.coli* 8879 samples were labelled with 300pm[^{3}H]PABA and subsequently treated with 2μg/ml TMP for 0, 2hr and 4hr. The radiolabelled *E.coli* 8879 cell lysates were spiked with 5-CH_3-THF and 5-CHO-THF and analysed on a HPLC using a 16% methanol mobile phase. Fractions were collected at 0.5ml/min and the elution profile of the radiolabelled intracellular folate co-factors were recorded following scintillation counting.
Peak 1: 5,10-CH^+-THF, Peak 2: *p*ABG, Peak 3: 10-CHO-THF and THF, Peak 4: THF, Peak 5: 5-CHO-THF, Peak 6: 5,10-CH_2-THF and DHF, Peak 7: Folic Acid, Peak 8: 5CH_3-THF

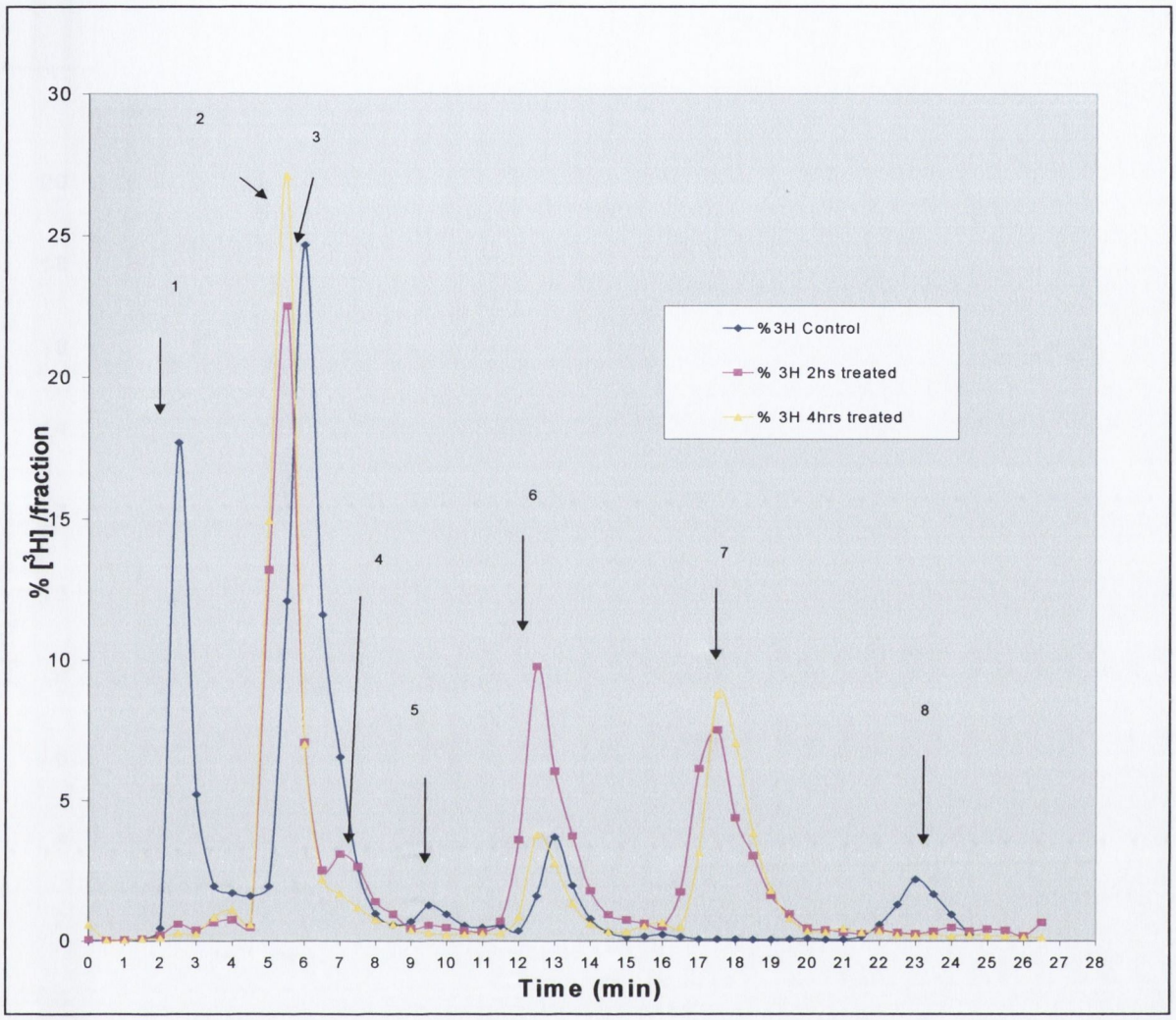


Figure 3.3: Effect of TMP on the intracellular folate co-factor distribution in *E.coli* 8879 using 16% (v/v) methanol mobile phase. *E.coli* 8879 samples were labelled with 300pm[^{3}H]PABA and subsequently treated with 2μg/ml TMP for 0, 2hr and 4hr. The radiolabelled *E.coli* 8879 cell lysates were spiked with 5-CH_3-THF and 5-CHO-THF and analysed on a HPLC using a 16% methanol mobile phase. Fractions were collected at 0.5ml/min and the elution profile of the radiolabelled intracellular folate co-factors were recorded following scintillation counting.
Peak 1: 5,10-CH^+-THF, Peak 2: *p*ABG, Peak 3: 10-CHO-THF and THF, Peak 4: THF, Peak 5: 5-CHO-THF, Peak 6: 5,10-CH_2-THF and DHF, Peak 7: Folic Acid, Peak 8: 5CH_3-THF

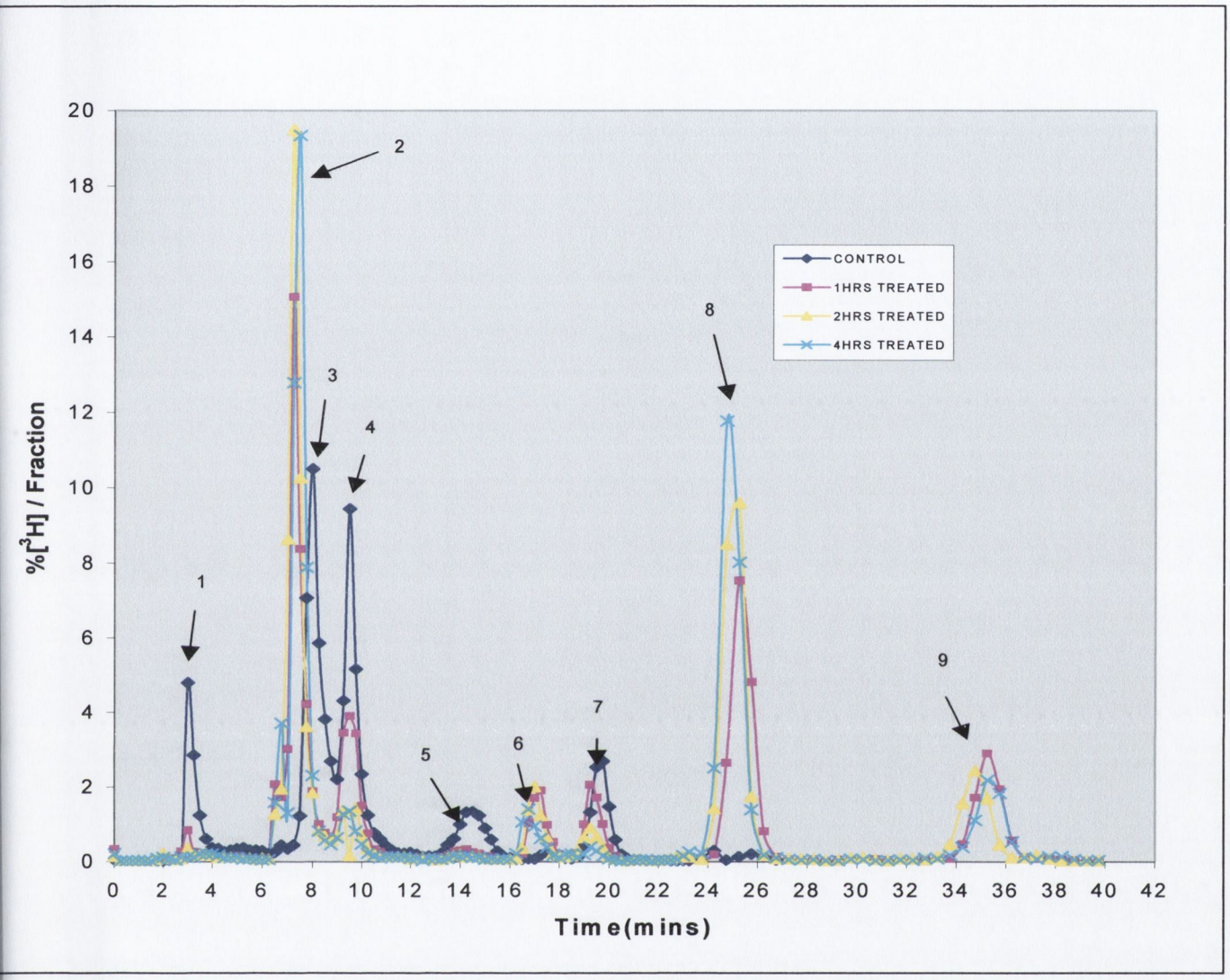


Figure 3.4: Effect of TMP on the intracellular folate co-factor distribution in *E.coli* 8879 using 14% (v/v) methanol mobile phase. *E.coli* 8879 samples were labelled with 300pm[^{3}H]*p*ABA and subsequently treated with 2µg/ml TMP for 0, 1hr, 2hr and 4hr. The radiolabelled *E.coli* 8879 cell lysates were spiked with 5-CH_3-THF and 5-CHO-THF and analysed on a HPLC using a 14% methanol mobile phase. Fractions were collected at 0.25ml/min and the elution profile of the radiolabelled intracellular folate co-factors were recorded following scintillation counting.
Peak 1: 5,10-CH^+-THF, Peak 2: *p*ABG, Peak 3: 10-CHO-THF, Peak 4: THF, Peak 5: 5-CHO-THF, Peak 6: DHF, Peak 7: 5,10-CH_2-THF, Peak 8: Folic Acid, Peak 9: 5-CH_3-THF

elution times and the sample itself was spiked with 5-CH_3-THF and 5-CHO-THF before injection. The duplicate radiolabelled folate co-factor results were recorded; see table 3.3. Approximately 87% of the radioactivity injected onto the column was recovered in the radioactive folate co-factor peaks, with very little variation in relation to the peak distribution between the duplicate samples. The remaining 13% radioactivity was found in the background radioactivity between peaks, as when the radioactivity results from each vial were added 100% recovery from the column was reported.

3.2.7 The effect of TMP on folate co-factor distribution in *E.coli* 8879 cells

Following on from the previous section the effect of TMP on folate co-factor distribution within the cell was investigated; see table 3.4 for results. *E,coli* 8879 was labelled with 300pmol[^{3}H]-PABA and incubated for 10hr in an orbital shaker at 37°C and subsequently treated in 5ml of media containing 2μg/ml of TMP as described in method 2.5.6 for varying time points from 0-24hr and prepared for assay as per method 2.5.7. The results were reported in table 3.4. The average percentage recovery of radioactivity in the folate co-factor peaks was 88%±10 (n=12).

The fractions containing each folate co-factor were added and the result was re-adjusted to 100% in order to make a comparison with the results published by Quinlivan *et al.,* (2000), see results table 3.4. Re-adjusting the folate co-factor peaks to 100% took into account the differences in recovery of radioactivity from the column for each sample.

Co-Factors	1	2	Mean
5,10-CH^+-THF	10	7	9
p ABG	0	0	0
10-CHO-THF	40	42	41
THF	11	9	10
5-CHO-THF	4	3	4
DHF	0	0	0
5,10-CH_2-THF	9	11	10
FA	0	0	0
5-CH_3-THF	13	12	13
% Recovery of DPM	88	86	87

Table 3.3: The intracellular folate co-factor distribution of *E.coli* 8879. A comparison of the percentage of radioactive folate co-factor peak elutions from duplicate control *E.coli* 8879 samples grown in glucose growth media containing 300pm[^{3}H]*p*ABA and incubated for 10hr at 37°C.

Co-Factors	Control		25 Mins		1 Hour		2 Hour		4 Hour		24 Hour	
	A	B	A	B	A	B	A	B	A	B	A	B
THF CO-ENZYMES	100	100	12	22	7	19	6	8	0	8	0	0
DHF			49	38	33	28	23	26	2	14	0	7
PABG			23	31	33	39	39	48	58	51	55	77
FA			16	9	27	14	32	18	40	27	45	13

Table 3.4: The effect of trimethoprim on the intracellular flux of DHF, *p*ABG, FA and THF co-enzymes in *E.coli* 8879. A comparison of the percentage radioactive folate co-factor peak elutions of *E.coli* 8879 samples labelled with 300pm[^{3}H]*p*ABA and treated for 0, 25min,1hr, 4hr, 4hr and 24hr with 2μg/ml TMP. The Original results reported by Quinlivan et al, (2000) (A) were compared with results obtained using the method described in section 3.2.5 (B).

The main reason for comparing the two sets of results was to verify that the results obtained by Quinlivan *et al.,* (2000) could be reproduced using the current HPLC system set-up. As discussed above, a few modifications were made to the original method to allow the results to be compared. The main differences between the two sets of results were: 1) The THF co-enzymes were completely depleted following 4hr incubation with TMP in the original report but in the present study there was still 8% present. 2) There was 12% more DHF present in this study than the original study which reported only 2% DHF present following 4hr incubation with TMP. 3) The percentage of *p*ABG and FA in the new study were not as high as originally reported following 4hr incubation with TMP. The new THF co-enzyme results obtained were further analysed by separating them into each their folate co-factor forms. This was possible by reducing the methanol concentration in the mobile phase to 14% (v/v), which allowed THF and 10-CHO-THF to be separated as shown in figure 3.5 and table 3.5. Using the modified method, when the TMP was removed and the *E.coli* re-grown, the level of *p*ABG was still increasing and the level of FA was decreasing whereas in the original results the level of *p*ABG was decreasing and the level of FA was increasing.

Once it had been established that the results reported by Quinlivan *et al.,* (2000) could be reproduced, the next step was to transfer the method from a bacterial cell model to a mammalian cell model. The robust and rapidly proliferating ECV304 cell line was chosen in order to determine the optimum conditions required for the separation of mammalian intra-cellular folate co-factors.

Co-Factors	*E.coli* 8879 2ug/ml TMP						
	Control	*25 mins*	*60 mins*	*120 mins*	*240 mins*	*24 hours*	*Treated & Untreated*
5,10-CH^+-THF	8	1	1	0	0	0	0
p ABG	0	31	39	50	49	78	58
10-CHO-THF	46	2	0	0	0	0	8
THF	16	6	13	0	0	0	3
5-CHO-THF	13	0	0	0	0	0	0
DHF	0	38	28	27	16	7	11
5,10-CH_2-THF	13	8	0	0	0	0	0
FA	0	10	14	19	27	15	21
5-CH_3-THF	14	4	5	4	8	0	5

Table 3.5: The effect of trimethoprim on the intracellular flux of DHF, *p*ABG, FA, 5,10-CH^+-THF, 10-CHO-THF, 5-CHO-THF, 5,10CH_2-THF and 5-CH_3-THF in *E.coli* 8879. The percentage radioactive folate co-factor peak elutions of *E.coli* 8879 samples labelled with 300pm[^{3}H]*p*ABA and treated for 0,25min,1hr,4hr,4hr and 24hr with 2μg/ml TMP. The HPLC analysis was carried out as per method 3.2.5 using a 12% methanol mobile phase.

3.2.8 The effect of folic acid on the growth of ECV304 Cells

Growth curves were set up as described in method 2.9.4 using medium 199, which contained 22.65nM folic acid and folate free medium 199. The cells were seeded $1x10^5$ cells/well in triplicate for each time point and counted at 24hr intervals over 5 days. Each experiment was set up in triplicate (n=3). The mean and standard deviation were calculated for each time point and plotted on a graph, see figure 3.5(a) and (b). The normal growth response for ECV304 cells was firstly established and then compared with the growth response in folate free media. There appeared to be no difference in cell growth or appearance between the two growth conditions.

3.2.9 The effect of folate depletion on ECV304 cell growth

Growth curves were set up as described in method 2.9.4 using cells that had been grown for 4 passages in folate free medium 199. The cells were seeded $1x10^5$ cells/well in triplicate for each time point and counted at 24hr intervals over 5 days and graphed, see figure 3.. A comparison was made between the growth rates of cells grown in full medium 199 containing 22.6nM folic acid and cells which had been depleted of folic acid for 4 passages prior to the growth curve. As shown there was no difference in the growth response or appearance of cells following the depletion of folic acid for four passages when compared with normal growth responses.

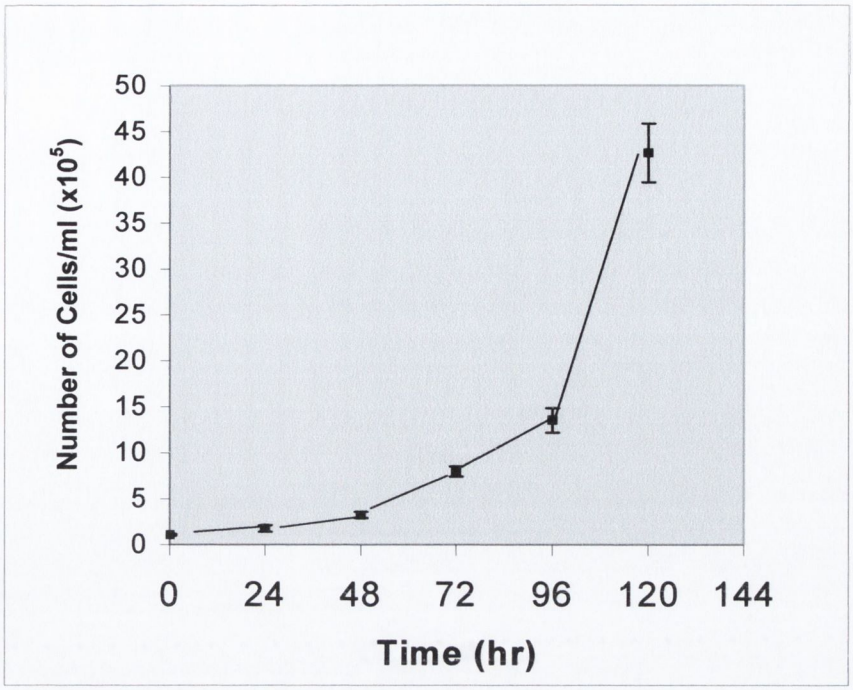


Figure 3.5(a)

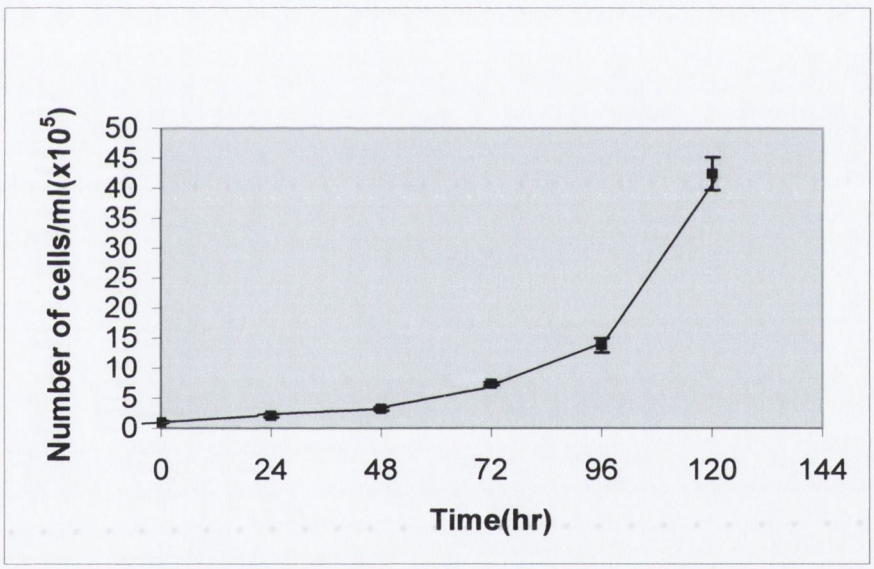


Figure 3.5(b)

Figure 3.5 The effect of folic acid on the growth response in ECV304 cells. ECV304 cells seeded 1x10^{5}cells/well and incubated at 37°C, 5% CO_2 for defined time points (0, 24hr, 48hr, 72hr, 96hr and 120hr) and counted. All values represent triplicate experiments and error bars are SD. A: Cells grown in medium 199. B: Cells grown in medium 199 without folic acid.

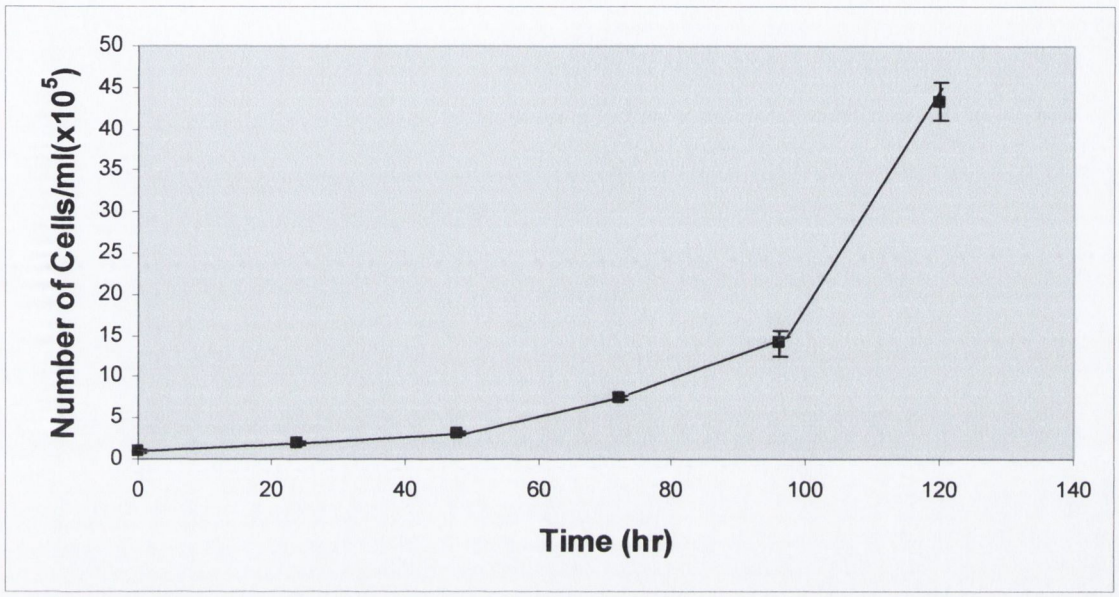


Figure 3.6 The effect of folic acid depletion on the growth response in ECV304 cells. ECV304 cells were seeded $1x10^5$cells/well and grown for four passages in medium 199 without folic acid at 37°C, 5% CO_2. The cells were then seeded at $1x10^5$cells/well and incubated in folate depleted medium 199 for defined time points (0, 24hr, 48hr, 72hr, 96hr and 120hr) and counted. All values represent triplicate experiments and error bars are SD.

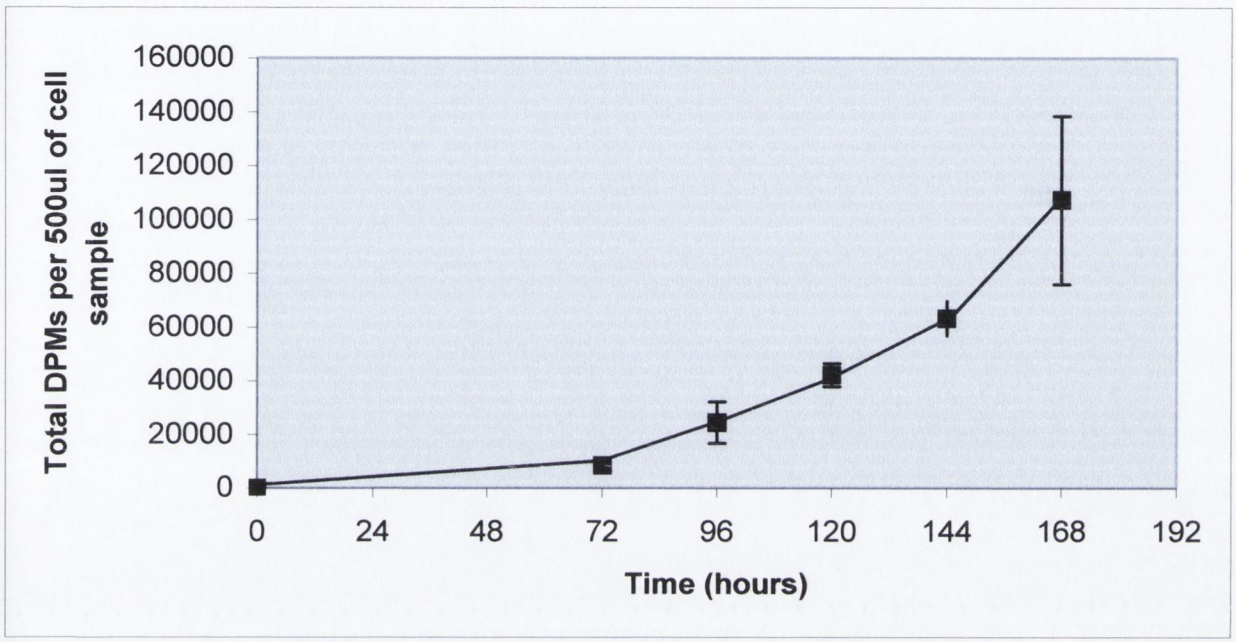


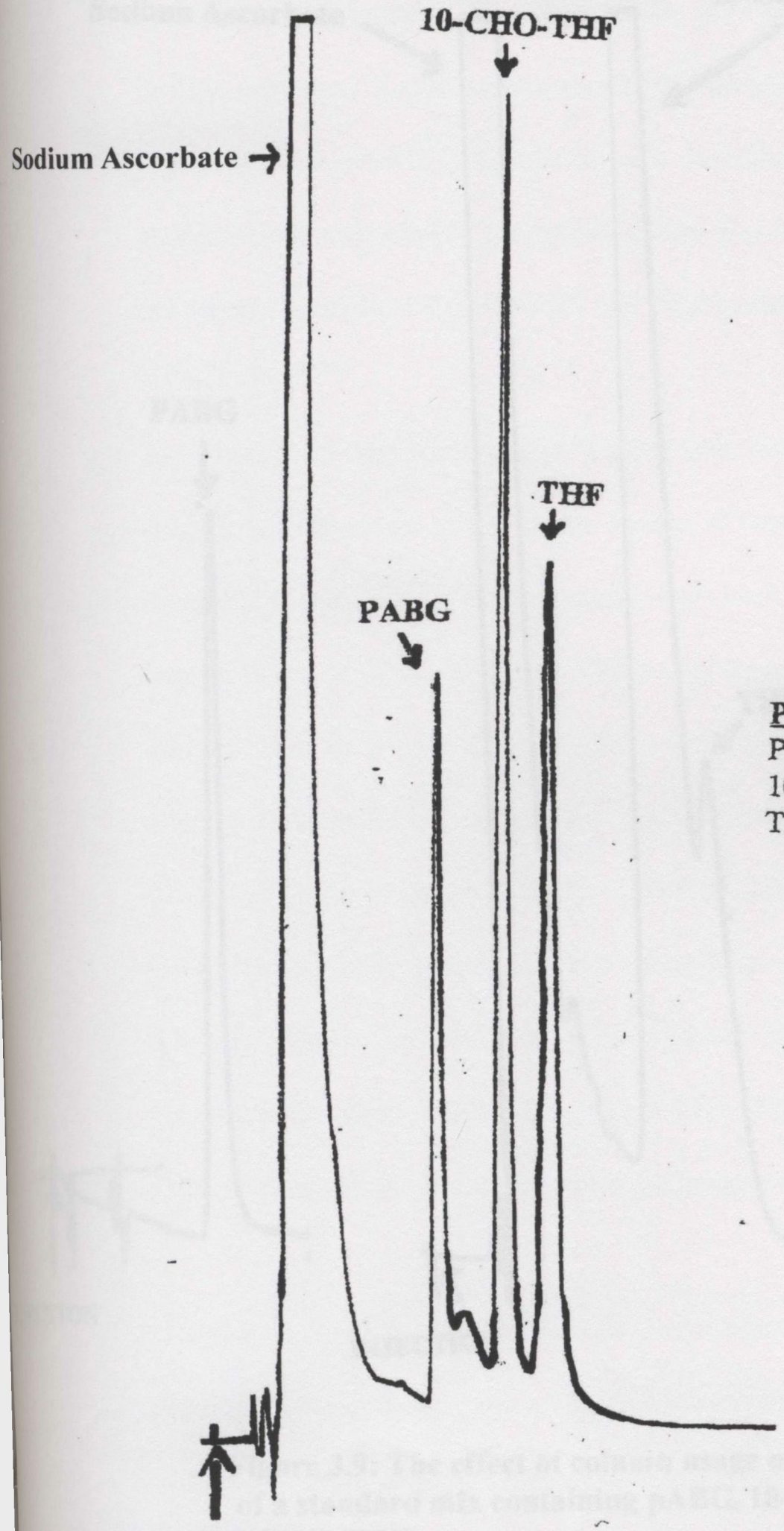
Figure 3.7: Uptake of [^{3}H]5CHO-THF by ECV304. ECV304 cells were seeded 1x10^{5}cells/well and grown for four passages in medium 199 without folic acid at 37°C, 5% CO_2. The cells were then seeded at 1x10^{5}cells/well and incubated in folate depleted medium 199 supplemented with 2.9nmol [^{3}H]5CHO-THF for defined time points (0, 72hr, 96hr, 120hr, 144hr and 168hr). The total number of dpm counts incorporated into the cells at each time point was determined by scintillation counting. All values represent triplicate experiments and error bars are SD.

3.2.10 Uptake of [^{3}H] 5-CHO-THF on cells, which have been depleted on folate for 4 passages

Cells which had been depleted of folate for 4 passages were seeded 1x10^{5}cells/well and grown in 4ml of folate free medium 199 supplemented with 0.5μCi [^{3}H] 5-CHO-THF which is the equivalent of 2.9nmol [^{3}H]5-CHO-THF. The cells were counted at varying time-points in triplicate and re-suspended in 500μl of PBS. Each cell suspension was sonicated and centrifuged as per method 2.10. 50μl of the supernatant obtained was suspended in scintillation fluid and counted in triplicate to determine the amount of [^{3}H] incorporated within the cells. The results were multiplied by 10 to determine the amount of radiolabel in the 500μl of cell supernatent, see figure 3.7. This experiment was carried out in triplicate (n=3). The uptake of [^{3}H]5-CHO-THF increased as the cell number increased demonstrating that as the cells divide they incorporated the radiolabelled folate.

3.2.11 Selection of HPLC system for the separation of folate co-factors: Reverse-phase chromatography versus Ion-pair reverse-phase chromatography

Initially, using the Phenomenex Kingsorb column and picA reagent mobile phase, as set up for the bacterial system, good separation of all folate co-factor peaks and *p*ABG was observed (see figure 3.8) but with the increasing number of cell samples injected onto the column the resolution of the 10-CHO-THF and THF peaks became very poor as shown in figure 3.10. In addition the back pressure of the pumps began to increase. The problem of co-elution began to re-occur between 10-CHO-THF and THF co-factors, but the other co-factors were being separated consistently. Changing the guard



PEAK	TIME
PABG	6mins 12
10-CHO-THF	7mins 45
THF	8mins 56

Figure 3.8: Chromatogram of pABG, 10-CHO-THF and THF standard mix.
A standard mix of pABG, 10-CHO-THF and THF was analysed on HPLC using a waters 510 pump, a new phenomenex kingsorb column and a 16% (v/v) methanol picA reagent mobil phase.

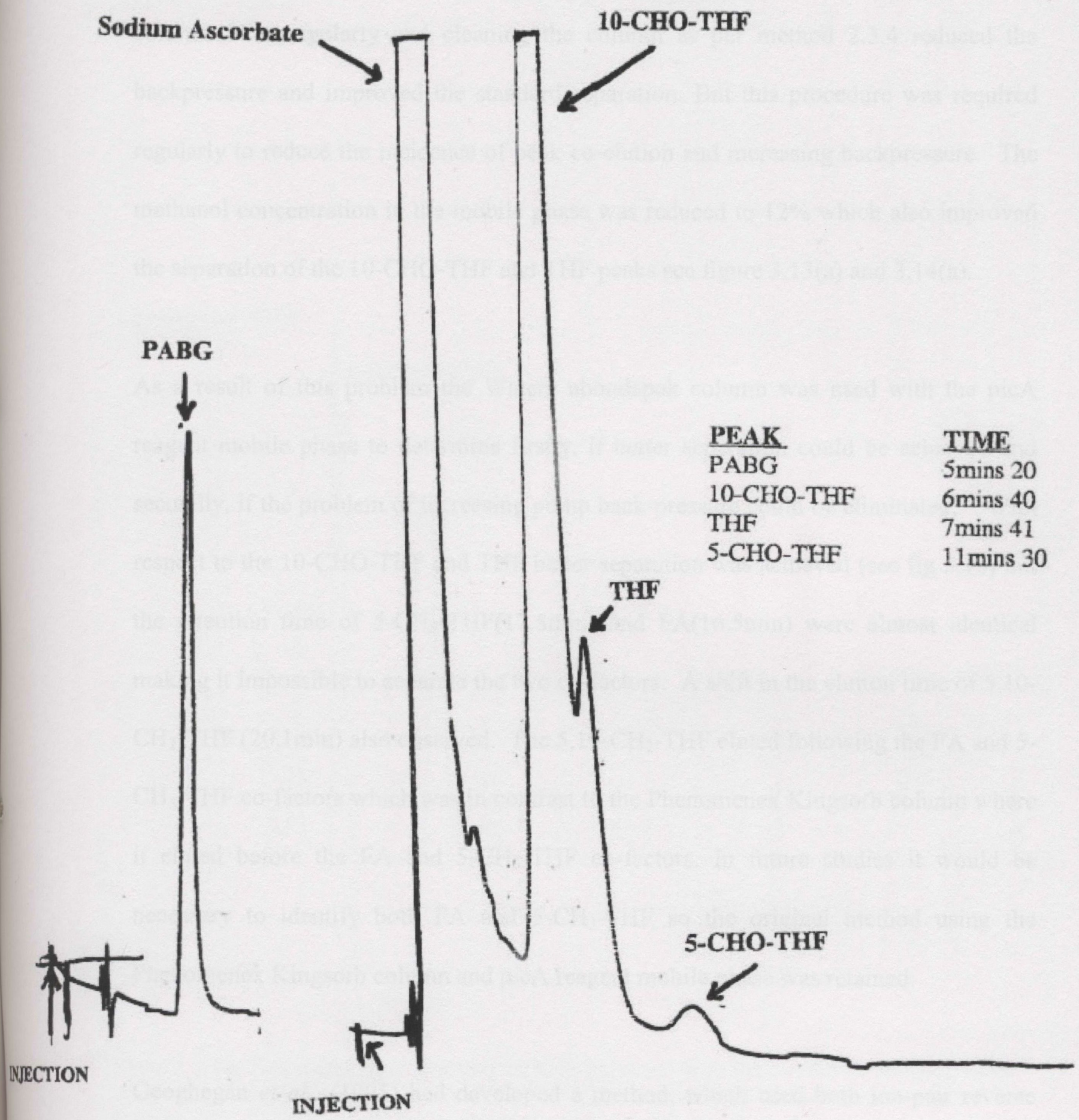


Figure 3.9: The effect of column usage on the peak elution profile of a standard mix containing pABG, 10-CHO-THF, THF and 5-CHO-THF.

A standard mix containing pABG, 10-CHO-THF, THF and 5-CHO-THF was analysed on HPLC using a waters 510 pump, a phenomenix kingsorb column following approximately 20 sample injections and a 16% (v/v) methanol picA reagent mobile phase. As can be seen the 10-CHO-THF and THF peaks are beginning to co-elute.

column filter regularly and cleaning the column as per method 2.3.4 reduced the backpressure and improved the standard separation. But this procedure was required regularly to reduce the incidence of peak co-elution and increasing backpressure. The methanol concentration in the mobile phase was reduced to 12% which also improved the separation of the 10-CHO-THF and THF peaks see figure 3.13(a) and 3.14(a).

As a result of this problem the Waters ubondapak column was used with the picA reagent mobile phase to determine firstly, if better separation could be achieved and secondly, if the problem of increasing pump back-pressure could be eliminated. With respect to the 10-CHO-THF and THF better separation was achieved (see fig 3.10) but the retention time of 5-CH_3-THF(17.5min) and FA(16.5min) were almost identical making it impossible to separate the two co-factors. A shift in the elution time of 5,10-CH_2-THF (20.1min) also observed. The 5,10-CH_2-THF eluted following the FA and 5-CH_3-THF co-factors which was in contrast to the Phenomenex Kingsorb column where it eluted before the FA and 5-CH_3-THF co-factors. In future studies it would be necessary to identify both FA and 5-CH_3-THF so the original method using the Phenomenex Kingsorb column and picA reagent mobile phase was retained.

Geoghegan *et al.,* (1995) had developed a method, which used both ion-pair reverse phase chromatography and reverse-phase chromatography to identify the folate co-factors and folate degradation products in the urine of rats. It was decided to test both these HPLC methods to determine if a more suitable HPLC system could be developed to separate intra-cellular folate co-factors in a mammalian cell model. The first was ion-pair reverse phase chromatography using a Phenomenex Kingsorb C18 (pore size 3μ) and picA reagent mobile phase with 12% methanol at pH 6.0 (see material and methods

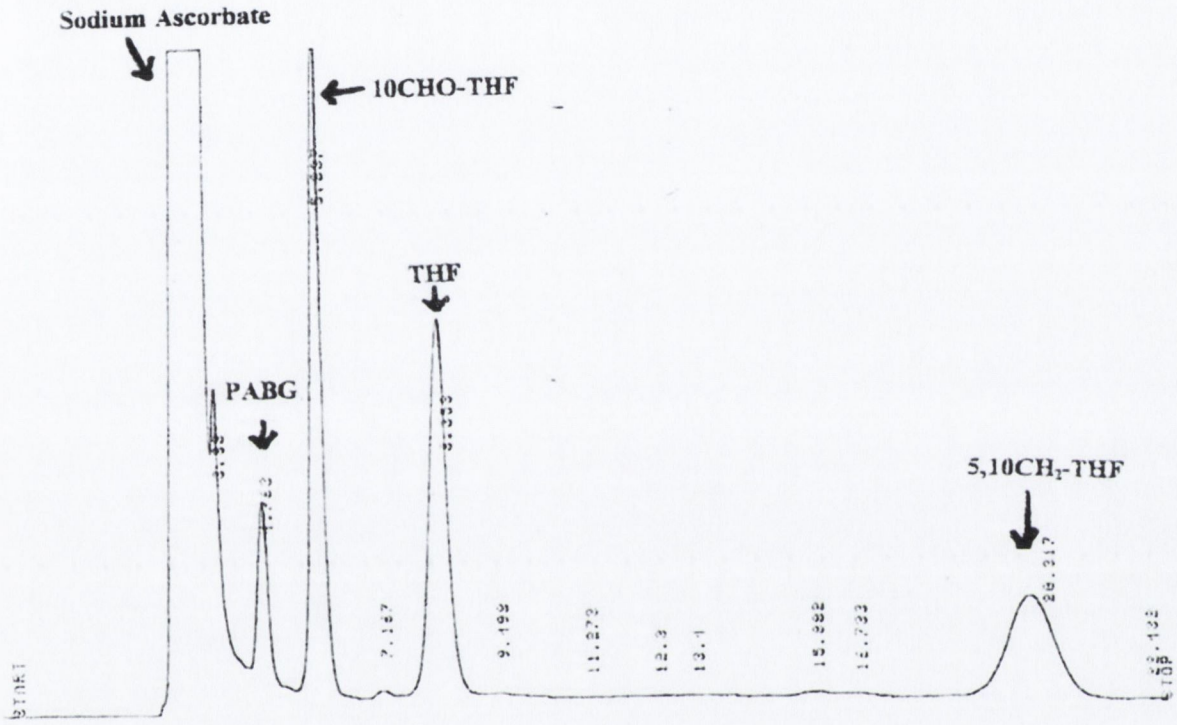


Figure 3.10: The effect of a ubondapak C18 column on folate co-factor peak elution. A standard mix of PABG, 10-CHO-THF, THF and 5,10-CH2-THF was analysed on HPLC using a waters ubondapak C18 column and a 12%(v/v) methanol picA reagent mobile phase. Better separation was observed between the 10-CHO-THF and THF peaks when compared to the phenomenex kingsorb column.

2.3.1 for mobile phase composition). This was the same method that was used in the bacterial cell system. The second involved reverse phase chromatography using a Waters μbondapak C18 (pore size 5μ) and a citrate phosphate mobile phase with 12.5 % methanol at pH 4.0 (see material and methods 2.3.1 for mobile phase composition). Folate standards were run on both columns using the two different HPLC systems. Following the examination of the folate co-factor peak distribution from the two systems it was clear that using the reverse-phase HPLC system resulted in the same problem of the co-elution of THF and 10-CHO-THF standards. The 5,10-CH_2-THF standard went undetected using this method. However, a very sharp peak for pABG was identified and the 5-CH_3-THF peak eluted as a sharp peak after the two co-eluted standards of 10-CHO-THF and THF.

Both these methods were used to analyse the intracellular folate co-factor distribution in radiolabelled ECV304 cells. This allowed the verification of the pABG and 5-CH_3-THF peaks, as both are stable at pH 4.0 and pH 6.0. The other folate standards were also verified using both these methods, except for 5,10-CH_2-THF which was undetectable using reverse phase chromatography.

Using both these methods in combination, as per Geoghegan *et al.,* (1995), presented the following problems; 1) using both methods was time consuming, which would eventually result in the breakdown of the more labile folates, 2) the number of radioactive scintillation vials would be doubled, making the analysis very cumbersome. As a result it was decided that using the modified ion-pair reverse phase HPLC method with the Phenomex Kingsorb column and picA reagent mobile phase was the most

suitable method to separate the intracellular folate co-factors in a mammalian cell model.

3.2.12 Development and validation of an ion-pair reverse phase discontinuous gradient HPLC system

The main problem with reducing the methanol concentration in the picA mobile phase from 16% down to 12% was that it took 5-CH_3 –THF over 60 min to elute from the column, see figure 3.13(a) and 3.14(a). As a result a discontinuous gradient system was set up to determine if the run time could be reduced without interfering with the separation of the earlier peaks. A standard mix was prepared containing *p*ABG, THF, 5-CHO-THF, 5,10-CH_2-THF, FA and 5-CH_3-THF and analysed using various gradient conditions. The system which gave the best overall results began with a methanol concentration of 12% for 17min, increased to 14% for 35min and 16% for 60min, see figure 3.12 for results. This discontinuous gradient system reduced the run time by 15-20min and resulted in much sharper peaks for FA and 5-CH_3-THF standards. The standard mix was run in triplicate on three consecutive days, to determine the intra-assay variation for each of the standards in the mix (see table 3.6). The percentage co-efficient of variation for each folate co-factor present in the standard mix was below 10%. Therefore, the new discontinuous gradient method gave results which were as reproducible as the original method results reported by Quinlivan *et al.,* (2000), see results table 3.1.

A double peak of equal area was occasionally observed for the 5,10-CH_2-THF standard, see figure 3.12. The 5,10-CH_2-THF standard was a Shircks (6R,S)-5,10-methylene-

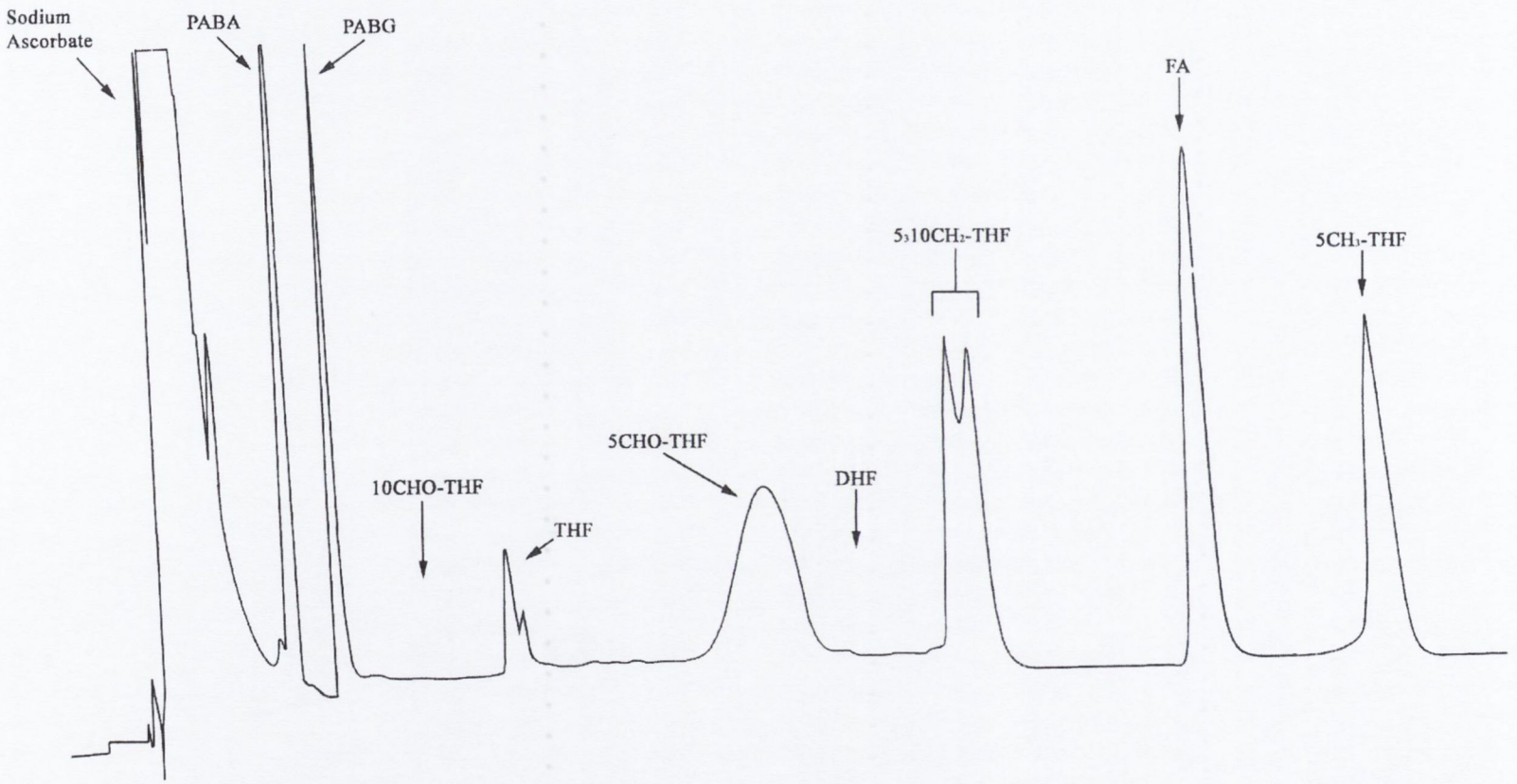


Figure 3.11: Chromatogram of a folate standard mix using discontinouus ion-pair reverse phase gradient HPLC.

Chromatogram of a folate standard mix containing pABA, pABG, THF, 5-CHO-THF, FA and 5-CH3-THF run using a discontinouus ion-pair reverse phase gradient HPLC. The 10-CHO-THF and DHF standards were run separately and their elution times have been marked on the chomatogram.

	Peak Elution Time(min)					
Result no.	pABG	THF	5-CHO-THF	5,10-CH_2-THF	FA	5-CH_3-THF
1	7.692	16.408	22.072	29.498	37.91	44.287
2	8.428	16.067	20.615	29.078	37.665	43.485
3	7.51	16.042	23.233	30.957	39.55	44.805
Mean	**7.9**	**16.2**	**22.0**	**29.8**	**38.4**	**44.2**
SD	**0.5**	**0.2**	**1.3**	**1.0**	**1.0**	**0.7**
%CV	**6.2**	**0.1**	**1.3**	**3.3**	**2.7**	**1.5**

Table 3.6: The elution profile of a folate co-factor standard mix using an ion-pair reverse phase discontinuous gradient HPLC system. The mean elution time of triplicate runs for the folate co-factor standard mix which was used to spike the cell samples were recorded on three consecutive days, using freshly made mobile phase each day. The mean, standard deviation and percentage coefficient of variation of the mean elution times was calculated in order to determine the inter-assay variation for the folate co-factor standard.

	Cell Pellet before Extraction (dpm)	Cell Pellet after Extraction (dpm)
	129721	128659
	122266	132561
	131706	125962
Mean	**127898**	**129061**
SD	**4977**	**3318**

Table 3.7: **The effect of folate extraction on the intracellular folate co-factors of ECV304 cells.** The amount of radiolabel incorporated into an ECV304 cell sample following incubation with 0.5μCi[^{3}H] 5-CHO-THF was determined by counting the amount of radioactivity present in three separate cell pellets The folates were extracted as per method 2.10 and the radioactivity present in the supernatant following extraction was calculated. As shown there was no loss of radiolabelled folates during the extraction of the folate co-factors from ECV 304 cells.

5,6,7,8 THF Magnesium salt standard and when analysed on a scanning spectrophotometer gave a λ_{max} of 294nm at pH 7.2. The λ_{max} corresponded with that recorded by Blakley and Benkovic (1984) and therefore, it would appear that the 5,10-CH_2-THF standard was being separated into it (R) and (S) form. The absorption spectra results eliminated the possibility that one of the double peaks was an unidentified degradation product or a contaminant in the standard. It was worth noting that the column used to develop the discontinuous gradient system was a new column and the double peak later merged into one a single peak which was slightly broader and higher following continuous usage of the column.

3.2.13 To investigate the time required for the complete folate deconjugation using rat serum using both HPLC and the microbiological assay.

It was reported by Quinlivan *et al.,* (2000) that an incubation time of 45min with rat serum at 37°C ensured the full deconjugation of bacterial folates with no apparent degradation of the folate co-factors except for DHF, which degrades by 13%, if present within the cell using this folate extraction method. The length of time required for the complete deconjugation folate co-factors in mammalian cells was examined.

A stock standard solution (2mg/20ml) of pteroyl-γ-L-glutamic acid, pteroyldi-γ-L-glutamic acid and pteroyltri-γ-L-glutamic acid was prepared as per method 2.2.3. Each standard was run on the HPLC singularly and then in a combined mix see figure 3.12. The elution time of pteroyl-γ-L-glutamic (35.51min) of pteroyldi-γ-L-glutamic acid (39.813min) and pteroyltri-γ-L-glutamic acid (42.475min). 100µl of the standard pteroyltri-γ-L-glutamic acid solution (0.0143µm) was mixed with 25µl of dialysed rat

serum and incubated at 37°C for 0,1 and 2hr incubation times. 100µl of this sample was run on the HPLC. Full deconjugation was observed when the entire pteroyltri-γ-L-glutamic acid peak was converted to a mono glutamic acid standard peak, which was identified the elution times of the standards. Therefore, an incubation time of 1hr is sufficient to allow for full deconjugation of the polyglutamated folates.

An ECV304 cell pellet labelled with 0.5µCi[^{3}H] 5-CHO-THF was resuspended in 1ml of 1% sodium ascorbate and split into two 500µl samples. 100µl rat serum was added to each cell sample and incubated at 37°C for 1 and 2hr respectively to determine if a difference in the HPLC profile could be determined, see figure 3.13(a and b) and 3.14(a and b) for results. There appeared to be complete deconjugation following 1hr incubation with rat serum as each folate peak appeared as one sharp with no tailing peaks present, which might suggest incomplete deconjugation of the folates.

The results reported from these two experiments described above demonstrate that the polyglutamted radiolabelled intracellular folate co-factors are fully deconjugated following an incubation with rat serum for 1hr at 37°C.

3.2.14 To investigate if any radioactive folate is lost during extraction and to determine the degree of breakdown for each of the folate co-factors following extraction and deconjugation.

The amount of radiolabel incorporated into an ECV304 cell sample following incubation with 0.5µCi[^{3}H] 5-CHO-THF was determined by counting the amount of radioactivity present in three separate cell pellets, see table 3.7. The folates were

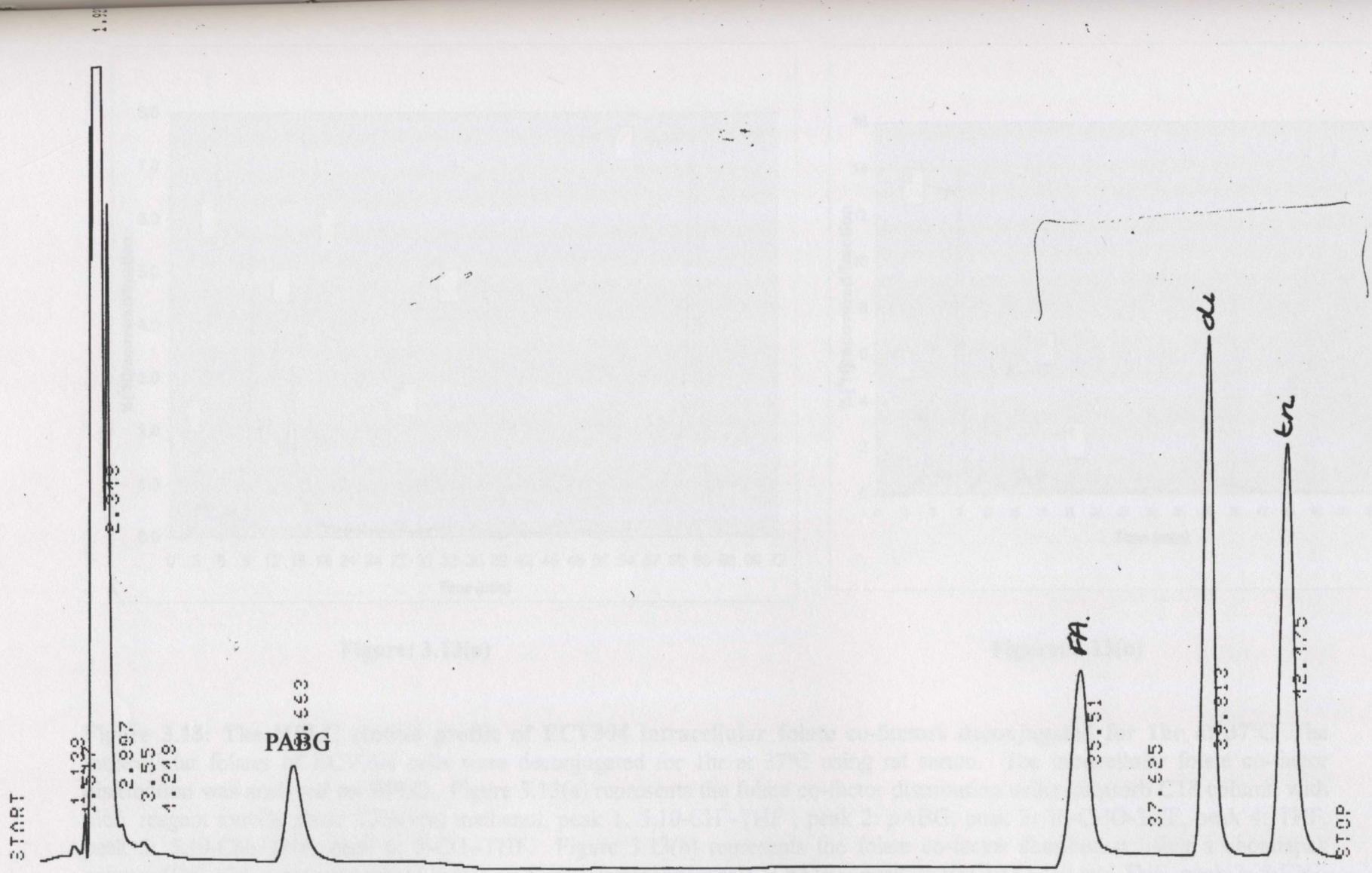


Figure 3.12: The HPLC elution profile of pteroyl-y-L-glutamic acid, pteroyldi-y-L-glutamic acid and pteroyltri-y-L-glutamic acid.

A standard mix of mono-, di- and tri-pteroylglutamic acid was analysed using HPLC. The elution times for each standard were pteroyl-y-L-glutamic acid (35.51min), pteroyldi-y-L-glutamic acid (39.813min) and pteroyltri-glutamic acid (42.475min).

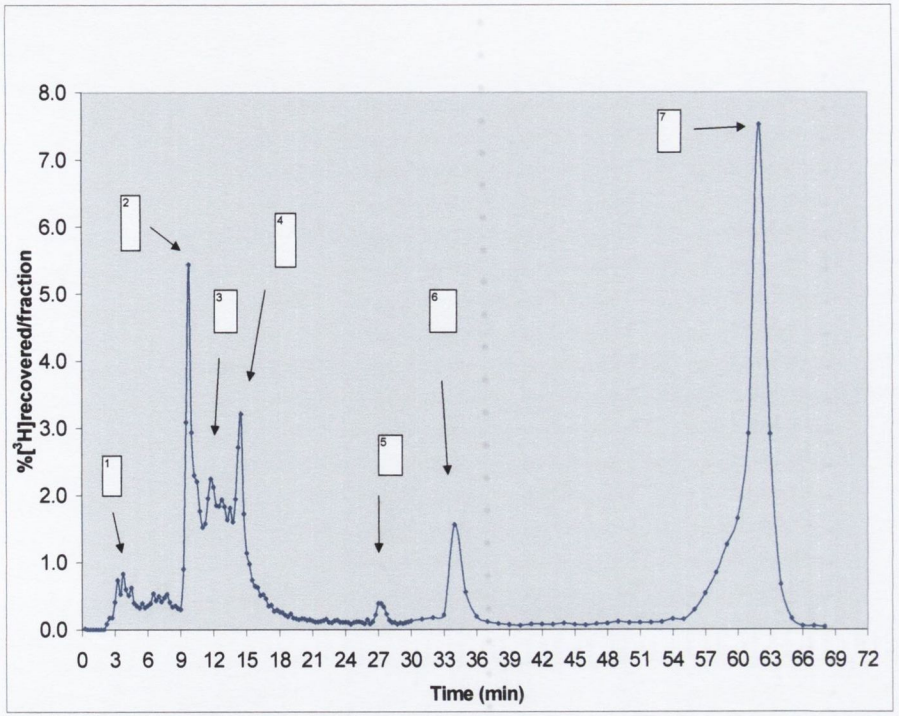


Figure: 3.13(a)

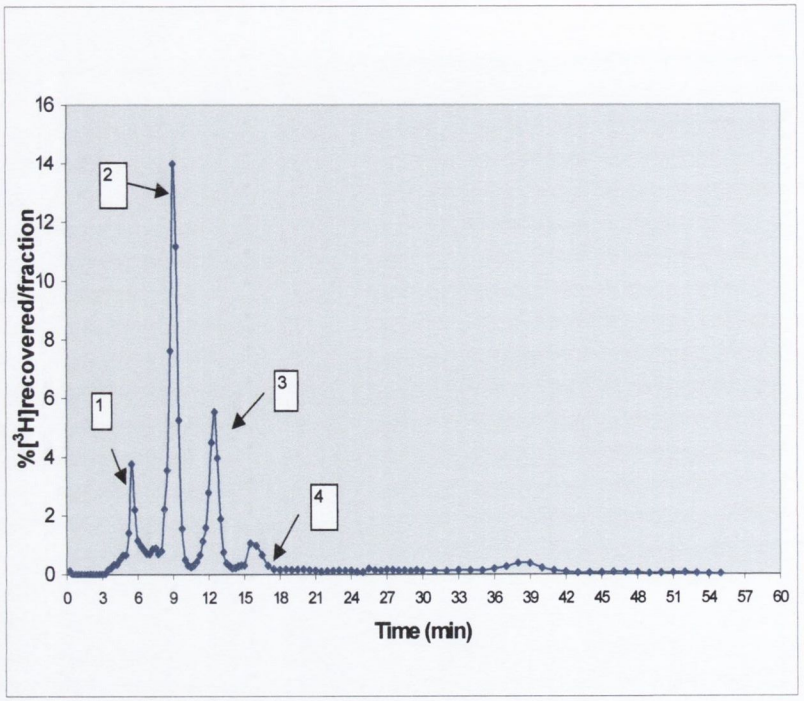
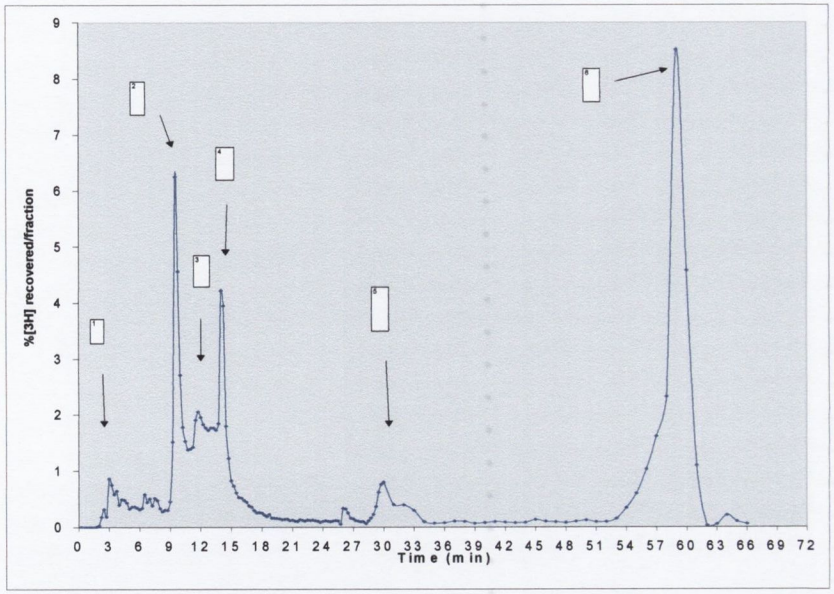


Figure: 3.13(b)

Figure 3.13: The HPLC elution profile of ECV304 intracellular folate co-factors deconjugated for 1hr at 37°C. The intracellular folates of ECV304 cells were deconjugated for 1hr at 37°C using rat serum. The intracellular folate co-factor distribution was analysed on HPLC. Figure 3.13(a) represents the folate co-factor distribution using kingsorb C18 column with picA reagent mobile phase 12%(v/v) methanol, peak 1: 5,10-CH^+-THF , peak 2: *p*ABG, peak 3: 10-CHO-THF, peak 4: THF, peak 5: 5,10-CH_2-THF, peak 6: 5-CH_3-THF. Figure 3.13(b) represents the folate co-factor distribution using a ubondapak column C18 with citrate phosphate buffer 12.5%(v/v)methanol, peak 1: *p*ABG, peak 2: 10-CHO-THF and THF, peak 3: 5-CH_3-THF and peak 4: 5,10-CH^+-THF.



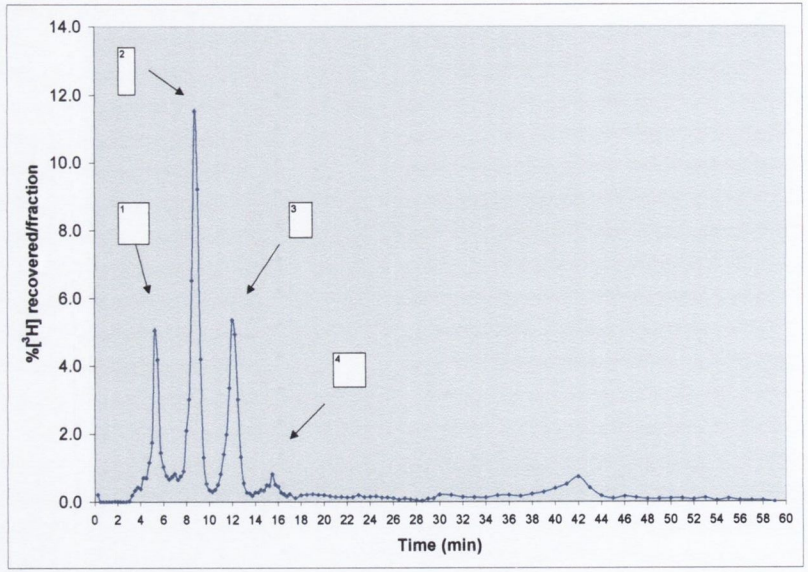


Figure : 3.14(a)

Figure : 3.14(b)

Figure 3.14: The **HPLC elution profile of ECV304 intracellular folate co-factors deconjugated for 2hr at 37°C.** The intracellular folates of ECV304 cells were deconjugated for 2hr at 37°C using rat serum. The intracellular folate co-factor distribution was analysed on HPLC. Figure 3.14(a) represents the folate co-factor distribution using kingsorb C18 column with picA reagent mobile phase 12%(v/v) methanol, peak 1: 5,10-CH^+-THF , peak 2: *p*ABG, peak 3: 10-CHO-THF, peak 4: THF, peak 5: 5,10-CH_2-THF, peak 6: 5-CH_3-THF. Figure 3.14(b) represents the folate co-factor distribution using a ubondapak column C18 with citrate phosphate buffer 12.5%(v/v)methanol, peak 1: *p*ABG, peak 2: 10-CHO-THF and THF, peak 3: 5-CH_3-THF and peak 4: 5,10-CH^+-THF.

extracted as per method 2.10 and the radioactivity present in the supernatant following extraction was calculated, see results table 3.7. As shown there was no radioactivity lost during the folate extraction method.

Folate co-factors are labile and readily undergo oxidative degradation therefore, it was essential to determine the percentage breakdown of each folate co-factor during extraction and deconjugation as described in method 2.10. The peak area of each standard before and after deconjugation was recorded and the percentage of peak area lost during the extraction method was determined, see table 3.8(a-h). Triplicate experiments were performed for each folate co-factor. As was expected, during the extraction and deconjugation method some of the more labile folates *i.e.* 10-CHO-THF, THF, DHF were lost during the extraction method. The more stable folates were fully recoverable.

The 5,10-CH^+-THF standard elutes from the column in the void volume due to its pI value. The intracellular folates are extracted and deconjugated in a 1% sodium ascorbate buffer, pH 6.0. The optimum pH for rat serum conjugase activity is pH 6.0, therefore to ensure the rat serum conjugase converts all the polyglutamated folates to monoglutamated folates this pH was required. Following deconjugation the 5,10-CH^+-THF standard is recovered not as 5,10-CH^+-THF but as 10-CHO-THF. As a result any intracellular 5,10-CH^+-THF present will be recovered as 10-CHO-THF following extraction. Therefore the small radioactive peak eluting in the void volume of some of the samples was not as previously reported 5,10-CH^+-THF but an unidentified radioactive peak. One possibility is that it is a folate degradation product with a positive electrostatic charge that cannot bind to the ion-pair reagent on the column.

p ABG	Before	After	% Recovery
1	367276	371766	101.2
2	360074	374663	104.1
3	357021	339539	95.1
Mean	**361457.0**	**361989.3**	**100.1**
SD	**5265.5**	**19496.4**	**4.6**
% CV	**1.5**	**5.4**	**4.6**

Table 3.8(a) : Peak area of *p* ABG before and after extraction and deconjugation and the percentage recovery

5-CHO-THF	Before	After	% Recovery
1	1082877	1092751	100.9
2	943633	1046537	110.9
3	996331	916642	92.0
Mean	**1007613.7**	**1018643.3**	**101.3**
SD	**70304.3**	**91307.9**	**9.5**
% CV	**7.0**	**9.0**	**9.3**

Table 3.8(b) : Peak area of 5-CHO-THF before and after extraction and deconjugation and the percentage recovery

5,10-CH_2-THF	Before	After	% Recovery
1	512816	437371	85.3
2	471211	429485	91.1
3	473821	471710	99.6
Mean	**485949.3**	**446188.7**	**92.0**
SD	**23303.8**	**22451.1**	**7.2**
% CV	**4.8**	**5.0**	**7.8**

Table 3.8(c) : Peak area of 5,10-CH_2-THF std before and afte extraction and deconjugation and the percentage recovery

FA	Before	After	% Recovery
1	584277	573229	98.1
2	535336	544181	101.7
3	531659	471710	88.7
Mean	**550424.0**	**529706.7**	**96.2**
SD	**29375.1**	**52284.4**	**6.7**
% CV	**5.3**	**9.9**	**6.9**

Table 3.8(d) : Peak area of FA before and after extraction and deconjugation and the percentage recovery

5-CH_3-THF	Before	After	% Recovery
1	850796	851243	100.1
2	790053	807690	102.2
3	793622	723775	91.2
Mean	**811490.3**	**794236.0**	**97.8**
SD	**34086.4**	**64790.3**	**5.8**
% CV	**4.2**	**8.2**	**6.0**

Table 3.8(e) : Peak area of 5CH_3-THF before and after extraction and deconjugation and the percentage recovery

10-CHO-THF	Before	After	% Recovery
1	1346709	1114583	82.8
2	1042262	807527	77.5
3	1098761	876497	79.8
Mean	**1162577.3**	**842012.0**	**80.0**
SD	**161945.6**	**48769.2**	**2.7**
% CV	**13.9**	**5.8**	**3.3**

Table 3.8(f) : Peak area of 10-CHO-THF before and after extraction and deconjugation and the percentage recovery

THF	Before	After	% Recovery
1	353233	294421	83.4
2	335560	309876	92.3
3	342601	308158	89.9
Mean	**343798.0**	**309017.0**	**88.5**
SD	**8897.1**	**1214.8**	**4.7**
% CV	**2.6**	**0.4**	**5.3**

Table 3.8(g) : Peak area of THF before and after extraction and deconjugation and the percentage recovery

DHF	Before	After	% Recovery
1	736796	648323	88.0
2	682974	633700	92.8
3	691094	631452	91.4
Mean	**703621.3**	**631452.0**	**90.7**
SD	**29015.6**	**1589.6**	**2.5**
% CV	**4.1**	**0.3**	**2.7**

Table 3.8(h) : Peak area of DHF before and after extraction and deconjugation and the percentage recovery

3.3 Discussion

By developing a method to determine the folate co-factor distribution in mammalian cells the effect of nutritional, genetic and pharmaceutical factors on folate homeostasis could be investigated as well the role of folate metabolism in vascular disease, colonic carcinoma and neural tube defects. The aim of this chapter was to develop a HPLC method to analyse the folate co-factor distribution in human cells in order to; 1) identify any differences in folate co-factor distribution in a mammalian cell model carrying the MTHFR C677T polymorphism and 2) to determine the effect of the antifolate drug methotrexate (MTX) on the intra-cellular folate co-factor distribution.

A number of HPLC methods have been developed to analyse folate concentrations and folate co-factor distribution in animal tissues (McMartin *et al.,* 1981; Wilson and Horne, 1984), human plasma (Kelly *et al.,* 1997; Sweeney *et al.,* 2003), urine (Geoghegan *et al.,* 1995) bacterial cells (Quinlivan *et al.,* 2000) and human red blood cells (Bagley an Selhub, 1998). These methods differed not only in the chemistry involved in separating these folate co-factors i.e reverse-phase HPLC, ion-pair reverse phase chromatography and affinity chromatography but also in the detection methods used to identify and quantify these folate co-factors. Therefore, choosing the most suitable method to analyse intracellular folate co-factors proved difficult as each had both advantages and disadvantages.

Folate co-factors have been detected following HPLC analysis by UV absorption (Horne *et al.,* 1981), fluorescence (Gregory *et al.,* 1984) or electrochemical oxidation

(Lucock *et al.,* 1995). UV detection has very limited sensitivity (Gregory, 1985) but combining HPLC using UV detection and a microbiological assay greatly increased the sensitivity (Belz and Nau, 1998; Sweeney *et al.,* 2003). Fluorescence detection was only sensitive to 5-CH_3-THF but following a post-column oxidation step it could separate the other folate co-factor forms (Lucock *et al.,* 1995). However, the introduction of this extra step made the HPLC method complicated and cumbersome. An affinity/ion-pair HPLC method using diode array UV detection had been developed to measure folate distribution in animal tissues (Varela-Moreiras and Selhub, 1992). The limit of detection using the diode array detector was in the range of approximately 1nmol, but this meant that gram quantities of tissue were required for analysis. Bagley and Selhub (2000) developed a HPLC method that allowed the measurement of folate co-factor distribution, in picomole concentrations in biological samples by using a fully automated combination of affinity chromatography and HPLC with electrochemical detection. The increased sensitivity of this method enabled folate co-factor forms to be separated in human mammalian cells. However, this method separated only four of the main folate co-factor forms THF, 5-CH_3-THF, 5,10-CH^+-THF and FA and the majority of formylated folates eluted as 5,10-CH^+-THF because of isomerization attributable to the acidic pH of the mobile phase.

Quinlivan *et al.,* (2000) developed an ion-pair reverse phase HPLC method, which assessed the relative distribution of intracellular folate co-factors in bacterial cells. This method was not quantitative but could separate out all eight folate co-factors and *p*ABG and it was for this reason the method was chosen. Initially, Quinlivan *et al.,* (2000) developed this ion-pair reverse phase chromatography method to investigate the effect the antifolate drug TMP had on the folate co-factor distribution in bacterial cells. The

method was set up and the study carried out by Quinlivan *et al.,* (2000) was repeated which ensured that the method was capable of giving reproducible results.

The first step in setting up this method was to verify the folate co-factor standards, which had been purchased from Schircks laboratory, Switzerland. The absorption spectrum for each of the standards was determined and their λ_{max} was compared with those reported by Blakley and Benkovic (1984). The λ_{max} result for each standard corresponded with those reported by Blakley and Benkovic (1984), thus verifying each of the folate co-factors standards.

The next step was to determine if these folate co-factor standards could be separated using the HPLC method developed by Quinlivan *et al.,* (2000). A set of folate co-factor standards were prepared as per method 2.3.2 and analysed using the ion-pair reverse phase chromatography method (Quinlivan *et al.,* 2000). Each folate standard was identified as a large peak on the recorded chromatogram. The elution time for each of the standard peaks was recorded in triplicate on three consecutive days and the intra-assay variation was calculated (see table 3.1). There was very little variation between elution times for each of the standards with the percentage coefficient of variation between assays being less than 5%. These results proved that the method was capable of giving reproducible elution times for each of the folate co-factor standards

A number of combined HPLC/microbiological methods were developed (McMartin *et al.,* 1981; Wilson and Horne,1984; Kelly *et al.,* 1996; Belz and Nau, 1998; Sweeney *et al.,* 2003) in order to identify and quantify folate co-factors eluting from

HPLC columns. Using these methods each folate co-factor was firstly identified by its elution time from the HPLC column and then quantified using the *L.casei* microbiological assay. The standards eluting from the column could not be quantified using the combined HPLC/microbiological method developed by Belz and Nau (1998) because of the large randomised background results obtained from the mobile phase eluting from the column. The large background counts would interfere with the microbiological assay results. Instead a microbiological assay was carried out on each of the prepared folate co-factor standards to ensure they were biologically active folates. The results obtained from these assays were compared with an in-house folic acid standard, as shown in figure 3.1. All the folate co-factors supported the growth of the *L.casei* but to varying degrees. These results in part verified that each large folate co-factor peak identified by its elution time using the HPLC method was a biologically active folate standard. The *p*ABG standard did not support the growth of *L.casei* which was expected as *p*ABG is a degradation product of folates.

Varying growth responses were reported for the folate co-factors using *L.casei,* as shown in figure 3.1 however, these assays were performed on different days and these differences could be due to intra-assay variation. The variation in the growth responses of the folate co-factor standards was not investigated further as the aim of this set of experiments was to demonstrate that each of the folate co-factor standards were biologically active.

Once the folate co-factor standards were separated using the ion-pair reverse phase chromatography method developed by Quinlivan *et al.,* (2000) the effect TMP had on the folate co-factor distribution in *E.coli* 8879 was determined and compared with the original results reported (Quinlivan *et al.,* 2000). All the experimental procedures were performed on *E.coli* 8879 in log phase growth. This was to ensure that maximum incorporation of [^{3}H]*p*ABA occurred before treating the cells with TMP. Once the bacterial harvest times were determined, see table 3.2, the minimum concentration of TMP required to give maximum inhibition was calculated, see figure 3.2. A concentration of 2μg/ml TMP was required to maximally inhibit the growth of the *E.coli* 8879 which corresponded with the concentration required by Quinlivan *et al.,* (2000).

The original method described by Quinlivan *et al.,* (2000) was performed on *E.coli* samples, see figure 3.3, but the results obtained could not be compared with those reported in Quinlivan *et al.,* (2000) because not all the co-factor peaks could be separated. As shown in figure 3.3, 10-CHO-THF and THF co-eluted as did 5,10-CH_2-THF and DHF. The co-elution of 10-CHO-THF and THF was not a major problem as the original paper reported the THF co-enzymes as one combined group (see table 3.4). The major problem arose with the co-elution of 5,10-CH_2-THF and DHF because following the treatment of *E.coli* 8879 with 2μg/ml of TMP there had been reported a transient accumulation of DHF, which later disappeared. Therefore it was necessary to modify the method to allow the clear separation of these two peaks.

In order to improve peak separation the methanol concentration in the mobile phase was reduced to 14% (v/v) and the fraction size collected was reduced to 0.25ml/min. On

examination of the results, see figure 3.4, separation of both DHF from 5,10-CH_2-THF and 10-CHO-THF from THF was achieved. An internal standard was run with each bacterial cell sample to verify the elution times of the co-factors. The internal standard was a mix of both 5-CH_3-THF and 5-CHO-THF .

The results reported for each sample was the mean result of duplicate assays, which took into account any variation that occurred within the bacteria and the assay conditions. As seen from the duplicate control results in table 3.3 only a small amount of variation occurred in the distribution of folate co-factor peaks. The mean recovery of counts from each injected sample was approximately 87%. The remaining radioactivity not present in the peaks could be accounted for in the background radioactivity. The results obtained for each folate co-factor were re-adjusted to 100% so as to compare them with the original results reported by Quinlivan *et al.,* (2000).

A comparison of results obtained using the method adapted from Quinlivan *et al.,* (2000) and the original results was essential as it determined if the results could be reproduced (see table 3.4). Only if the results were reproducible could the method be transferred from the bacterial cell model to a mammalian cell model. As shown in both cases there was an accumulation of DHF within the first 25min of treatment with TMP, which reduced with time. Folic acid and *p*ABG appeared within the cells upon treatment and there was an eventual decline in all the THF co-factors within 24hr. The main differences between the new results was that the accumulation of DHF following 25min of treatment was 11% lower than the original study and it still appeared as a 7% peak following 24hr of treatment. This leads us to believe that either the cells redox

state was protecting the DHF from degrading or residual DHF was being supplied to the cell. The latter would seem to be the most likely as the complete depletion of THF co-enzymes only occurred following an incubation period of 24hr, unlike the original results where complete depletion occurred after a 4hr incubation. The cells would still be utilising the small amounts of THF co-factors present and therefore the small amount of DHF would be expected to be present as this is produced during thymidylate synthesis.

Folic acid accumulated within the cells with the level increasing over 4 hours. In the original results the levels were still increasing following 24hr of treatment whereas the level was decreasing at 24hr in this study. This suggests that the folic acid was competing with the TMP for binding sites and was slowly being re-utilised. Another difference between the two sets of results is that the level of pABG in our study was increasing over time as opposed to Quinlivan *et al.*, (2000) which decreased at 24hr. This suggests that any residual DHF being supplied or being produced by the cell is more likely to be catabolised rather than converted to folic acid.

Using the method described in section 3.2.5 the separation of 10-CHO-THF and THF was achieved. 10-CHO-THF accounted for 46% and THF accounted for 16% of the total radiolabelled folate co-enzymes in the control cell. There was a dramatic reduction in the level of 10-CHO-THF to just 2% following 25min of treatment with TMP and by 60min it is undetectable. The level of THF reduced at a slower rate, requiring 2hr of TMP treatment before it disappeared. THF is produced following purine synthesis therefore it would be expected that the level within the cell would diminish at a slower rate than that of 10-CHO-THF. The consequences of THF depletion include the

cessation in biosynthesis of purines and pyrimidines, the metabolic block of greatest severity in animals and bacteria is thought to be the interruption of thymidylate synthesis, the so-called "thymine-less death" (Blakley, 1969, Van Triest, 2000). Within 60 min of TMP treatment the supply of 5,10-CH_2-THF was exhausted, which would result in the cessation of thymidylate synthesis. Following 240min of treatment of TMP there was still traces of 5-CH_3-THF present. 5,10-CH_2-THF was reduced to 5-CH_3-THF by methylene tetrahydrofolate reductase (MTHFR). This reaction is physiologically irreversible and therefore the small amount of 5-CH_3-THF left following the 240min treatment with TMP cannot be converted back to 5,10-CH_2-THF to restart thymidylate synthesis. 5-CH_3-THF is necessary to supply methyl groups for the methylation cycle.

Folic acid appeared within 25min of TMP treatment with the level increasing upon incubation as the level of DHF decreased. This suggested that some of the DHF was perhaps catabolised to *p*ABG and pteridines and some re-oxidised to folic acid (Chippel and Scrimgeour, 1970; Archer and Reed, 1980) and stored in the cell. The accumulation of folic acid was also observed by Quinlivan *et al.,* (2000) and may perhaps be one of the reasons why TMP is bacteriostactic as opposed to bactericidal. Once the inhibition of DHFR was removed the folic acid was able to re-enter the folate cycle and the cells began to replicate again. This was evident because following the removal of TMP, the level of THF co-factors slowly increased following the cells re-growth in TMP free glucose media for 4 hours.

The ultimate aim of this chapter was to transfer the method developed by Quinlivan *et al.,* (2000) from a bacterial cell model to a mammalian cell model. Some modifications had been made to the original method to ensure all the intracellular folate

co-factors were separated using the HPLC method and the results reported by Quinlivan *et al.,* (2000) were very similar when compared with those using the modified method as discussed above. This established that the results were reproducible which was essential before the method could be transferred from a bacterial cell model to a mammalian cell model. The robust and rapidly proliferating mammalian ECV304 cell line was used to develop and validate the methodology.

A normal growth response was established for ECV304 in full medium 199 which contained 22.66nM folic acid, see figure 3.5(a). The cells were grown in folate free medium 199 to determine the effect folic acid had on the growth response of ECV304 cells. No difference was reported in the growth rate or appearance of these cells as was shown in figure 3.5(b). It appeared that the cells previously grown in medium 199 containing 22.66nM folic acid accumulated an intracellular folate store, which allowed the cells to sustain normal cell proliferation or perhaps the medium contained an ample supply of purine and pyrimidine precursors for DNA synthesis, reducing the requirement for folate. ECV304 cells were sub-cultured 4 times in folate free medium 199 to try and deplete the intracellular store of folates. The growth response was again determined in folate free medium 199 to establish if any changes could be detected in the growth response or appearance of the cells. As shown in figure 3.6 normal cell proliferation and morphology was maintained even though the cells had been sub-cultured four times in folate free medium 199 prior to analysis. Therefore folic acid appeared not to be a rate-limiting factor in the growth of ECV304 cells using folate free medium 199.

Prior to HPLC analysis it was necessary to radiolabel the intracellular folate co-factors. [^{3}H]5-CHO-THF was the exogenous source of radiolabelled folate used to label the intracellular folate co-factors. As discussed in section 3.1 the [^{3}H]5-CHO-THF was influxed into the cell by a RFC and rapidly converted to 5,10-CH^+-THF by the enzyme MTHFS in the cytoplasm. Monoglutamated folates can either be converted to folate polyglutamates in the cytoplasm by the enzyme FPGS, or enter the mitochondria where they are converted to polyglutamates and sequestered in that compartment (Anguera *et al.,* 2003). 5-CHO-THF is not an effective substrate for FPGS, therefore these newly imported folate derivatives are unlikely to receive a polyglutamate tail prior to equilibration in the folate pool (Garrow and Shane, 1993). Prior to labelling the ECV304 cells with [^{3}H]5-CHO-THF ECV304 cells were sub-cultured four times in folate free medium 199. The rationale behind this step was to maximize the amount of radiolabel incorporated into the cell. The cells were grown in folate free medium 199 supplemented 2.9nM [^{3}H]5-CHO-THF. As shown in figure 3.8 the cells incorporated the radiolabelled folate as they proliferated.

It was essential that the HPLC method was capable of separating all eight folate co-factors; 5,10-CH^+-THF, 10-CHO-THF, THF, 5-CHO-THF, DHF, 5,10-CH_2-THF, FA, 5-CH_3-THF and also pABG. As discussed previously numerous methods had been developed to separate folate co-factors using HPLC (McMartin *et al.,* 1981; Wilson and Horne,1984; Geoghegan *et al.,* 1995; Kelly *et al.,* 1996; Belz and Nau, 1998; Quinlivan *et al.,* 2000; Sweeney *et al.,* 2003). These methods differed with respect to the choice of column, mobile phase, sample preparation and conjugase source. McMartin *et al.,* (1981) and Duch *et al.,* (1983) used a μbondapak C_{18} column and an ion-pair reagent (picA) in the mobile phase. Geoghegan *et al.,* (1995) used both reverse phase HPLC

and ion-pair reverse phase HPLC on a μbondapak C_{18} column. The reverse phase buffer was a citrate phosphate buffer and the ion-pair reverse phase buffer contained a picA reagent. Belz and Nau, (1998) used a spherisorb ODS-2.3μm column and picA reagent mobile phase. Kelly *et al.,* (1996) and Sweeney *et al.,* (2003) used a Waters μbondapak C_{18} column and a citrate phosphate buffer.

To determine which column achieved optimal separation of the folate co-factors, the folate co-factor standards were separated using a picA reagent mobile phase at pH 6.0 and 12% (v/v) methanol on both a Waters μbondapak C_{18} (pore size 3μ) column and a Phenomenex Kingsorb C_{18} column (pore size 5μ). The kingsorb column initially separated all eight folate co-factors and *p*ABG sufficiently using a picA reagent mobile phase at pH 6.0 and 14% (v/v) methanol (see figure 3.5) but continual column usage resulted in the co-elution of 10-CHO-THF and THF as shown in figure 3.8 and 3.9. This was a major problem because the systems ability to determine the percentage of formyl folates present will be a key feature in the future studies in this thesis. Following column cleaning the folate co-factor separation improved but cleaning was required regularly. Reducing the methanol concentration of the mobile phase to 12% (v/v) also improved the separation of these peaks, as shown in figure 3.13(a) and 3.14(a). Using the Waters ubondapak C18 (pore size 5μ) with the picA reagent mobile phase at pH 6.0 and 12% (v/v) methanol clearly separated both the 10-CHO-THF and THF, as shown in figure 3.10. However, the folate co-factors which had longer retention times such as 5-CH_3-THF and FA co-eluted. The reasons for developing a HPLC method which separated intracellular folate co-factors was to carry out future studies on mammalian cell lines in order to; 1) identify any differences in folate co-factor distribution in a mammalian cell model carrying the MTHFR C677T

polymorphism and 2) to determine the effect the antifolate drug methotrexate (MTX) would have on the intra-cellular folate co-factor distribution. The results reported by Quinlivan *et al.,* (2000) and in first section in this chapter demonstrated that treating bacterial cells the DHFR inhibitor TMP resulted in some of the accumulated DHF being re-oxidised to FA and therefore if the same mechanism of action was to happen in mammalian cells treated with the DHFR inhibitor MTX it is essential that the FA peak would be clearly identified as an individual peak. Bagley *et al.,* (1998) demonstrated that the concentration of 5-CH_3-THF was reduced in the red blood cells of the mutant MTHFR C677T genotype and the formylated folates increased and therefore it would be essential that the method developed in this chapter clearly separated the 5-CH_3-THF co-factor. As a result the Phenomenex Kingsorb C_{18} column would appear be the most suitable column to achieve the folate co-factor separation that in required for future studies in this thesis.

Reverse-phase chromatography had been used to separate folate co-factors (Geoghegan *et al.,* 1995; Kelly *et al.,* 1996). This method was tested to determine if improved 10-CHO-THF and THF separation could be achieved using the waters µbondapak column with a citrate phosphate buffer pH 4.0. It was clear that using this method resulted in the same THF and 10-CHO-THF peak co-elution problem, while the 5,10-CH_2-THF standard went undetected. The results demonstrated a very sharp peak for *p*ABG and 5-CH_3-THF therefore the use of the two methods on the same sample would allow the verification of both pABG and 5-CH_3-THF as both are stable at pH 4.0 and pH 6.0 see figure 3.13(a and b) and figure 3.14(a and b). However, using both these methods on the same sample proved very time consuming and one of the main advantages of the original Quinlivan *et al.,* (2000) method was the speed in which the folate co-factors

were identified because it reduced the amount of folate co-factor breakdown in the sample prior to analysis. Hence the original ion-pair reverse phase method was the chosen to analyse future samples.

As well as the problem of peak co-elution using the Quinlivan *et al.,* (2000) method another problem emerged during the assay development, which was an increase in the pump back-pressure. This was due to the blocking of the guard column and eventually resulted in the column becoming blocked. Once the column became blocked tailing peaks appeared at the end of the folate co-factor peaks. Column blocking was to be expected using the Quinlivan *et al.,* (2000) method as the cell sample was diluted 1/5 with rat serum in order to deconjugate the intracellular polyglutamated folates. Rat serum contains a lot of large molecular weight molecules and proteins, which in time block both the guard column and eventually the column resulting in high pump back pressures being observed and poor peak resolution. Regular changing of the guard column and column cleaning reduced these problems but did not eliminate them completely. Chicken pancreas is another source of conjugase, which is available to buy in a freeze-dried powder form and does not contain the large molecular weight molecules present in the rat serum. The problem with using chicken pancreas is that following deconjugation both mono and di- glutamate folate co-factor forms are present in the sample. As shown in figure 3.13(a) the retention time of pteroyl-γ-L-glutamic acid and pteroyldi-γ-L-glutamic acid are different and therefore if both mono and di glutamate forms were present for each intracellular folate co-factor it would be impossible to identify each individual folate co-factor in their mono and di-glutamate forms because the elution times of the monoglutamated folate co-factor forms are relatively close.

Usually a clean-up step is performed on a sample to remove the larger molecules and proteins before injection onto a HPLC column, which was the case in most of the studies to date (Wilson and Horne, 1984: Allegra *et al.,* 1986: Baram *et al.,* 1987 and Zhoa *et al.,* 2001). It would appear cleaning up the sample following deconjugation before injection would be an ideal solution to the problems being experienced with the Phenomenex Kingsorb column i.e column blocking, increased pump back-pressure and peak co-elution. Large proteins can be removed from a sample by number of methods including boiling, protease treatment and perchloric acid precipitation. Although these procedures would have no effect on some of the more stable folate co-factors the more labile folates such DHF, THF and 10-CHO-THF, if present in a sample, would be degraded by the use of these harsh methods and therefore could not be used . Another method of sample clean up would be to run the sample through a sep-pak column before injection onto the HPLC column. Using a Sep-Pak column the sample is eluted in methanol and the methanol then evaporated from the sample under a continuous stream of nitrogen gas or by air exposure (Kelly *et al.,* 1996; Sweeney *et al.,* 2003). Both these procedures would prolong the time between folate extraction and HPLC analysis, thus resulting in the more labile folates being degraded. Regular changing of the guard column and cleaning of the column was decided upon to tackle the problem of increasing pump back-pressure in the system and peak co-elution.

The methanol concentration in the picA mobile phase was reduced to 12% (v/v), which improved the separation of the radiolabelled 10-CHO-THF and THF and also 5,10-CH_2-THF and DHF co-factors within the cell extracts, see figure 3.13(a) and 3.14(a).

However, reducing the methanol concentration in the mobile phase increased the retention time of the folate co-factors especially FA and 5-CH_3 –THF. It took over 60min for 5-CH_3 –THF to elute from the column using this mobile phase, see figure 3.13(a) and 3.14(a). Therefore, to reduce the overall run time of the assay a discontinuous gradient system was set up. A standard mix was prepared containing *p*ABG, THF, 5-CHO-THF, 5,10-CH_2-THF, FA and 5-CH_3-THF and analysed on the HPLC using various gradient conditions. The system which gave the best overall results, began with a methanol concentration of 12% (v/v) for 17min, increased to 14%(v/v) for 35min and 16%(v/v) for 60min, see figure 3.11. The discontinuous gradient system reduced the run time by 15-20 minutes and resulted in sharper FA and 5-CH_3-THF peaks. This standard mix was used to spike cell samples to allow the identification of the radiolabelled peaks. The standard mix was run in triplicate on three consecutive days and the mean elution times of the triplicate runs were reported and from these results the intra-assay variation was calculated, see table 3.6. The percentage co-efficient of variation in elution times between each assay was low, proving that the discontinuous gradient gave reproducible results.

Following the development and validation of the discontinuous gradient HPLC system for the separation of folate co-factors the next step was to determine the length of time required for the complete deconjugation of intra-cellular folate co-factors in mammalian cells. It was reported by Quinlivan *et al.,* (2000) that an incubation time of 45min with rat serum at 37°C ensured the full deconjugation of intra-cellular folates in bacterial cells with no apparent degradation of the folate co-factors except for DHF, which degrades by 13% if present within the cell using this folate extraction method.

Two different HPLC experiments were used to determine the length of time required for the complete deconjugation of intra-cellular folates in mammalian cells.

A set of folate standards pteroyl-γ-L-glutamic acid, pteroyldi-γ-L-glutamic acid and pteroyltri-γ-L-glutamic and their elution times recorded. 100µl of the standard pteroyltri-γ-L-glutamic acid solution was mixed with 25µl of dialysed rat serum and incubated at 37°C for varying time points 0-2hr and 100µl of the standard was run on the HPLC. An incubation time of 1hr allowed the full deconjugation of polyglutamated folates into their monoglutamated forms as was identified by their elution times from the HPLC.

As intracellular mammalian folate co-factors can have more than three glutamate residues attached a mammalian cell line ECV304 labelled with [^{3}H]5-CHO-THF was split in two which allowed one half to be incubated with a 1/5 dilution of rat serum for 1hr and the other half for 2hr to determine if a difference between the two treatments could be detected using HPLC analysis, see figure 3.13(a and b) and 3.14(a and b). There appeared to be complete deconjugation following a 1hr incubation with rat serum as each folate peak appeared sharp with no tailing peaks, which might suggest incomplete deconjugation of the folates.

The next step was to determine if any [^{3}H]folate was lost during the extraction method. As shown in table 3.7 there was no loss of radioactivity during the folate extraction method and therefore it is clear that no [^{3}H]folate is lost during the extraction method.

Each folate co-factor standard was analysed on the HPLC before and after extraction and deconjugation in triplicate in order to determine the amount of labile folate lost during extraction and deconjugation, see table 3.8(a-h). During the extraction and deconjugation method some of the more labile folates *i.e.* 10-CHO-THF, THF, DHF were lost. The more stable folates were fully recoverable. Breakdown of the more labile folates is a major problem associated with analysing folate co-factors. However, this method developed for the extraction and deconjugation of intracellular folate co-factors appeared to be one of the less aggressive methods but still some breakdown occurs. As discussed in the results section 3.2.14 the 5,10-CH^+-THF standard is not recovered 5,10-CH^+-THF but as 10-CHO-THF. Therefore the small radioactive peak which elutes in the void volume of some of the samples, see figure 3.13(a) and 3.14(b) was not as originally reported 5,10-CH^+-THF but an unidentified radioactive peak. It may perhaps be a radiolabelled folate degradation product, which possesses a positive electrostatic charge and therefore elutes in the void volume.

In conclusion, the modifications made to the ion-pair reverse phase HPLC method developed by Quinlivan *et al.,* (2000) produced a method which separated all eight intracellular folate co-factors and pABG in a mammalian cell model namely; 5,10-CH^+-THF/ 10-CHO-THF, THF, 5-CHO-THF, DHF, 5,10-CH_2-THF, FA and 5-CH_3-THF. The main advantages of this method were that it was quick, reliable and reproducible. Prior to HPLC analysis, the samples are sonicated, centrifuged and incubated for 1hr rat serum at 37°C to deconjugate the folates and as shown in table 3.8(a-h) very little breakdown in the folate co-factors occurs which is one of the main problems associated with HPLC methods used for folate co-factor analysis. Using this newly modified HPLC method the effect the antifolate drug MTX would have on the folate co-factor

distribution in both MTHFR 677CC and TT mammalian cell lines could be investigated.

Chapter 4:

The use of human derived umbilical vein endothelial cells (HUVEC's) to investigate folate metabolism in relation to the MTHFR C677T mutation.

4.1 Introduction

The linings of all blood vessels contain endothelial cells. These cells are involved in a number of physiological and pathophysiological processes including haemostasis, vasoregulation, inflammation, angiogenesis and the extravasation of fluid macromolecules, hormones and leukocytes. Endothelial dysfunction is a key process in arteriosclerosis (Ross, 1993) and independently predicts cardiovascular events (Schachinger *et al.,* 2000). Mildly elevated plasma homocysteine has also been associated with an increased rate of coronary artery disease (Boushey *et al.,* 1995). Doshi *et al.,* (2001) have reported an improvement in the endothelial function of patients with coronary artery disease (CAD) as a result of an increased intake of folic acid (5mg daily) alone or in combination with B-group vitamins (Doshi *et al.,* 2001).

Jaffe *et al.,* (1973) were the first to successfully isolate and sub-culture human umbilical vein endothelial cells (HUVEC). These cells were shown to synthesize and secrete von Willebrand factor (Jaffe *et al.,* 1973), tissue factor (Maynard *et al.,* 1977) and factor V (Cerveny *et al.,* 1984) and interact at their surface with procoagulation proteins. They were also shown to participate in several anti-thrombotic reactions including vasodilatation and the inhibition of platelet aggregation (Weksler *et al.,* 1977). More recently, these cells have been used to investigate the effect of folic acid on homocysteine metabolism (van der Molen, 1996), the effects of homocysteine on endothelial nitric oxide production (Zhang *et al.,* 2000) and endothelial dysfunction (Outinen *et al.,* 1999).

As discussed in chapter 1, the MTHFR C677T mutation results in reduced MTHFR activity, due to a thermolabile form of the enzyme. Upon isolation of the human cDNA for MTHFR (Goyette *et al.,* 1994), the *in vitro* properties of the thermolabile enzyme were found to be due to a homozygous cytosine-to-thymidine transition at nucleotide 677C→T, resulting in an alanine-to-valine substitution (Frosst *et al.,* 1995). Homozygosity for the MTHFR C677T polymorphism is associated with mild hyperhomocysteinemia in humans with plasma folate concentrations below the median (Jacques *et al.,* 1996) and is the most common genetic cause of mild elevations in plasma homocysteine in humans (Schwahn *et al.,* 2001). It is believed that 5-20% of the population are homozygous for the MTHFR thermolabile gene (Pepe *et al.,* 1998). HUVEC cell lines were chosen as the mammalian model for this study because their function has been closely linked with folate metabolism and homocysteine metabolism (Lobo *et al.,* 1999; Doshi *et al.,* 2001).

The main aim of this chapter was to explore the change in folate co-factor distribution that might result from the common MTHFR C677T mutation using the HPLC method developed in chapter 3.

4.2 Results

4.2.1 Isolation and culture of Human Umbilical Vein Endothelial Cells (HUVEC)

Sterile 400ml collection jars containing 250ml of 1% sterile PBS (cord buffer) were stored in a fridge at 4°C in the delivery suite of the Coombe Womens Maternity Hospital. Umbilical cords were obtained from normal vaginal deliveries by consenting women of Irish nationality and stored at 4°C in cord buffer.

The work-up toward achieving a regular and consistent culture of HUVEC cells was technically intricate and only achieved by paying particular attention to several details that were established during the course of this project.

a) Cord Quality

The cords were required to be approximately 20cm in length in order to successfully canulate both ends of the umbilical vein as per method 2.9. Upon inspection any damaged areas *i.e.* cord clamp marks or tears were cut away using a sterile scalpel blade. If these damaged areas were not cut away, the collagenase solution, which is required to digest the endothelial cells from the vein, would leak from the vein before the endothelial cells could be digested. This step was crucial as a hole the size of a pinprick was enough to cause the collagenase to leak from the vein, resulting in the cord having to be discarded.

b) Removal of Blood Clots

It was essential to flush the umbilical vein with sterile cord buffer prior to endothelial cell isolation in order to dislodge any remaining blood clots from the vein. The blood clots interfered with the endothelial cell isolation by blocking the collagenase from running the whole length of the vein and therefore reducing the surface area of endothelial cells that the collagenase could digest. Following collagenase treatment the collagenase solution which also contained the endothelial cells could not be successfully flushed from the cord because the clots blocked the needle tip used on the collection syringe. The red blood cells flushed from the vein along with the endothelial cells reduced the number of endothelial cells that attached to the $25cm^2$ pre-gelatinised flask surface because they formed a layer of suspended cells on the flask surface which made it difficult for the endothelial cells to attach.

c) Initial Culture Conditions

Upon isolation the cells were grown overnight in a $25cm^2$ flask, which had been gelatinised with a sterile 0.2% gelatin solution and contained 4ml of medium 199 supplemented with 10% FCS. The flask was incubated overnight at 37°C and 5% CO_2. The HUVEC cells attached to the gelatinised flask surface. The media was replaced with 4mls of fresh media and 133µl of endothelial cell growth supplement (ECGS). Following this the cells began to proliferate and within approximately 4 days the flask had a confluent monolayer of HUVEC cells of approximately $1x10^6$cells/flask. The HUVEC cells appeared as a tightly packed cobble-stoned monolayer attached to the surface of the pre-gelatinised flask. The initial incubation time required for a confluent

flask varied from cord to cord, sometimes taking 1-2 days, other times taking 5-7days, and some never becoming confluent because the cell density was too low.

Incubating the vein with collagenase not only yields the endothelial cell layer but some fibroblast cells from the next layer also. This usually occurs only when veins are incubated with collagenase for longer than the specified 7min incubation time. Fibroblast cells can rapidly take over the flask yielding a confluent flask of fibroblasts. They appeared elongated, dendritic and thin and grew in a swirling motion. The ECGS supplement added to the flasks stimulated HUVEC cell growth and reduced the risk of fibroblast cells outgrowing the HUVEC cells.

d) Age of Cords

HUVEC cells could be successfully isolated from cords that had been stored for up to 48hr in sterile cord buffer at 4°C. The success rate decreased with increased storage time. Cords stored for more than 48hr at 4°C were discarded as the number of viable isolated cells that attached to the pre-gelatinised flask surface reduced and as a result the seeding cell density number was too low and did not support cell proliferation.

e) Limitation of Subculture

Healthy cells appeared as a cobble-stoned monolayer when confluent but following 5-6 sub-cultures a change in their morphology occurred. The cells appeared elongated, dendritic and their proliferation rate slowed down until eventually, they stopped growing, became detached from the flask surface and died. As a result it was necessary to carry

out all the analysis between passages 1-5 to ensure healthy proliferating cells. A change in cell morphology may result in altered intracellular folate metabolism and therefore, would not be a true representation of normal intracellular folate metabolism.

4.2.2 To investigate the effect freezing has on HUVEC cell lines

Cells were frozen as per method 2.9.3 and revived to investigate the possibility of storing the HUVEC cell lines and analysing these cell lines at some later date. There were two reasons for preparing a large stock of HUVEC cell lines, the first being that only MTHFR CC and TT cell lines were of interest in this study. Therefore, having a stock of genotyped cell lines would avoid the necessity of analysing samples from heterozygous MTHFR C677T cell lines. This would greatly reduce the number of cell lines to be cultured. The second reason was, that if any novel polymorphisms similar to the MTHFR C677T polymorphism were identified in the enzymes involved in folate metabolism the DNA from the stock cell lines could be genotyped and further analyses carried out to see if a phenotype could be identified for that particular genotype using the HPLC method which was developed in chapter 3.

The cells were frozen at passage 2 and 3 at an original density of $1x10^6$cells/stock, which was the recommended freezing density of HUVEC cells by the American ype ulture Collection (ATCC). However, the survival rate following revival was very low. The cells that did adhere to the flasks appeared elongated, large, dendritic and did not proliferate adequately to give a confluent flask even following incubation in medium

supplemented in 20% (v/v) FCS and doubling the concentration of the ECGS growth supplement. The frozen stock cell density was increased to $3x10^6$cells/stock but this did not improve the survival rate. The stocks were originally frozen in a solution containing 10% (v/v) DMSO which was reduced to 7.5% (v/v) but this did not improve the survival rate of the cell stocks. It became apparent that freezing and reviving the cell lines was not a viable option and therefore, every experiment was carried out on newly isolated HUVEC cells on as low a passage number as possible. This required the mid-wife in the Coombe Womens Maternity Hospital to collect 2-3 cords per week on an on-going basis until the study was completed.

4.2.3 Isolation of DNA from HUVEC cells

Once a confluent monolayer of cells was achieved from the cells isolated from the cord they were split 1/3 and grown in medium depleted of folic acid for 4-6 days until confluent again. Normally the cells still appeared as a healthy, tightly packed cobble-stoned monolayer. The cells needed to be seeded at a density of $3x10^5$cells/flask to ensure a healthy culture. DNA was isolated from one of these confluent flasks as per method 2.11 and genotyped for the MTHFR C677T variant as per method 2.13 and 2.14. A confluent flask of HUVEC cells contained approximately $1x10^6$cells/flask, which was sufficient to successfully extract an adequate amount of DNA to PCR and genotype. DNA could be isolated from cell numbers as low as $1x10^4$cells and successfully genotyped provided the recovered DNA was freeze dried and concentrated into 50µl of PBS prior to PCR and genotyping. The other two flasks that had been split and passaged

once in media 199 depleted of folic acid were subsequently, grown in folate free medium 199 supplemented with 2.9nmol [^{3}H]5-CHO-THF for 96hr which is the equivalent of 0.5μCi [^{3}H]5-CHO-THF.

4.2.4 Uptake of [^{3}H] 5-CHO-THF in HUVEC cells lines

A confluent flask of cells initially grown from cords was split 1/3 and passaged once in folate free medium 199 until confluent. This confluent flask was again split 1/3 and grown in folate free medium 199 supplemented with 2.9nmol [^{3}H]5-CHO-THF for 96hr. The radiolabel incorporated within the cells in each flask was determined by re-suspending a cell pellet from each flask in 100μl of sterile PBS and 10μl of the cell suspension was counted in triplicate on a scintillation counter. This was carried on three separate cell lines; see table 4.1 for results. Each confluent flask of cells incorporated a mean value of 30,234(±SD 4209) dpm counts. In order to successfully estimate the intra-cellular folate co-factors using the HPLC method developed in chapter 3 approximately 100,000dpm counts were required to be injected onto the column. Therefore, in order to investigate the HUVEC intra-cellular folate co-factor distribution four flasks of radiolabelled [^{3}H]5-CHO-THF cells were combined before folate extraction and analysis.

4.2.5 Folate co-factor distribution in HUVEC cell lines

The cells from four confluent flasks of same cell line having firstly, been sub-cultured and grown in folate free medium 199, then sub-cultured and grown in folate free medium

	Experiment 1	Experiment 2	Experiment 3
DPM/ cell pellet	26031	27279	26931
DPM/ cell pellet	32107	31711	31122
DPM/ cell pellet	34431	37533	24959
Mean	**30856**	**32174**	**27671**
SD	**4337**	**5143**	**3147**

Table 4.1: **To determine the amount of dpm counts incorporated into HUVEC cell lines.** Following a 96hr incubation with 2.9nmol [^{3}H]5CHO-THF the amount of radiolabelled folates incorporated into HUVEC cell lines (n=3) was recorded as dpm counts/cell pellet.

199 supplemented with 2.9 nmol [^{3}H]5-CHO-THF for 96hr were combined. The folates were extracted and analysed as per method 2.10 (see results table 4.2). The experiment numbers were limited greatly by the random nature of MTHFR TT cords. Thus, three cell lines from each MTHFR CC and TT genotypes were analysed in this study, see table 4.2 and figure 4.1. The folate co-factor distribution of the MTHFR CC and TT cell lines were compared to examine possible changes in distribution resulting from the MTHFR C677T genotype. Upon examination of the folate co-factor distribution it appeared that both genotypes had very little 5,10-CH_2-THF present, with 4% in the MTHFR CC genotype and 3% in the MTHFR TT genotype. When 10-CHO-THF, 5-CHO-THF and 5,10-CH_2-THF peaks were combined to form a formyl group, the MTHFR CC cell lines had 21% of their radiolabelled folates present as formyl folates while the TT cell lines had 27% of their radiolabelled folates present as formyl folates. A large difference was found the level of THF present in the two genotypes, the MTHFR CC cell lines had 45% of their folates present in the THF form whereas the TT cell lines have 29% of their folates present in the THF form. There was no difference in the *p*ABG or DHF level present within the cells for both genotypes. The 5-CH_3-THF levels present in the two genotypes was surprising as, contrary to what would have been expected, the MTHFR TT contained a much higher level when compared with the CC cells. There was no difference reported in the *p*ABG levels in either MTHFR C677T genotype.

The sample numbers in this study were too small to make any comment from a statistical test and a larger sample number for each MTHFR C677T genotype would be required in order to verify these results. However, due to the difficulties associated with the use of

Folate co-factors	MTHFR 677 CC Mean (SEM), [n=3]	MTHFR 677 TT Mean (SEM), [n=3]
Unknown	9(0)	7(2)
*p*ABG	5(2)	5(1)
5,10-CH^{+}-THF/10CHO-THF	16(5)	19(3)
THF	45(1)	29(9)
5-CHO-THF	1(1)	5(4)
DHF	3(1)	4(2)
5,10-CH_2-THF	4(1)	3(1)
FA	ND	ND
5-CH_3-THF	18(1)	29(6)

Table 4.2: The effect of MTHFR C677T genotype on folate co-factor distribution in human derived endothelial cell lines. Human derived endothelial cells were isolated from umbilical cords and grown in medium 199 plus ECGS. Each cell line was sub-cultured in folate free medium 199 plus ECGS until confluent and then sub-cultured in folate free medium 199 plus EGCS and supplemented with 2.9nmol [^{3}H]5CHO-THF for 96hr. The intracellular folate co-factors were extracted and separated using the HPLC method described in section 2.9 and the radioactivity corresponding to each folate co-factor was expressed as a percentage of the total radiolabelled folate.

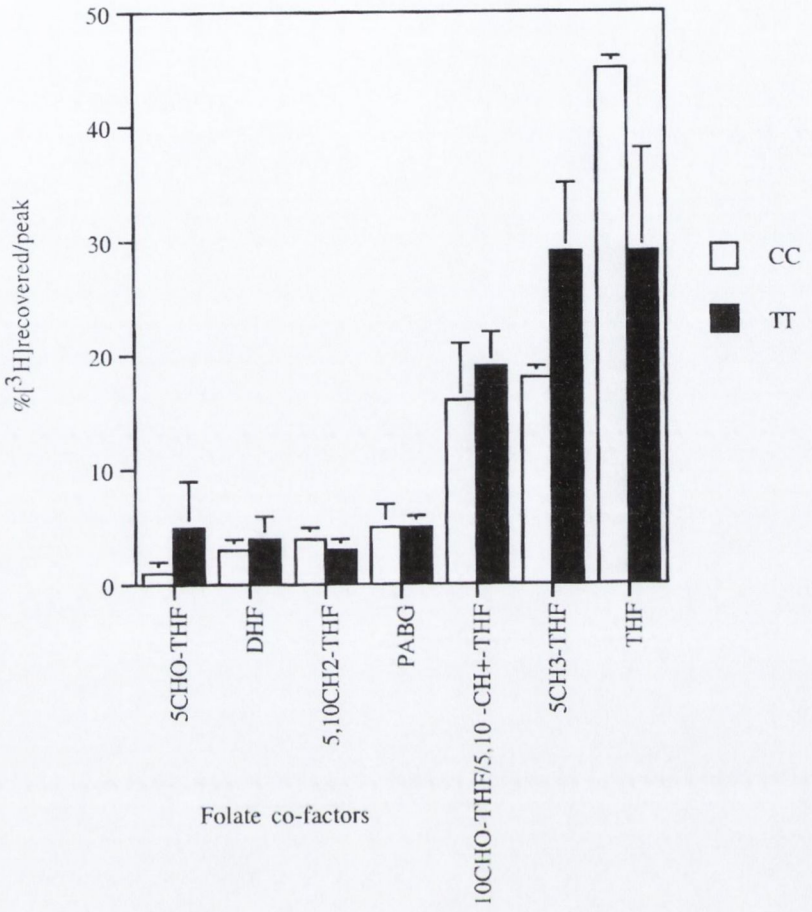


Figure 4.1: The effect of MTHFR C677T genotype on the intracellular folate co-factor distribution in HUVEC cell lines.
Cells from four confluent flasks of same cell line having firstly, been sub-cultured and grown in folate free medium 199, then sub-cultured and grown in folate free medium 199 supplemented with 2.9 nmol [^{3}H]5-CHO-THF for 96hr were combined. The folates were extracted and analysed using HPLC. Error bars represent the SEM of 3 cell lines (n=3).

□ = MTHFR 677 CC genotype
■ = MTHFR 677 TT genotype

the HUVEC cell lines no further analysis was carried out using these particular cell lines. The main difficulty associated with HUVEC primary cell lines was the low level of intracellular [^{3}H]5-CHO-THF incorporation. The intracellular folate co-factor distribution for the human derived primary endothelial cell lines was determined in their second passage in order to eliminate the possibility of cell morphology changes, as discussed in section 4.2.2. The second passage of a HUVEC cell line provided 6 x 25cm^{2} available for radiolabelling and subsequent HPLC analysis. Combining four of the confluent flasks of radiolabelled cells resulted in the folate co-factor distribution of each HUVEC cell line being analysed only once. Another difficulty with these primary cell cultures was that sub-culturing was unreliable and therefore it was decided a more robust mammalian cell model was needed to investigate the genotype-phenotype relationship between intracellular folate co-factor distribution.

4.3 Discussion

The main aim of this chapter was to determine the intracellular folate co-factor distribution in mammalian cell lines using the HPLC method that was developed and validated in chapter 3. Initially it was thought that the primary culture of HUVEC cells would be an ideal mammalian cell model to investigate the intracellular flux of folate co-factors because of their reported sensitivity to homocysteine (Hultberg *et al.,* 1998) and the relationship between folate concentration and cardio-vascular disease (Doshi *et al.,* 2001). The HUVEC cells were cultured using a method initially devised by Jaffe, (1973). They had reported a relatively high success rate in isolating HUVEC cells from cords and as a result numerous cell lines could be grown simultaneously. HUVEC cells are fastidious cells with only limited proliferative potential in serum-supplemented medium (Grimbone, 1974). An endothelial cell growth supplement (ECGS) partially purified from bovine hypothalmus was reported to stimulate HUVEC growth in-vitro in the presence of foetal calf serum (FCS) (Maciag, 1979). This growth supplement allowed the serial propagation of the HUVEC cells (Maciag, 1981).

The main advantages of using HUVEC cell lines as primary cultures in this study were; 1) The relative ease at obtaining samples, a supply of umbilical cords from consented mothers in a large Dublin Maternity Hospital (the Coombe Women's Hospital) was available for use in this study. Ordinarily these cords would be disposed of and so this was an ideal cell line to use, as with other primary cell lines some invasive techniques need to be employed to obtain the sample. 2) The cells are not transformed and therefore

are considered to be a primary culture. Transforming cells may affect intracellular folate metabolism and therefore a primary culture would give a true representation of intracellular folate co-factor distribution in human cells.

In order to successfully isolate endothelial cells from the umbilical vein particular attention was needed to be paid to the cord length and its condition. As discussed in the results section 4.2.1 it proved impossible to isolate HUVEC cells from umbilical cords that were damaged or too short. The vein needed to be washed free of blood clots prior to the distal end of the vein being canulated. This was required firstly, to ensure the syringe needle did not get blocked by the blood clots and secondly, to ensure as few red blood cells as possible were collected along with the digested endothelial cells as they interfered with the number of endothelial cells attaching to the culture flask surface. The cords could be stored for up to 48hr in 250ml sterile cord buffer at 4°C. The number of viable endothelial cells isolated from cords stored for longer than 48hr was greatly reduced and as a result the success rate for establishing a healthy culture was low. In this study all the cords were processed within 24hr of delivery to eliminate the possibility of unsuccessful isolations.

Primary cells are very susceptible to infections so it was essential that a broad-spectrum antibiotic was used during culturing. In the case of HUVEC cells an antibiotic/antimycotic was used. Cord collection and HUVEC cell isolation was very time consuming and therefore reducing the number of contaminated cultures discarded by supplementing the growth medium with antibiotic was a major time-saving advantage.

HUVEC cells were very sensitive to trypsin, which they are exposed during sub-culturing, see method 2.9.1. The trypsin was required to detach the cells from the flask surface. If HUVEC cells were exposed to the trypsin for too long the cells became damaged and as a result not enough healthy cells attached to the new pre-gelatinised flask surface to begin a healthy culture. If the cells were not exposed to trypsin for long enough not all the cells became detached from the flask and therefore the seeding density of the cells was too low to support healthy growth. It was essential during trypsinisation that the flask was monitored under the microscope to ensure the cells were not left floating in the trypsin solution following detachment. The cell lines that were damaged during trypsinistion did not recover fully and were unable to yield a healthy monolayers of cells with a small tightly packed cobble-stoned appearance and therefore were discarded. To successfully culture the HUVEC cells a seeding density of approximately 3×10^5 cells/flask was required. If the cells were seeded at a low a density the cells appeared as elongated dendritic cells and lost their ability to proliferate.

Originally it was thought that cords would only need to be collected from the Coombe Women's Maternity Hospital for a period of 2-3 months and within that time a large bank of cell line stocks would be frozen. It was thought that freezing the cell lines following isolation would eliminate the unnecessary culture and analysis of heterozygote cell lines. But, the reality of working with these cell lines proved very different as discussed below.

Reviving the cells stocks was very unreliable, approximately 60% of the cells from the revived stocks attached to the pre-gelatinised flask surface. These cells appeared elongated, large and dendritic in contrast to the tightly packed cobble-stoned appearance prior to freezing. The change in the cell morphology raised the question of how this change would affect the function of the cell and folate metabolism. Analysing the intra-cellular folate co-factors of these cells might not give a true representation of a normal HUVEC cell. However, analysis proved impossible as once the cell morphology changed, the cells ability to proliferate reduced and eventually ceased and therefore, cell numbers were never high enough to analyse their intracellular folate co-factor distribution. In conclusion the HUVEC cells isolated from the cords needed to be genotyped and analysed immediately. This meant the cords needed to be collected on an on-going basis from the Coombe Womens Maternity Hospital. For each successful HUVEC cell line isolated the cells required passaging, genotyping and intra-cellular folate co-factors analysis to be carried out by the cell lines second passage.

The intracellular folate co-factor distribution was analysed in HUVEC cell lines using the HPLC method previously developed in chapter 3. This method required the incorporation of [^{3}H]5-CHO-THF into the intracellular folate co-factor pool to allow the intracellular folate co-factor flux to be analysed using the ion-pair reverse-phase HPLC method. The incorporation of [^{3}H]5-CHO-THF by HUVEC cells was low (see results table 4.1) and as a result four confluent flasks of radiolabelled cells needed to be combined to ensure the radiolabelled folate co-factors peaks could be identified by HPLC. Approximately 100,000dpm counts were required per HPLC injection to ensure

all the radiolabelled intracellular folate co-factors peaks could be identified. In this study the sample size for each genotype was small (n=3) and the variability between cell lines within each genotype was relatively high which makes it impossible to make any comment from a statistical test. Increasing the sample number would reduce the variability and allow a better comparison of the two genotypes. As discussed in theory the HUVEC cell lines appeared to be the ideal choice to investigate intracellular folate metabolism using the HPLC method developed in chapter 3 but due to the difficulties discussed above a more robust mammalian cell line model was sought to investigate intracellular folate metabolism.

The HPLC results for each cell line were grouped in accordance with their MTHFR C677T genotype. Three cell lines for each of the MTHFR C677T genotypes were analysed; see table 4.2 and figure 4.1. Combining the percentage intracellular radioactive folate co-factor peaks of 10-CHO-THF, 5-CHO-THF and 5,10-CH_2-THF in the MTHFR CC cell lines resulted in 21% of the intracellular radioactive folate being present in this formyl folate group and 27% of the intracellular radioactive folate in the TT cell lines were formyl folates. Therefore there is a higher percent of these formylated folate co-factors in the MTHFR TT cell lines. A difference in the level of methylated folate was also reported, 29% being present in the MTHFR TT cell lines and only 18% present in the MTHFR CC cell lines. This was not in agreement with results previously reported for human red blood cells and human immortalised lymphocyte cell lines (Bagley and Selhub, 1998; Lathrop-Stern *et al.,* 2003). In 1998, Bagley and Selhub identified a phenotype for this common MTHFR C677T polymorphism in human red blood cells.

Folates were present in the 5-CH_3-THF polyglutamate form in red blood cells of individuals with the wild-type genotype whereas homozygous mutant individuals contained 71% of their folates in the 5-CH_3-THF polyglutamate form with the remaining folates were in formylated THF polyglutamate forms (Bagley and Selhub, 1998). More recently, Lathrop-Stern *et al.,* (2003) demonstrated in immortalised lymphocyte cell lines that although the concentration of intracellular folates did not differ with respect to the MTHFR C677T genotype in folate adequate people, the intracellular folate co-factor distribution differed. There was a significant increase in formylated folates and a significant decrease in methylated folates in MTHFR TT immortalised lymphocyte cell lines when compared with MTHFR CC cell lines. However, despite the lower MTHFR enzyme activity, the mutant enzyme provided sufficient 5-CH_3-THF to meet the demands for methionine synthesis and homocysteine remethylation (Lathrop-Stern *et al.,* 2003).

In conclusion, the HUVEC cell lines would be an ideal cell line to investigate the intracellular folate co-factor distribution using a HPLC method that did not require initial radiolabelling of intracellular folate co-factors prior to analysis. Radiolabelling the intracellular folate co-factors was a crucial step required for the identification of the folate co-factors using the HPLC method that had been developed and validated in chapter 3 and therefore these cell lines proved not to be ideal. One such method which did not require pre-labelling the folate co-factors with [^{3}H]5-CHO-THF was the method developed by Bagley and Selhub (1998). They had developed a method to separate folate co-factor distribution in mammalian cell models using affinity chromatography followed

by a two-column HPLC system with a four-channel coulometric electrochemical detection system. The multi-channel electrochemical detector provided a lower detection limit than UV detection but maintained the advantages of the diode array detection system *i.e.* the ability to distinguish between folates that elute at the same retention time by use of an algorithm (Selhub, 1989). The main advantages of this method were that it did not require the intracellular folate co-factors to be radiolabelled prior to HPLC analysis and that the folate co-factors were separated in their polyglutamate forms. The limit of detection using this method was reported to be very low at 0.2pmol for each folate co-factor. Because of the lower detection limit, this method allowed the folate co-factor distribution to be investigated in small specimens, such as human red blood cells (Bagley and Selhub, 1998) and lymphocytes (lathrop-Stern *et al.*, 2003).

Perhaps the next step might be to analyse the folate co- factor distribution in the HUVEC cell lines using this method and comparing the primary cell culture results with those reported for the immortalised human lymphocyte cell lines (Stern *et al.*, 2003) to determine if the folate metabolism in immortalised cells behaves differently than that in primary cells. Unfortunately this system could not be set up in the laboratory because of the lack of a multi-channel electrochemical detector and therefore choosing a more robust cell line was the only option available to complete the investigation into intracellular folate metabolism using the ion-pair reverse-phase chromatography method developed in chapter 3. One of the disadvantages associated with the Bagley and Selhub method was that the method only identified four of the intracellular folate co-factors; THF, FA, 5,10-CH^{+}-THF and 5-CH_3-THF (Bagley and Selhub, 1998). The overall aim of this thesis was

to investigate the flux of intracellular folate co-factors in a mammalian cell lines and the effect the antifolate drug MTX would have on the flux in relation to their MTHFR C677T genotype, therefore the HPLC method developed in chapter 3 was the preferred method as it allows all eight intracellular folate co-factors to be identified and compared with relation to MTHFR C677T genotype as shown by the results of this study.

PABG could also be identified using the HPLC method developed in chapter 3, which is a folate degradation product. It was thought that if an increase in the intracellular pABG levels could be identified in MTHFR TT individuals it would result from an increase in folate catabolism caused by the increased levels of formylated folates. Increased levels of formylated folates have been reported in both MTHFR TT human red cells (Bagley and Selhub, 1998) and MTHFR TT immortalised human lymphocyte cell lines (Lathrop-Stern *et al.,* 2003). Molloy *et al.,* (1997) reported that the MTHFR C677T polymorphism was associated with a lower than expected red cell folate concentrations in both pregnant and non-pregnant women. It was thought that the altered blood folate levels may, be due in part, to the thermolabile homozygous TT genotype. An increase in intracellular folate catabolism resulting from the increased formyl folates present in MTHFR TT cells may explain the reported reduction in red blood cell folate concentrations in MTHFR TT individuals and therefore it was crucial to measure the percentage of intracellular radiolabelled PABG levels present in both MTHFR C677T genotypes. A higher than expected prevalence of the TT genotype was reported among mothers of NTD cases (Whitehead *et al.,* 1995 and Van der Put *et al.,* 1995) and an altered blood folate levels had also been observed in NTD mothers (Kirke *et al.,* 1993, Mills *et al.,* 1995, Molloy *et*

al., 1998). Therefore, if increased folate catabolism could be identified in MTHFR TT cells it may in part explain the increased prevalence of TT genotype among mothers of NTD cases.

The limited results obtained from the HUVEC cell lines verified that the method developed in chapter 3 was capable of separating the intracellular folate co-factors in a mammalian cell model and observing differences in the folate co-factor distribution between the MTHFR 677 CC and TT cell lines if present.

Chapter 5:

The use of immortalised human lymphocyte cell lines to investigate folate metabolism in relation to the MTHFR C677T mutation.

5.1 Introduction

The metabolic role of 5-CHO-THF is not fully understood, however, it is a potent inhibitor of several folate-dependent enzymes including phosphoribosylaminoimidazole carboxamide formyltransferase and SHMT and may regulate folic acid-dependent metabolic pathways (Stover and Schirch, 1993). 5-CHO-THF polyglutamates are slow tight binding inhibitors of SHMT and therefore, may have a significant physiological relevance because slow binding inhibitors are generally more effective enzyme inhibitors *in vivo* (Stover and Schirch, 1991). *E.coli* lacking this enzyme do not contain 5-CHO-THF which suggests that SMHT catalyses this reaction *in vivo* also (Stover and Schirch, 1990). 5-CHO-THF is not thought to accumulate in mammalian cells because of the enzymatic activity of methenyltetrahydrofolate synthetase (MTHFS) (Holmes and Appling, 2002).

MTHFS is the only known enzyme to metabolise 5-CHO-THF and catalyse the conversion of 5-CHO-THF to 5,10-CH^{+}-THF *in vivo* (Maras *et al.,* 1994). MTHFS is located in the cytoplasm of rabbit liver, whereas in humans it is located both in the cytoplasm 85% and in the mitochondria 15% (Girgis *et al.,* 1997). It is expressed in many organisms ranging from bacteria to humans, which suggests that it may play an important role in one-carbon metabolism (Holmes and Appling, 2002; Roje *et al.,* 2002). Girgis *et al.,* (1997) demonstrated that increased MTHFS expression decreased the cellular concentrations of 5-methyl-THF and 5-formyl-THF, two of the most stable forms of folate, and increased the level of 10-formyl-THF and THF, which are the folate cofactors most susceptible to nonenzymatic oxidative degradation. Anguera *et al.,* (2003) demonstrated that MTHFS activity regulated intracellular folate concentrations by accelerating the rate of catabolism, and that

newly imported folate, which are in their monoglutamate forms and not protein-bound, were more susceptible to catabolism. The method used by Anguera *et al.,* (2003) involved culturing MCF-7 cells and cells over-expressing human MTHFS cDNA in medium containing [^{3}H]5-CHO-THF and analysing aliquots of the culture medium using an ion-pair reverse phase HPLC method very similar to that used by Quinlivan *et al.,* (2000). The folate degradation product *p*ABG was detected using a diode array UV spectrophotometric (Anguera *et al.,* 2003). An increase in the rate of folate catabolism was identified by an increase in the *p*ABG concentration present the medium. They suggested that MTHFS might accelerate folate catabolism by altering the distribution of folate co-factors, resulting in the accumulation of more labile folate co-factors (Anguera *et al.,* 2003). Therefore, MTHFS-mediated catabolism may modulate tissue or cellular folate concentrations independent of whole-body folate status, and thereby regulate folate metabolic pathways.

As discussed in chapter 1, homozygosity for the MTHFR C677T polymorphism results in reduced enzyme activity. It has also been associated with a lower than expected red cell folate concentration in both pregnant and non-pregnant women (Molloy *et al.,* 1997), however, the reason for this reduction in red cell folate is still unclear. Bagley and Selhub (1998) demonstrated that intracellular folate co-factor distribution differed with respect to the MTHFR C677T in human red blood cells. The folates present in red blood cells of individuals homozygous for the wild type variant CC were all in their methylated co-factor form. Red blood cells of individuals homozygous for the mutant TT had 71% of their folates present in a formylated folate form and 29% present in a methylated form (Bagley and Selhub, 1998). Formylated folates are more susceptible to folate degradation and an increase in folate catabolism

due to the presence of formylated folates may in part explain why MTHFR TT individuals have a lower concentration of red cell folates (Molloy *et al.,* 1997).

The aim of this chapter was to elaborate on the work of Bagley and Selhub (1998) by using the HPLC method developed in chapter 3 to demonstrate changes in individual folate co-factors in relation to the MTHFR C677T mutation. The objective was to examine the intra-cellular co-factor distribution in MTHFR CC human immortalised lymphocyte cell lines and compare them to MTHFR TT cell lines to determine if any alterations in folate co-factors between genotypes could be identified. The HPLC method developed in chapter 3 would also identify any intracellular *p*ABG present, and if an increase in *p*ABG levels was to be detected in MTHFR TT individuals it would suggest an increased rate of folate catabolism in this genotype. The folate degradation product *p*ABG was also determined in the growth medium from the cells to determine in there was an increase in the extracellular *p*ABG levels. This method was similar to that used by Anguera *et al.,* (2003) to determine the effect over-expressing MTHFS would have on the rate of folate catabolism in MCF-7 cells. An increase in *p*ABG levels would indicate an increased rate of folate catabolism, which, as suggested earlier, may explain why MTHFR TT individuals have lower concentrations of red cell folates.

5.2 Results

5.2.1 The effect of folic acid on the growth of immortalised lymphocyte cell lines

It was necessary to establish the growth conditions for human immortalised lymphocyte cell lines in which maximal labelling with radioactive folate could occur. Preliminary experiments were set up to determine the effect growth medium depleted of folates would have on the growth characteristics of these cells. Growth curves were set up as described in method 2.6.4 using normal RPMI 1640 medium which contained 22.65nM folic acid and folate free RPMI 1640 medium. The cells were seeded $2x10^5$ cells/ml into a $25cm^2$ flask containing 10ml of medium in triplicate for each time point and counted at 24hr intervals over 4 days. Each experiment was set up in triplicate (n=3). The mean and standard deviation was calculated for each time point and plotted on a graph, see figure 5.1. The normal growth response for immortalised lymphocyte cells was established. There appeared to be no difference between the two growth conditions, which suggested that either lymphocyte cells had an intracellular store of folates and that the cell required very little folate to function normally or that sufficient purine and pyrimidine precursors were available in the medium to support cell proliferation in the absence of folate. In order to maximise the incorporation of radioactive folate any intracellular folate stores would need to be depleted. To investigate if folic acid is a rate-limiting step on cell proliferation the cells were sub-cultured in folate free medium for one passage and their growth response again determined.

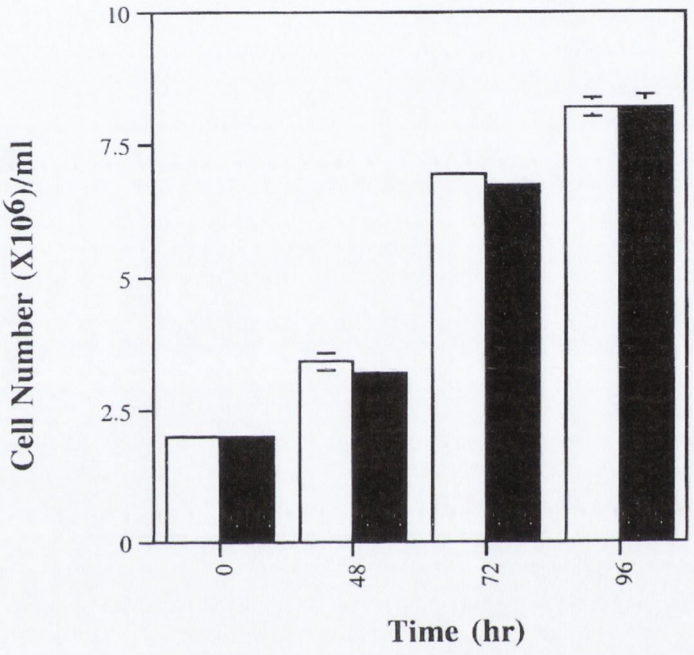


Figure 5.1: The effect of folic acid on the growth of immortalised lymphocyte cell lines.

Immortalised lymphocyte cells were seeded $2x10^5$ cells/ml into a $25cm^2$ flask containing 10ml of normal RPMI 1640 medium which contained 22.65nM folic acid and folate free RPMI 1640 medium in triplicate for each time point and counted at 24hr intervals over 4 days (n=3). The mean and standard error of the mean was calculated for each time point and plotted. Error bars represent the SEM of three flasks.

□ = RPMI 1640 medium containing 22.65nM folic acid.

■ = Folate free RPMI 1640 medium.

5.2.2 The effect of folate depletion on immortalised lymphocyte cell growth

Growth curves were set up as described in method 2.6.4 using cells that had been grown for 1 passage in folate free RPMI 1640 medium. A concentration of 0.5μCi [^{3}H]5-CHO-THF, which is the equivalent to 1.19nmol 5-CHO-THF, was to be supplemented into the growth medium in order to radiolabel the intracellular folate co-factors. For that reason the cells were seeded 2x10^5 cells/ml into a 25cm^2 flask containing 10ml of folate free RPMI 1640 medium supplemented with 1.19nmol 5-CHO-THF and counted at 24hr intervals over 4 days. Each experiment was set up in triplicate (n=3) for each time-point, see figure 5.2 for results. A comparison was made with the growth response of cells grown in folate free medium. There was a trend towards lower growth in the folate free medium but this was not significant. Nevertheless growing the cells for one passage in folate free medium was considered to be a suitable pre-treatment regime as the aim was to deplete the cells of their intracellular folate stores in order to maximise the incorporation of radioactive folate but keep their normal growth response and cell morphology. This ensured the cells were normal and healthy.

5.2.3 Uptake of [^{3}H] 5-CHO-THF on cells, which have been depleted on folate for one passage

Cells which had been depleted of folate for 1 passage were seeded 2x10^{5}cells/ml and fed with 10ml of folate free medium 1640 supplemented with 1.19nmol [^{3}H]5-CHO-

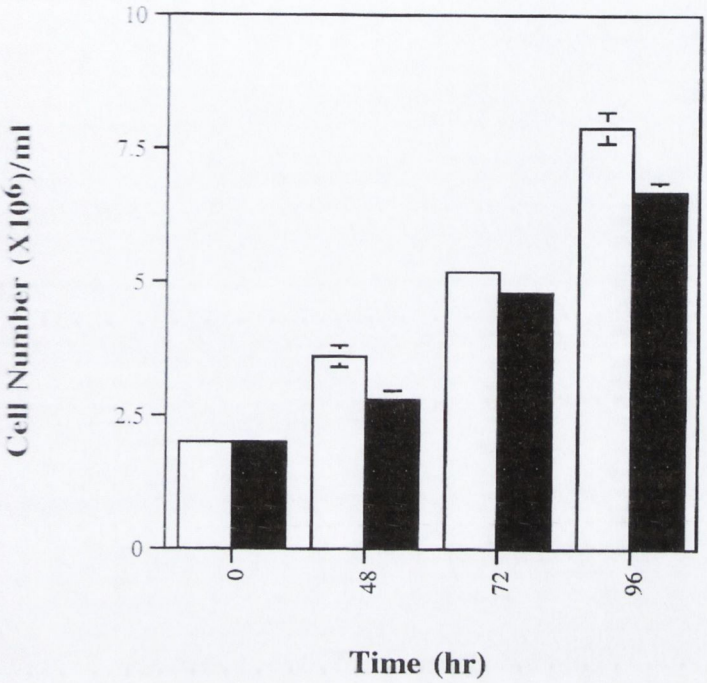


Figure 5.2: The effect of 5-CHO-THF on the growth response of immortalised lymphocyte cell lines.

Immortalised lymphocytes cells were passaged once in folate free medium and then seeded $2x10^5$ cells/ml into a $25cm^2$ flask containing 10ml of folate free RPMI 1640 medium and folate free RPMI 1640 medium supplemented with 1.19nmol 5-CHO-THF. The cells were counted at 24hr intervals over 4 days for each time-point (n=3). The mean and standard error of the mean was calculated for each time point and plotted. Error bars represent the SEM of three flasks.

□ = Folate free RPMI 1640 medium supplemented with 1.19nmol 5-CHO-THF.
■ = Folate free RPMI 1640 medium.

THF. The cells were grown for 72hr and then harvested. The radiolabel incorporation in each cell pellet was determined as per method 2.7. 10µl of the cell suspension was counted in a scintillation counter to determine the amount of [^{3}H]5-CHO-THF incorporated within the cells. The results were multiplied by 10 to determine the amount of radiolabel in the 100µl of cell suspension. This procedure was carried out on 12 cell lines, six of which were MTHFR CC and six MTHFR TT, see results table 5.1 and 5.2. A comparison of the [^{3}H]5-CHO-THF incorporation between the two MTHFR genotypes was carried out, see figure 5.3. The MTHFR CC genotype cell lines appeared to incorporate a higher level of [^{3}H]5-CHO-THF however, there was more variation in radiolabel incorporation into these cells. The difference between MTHFR C677T genotypes was not considered significant using a student t-test ($p=0.16$), a difference is considered significant when $p<0.05$.

5.2.4 Folate co-factor distribution in lymphocyte cell lines

The cell samples were prepared as per method 2.7 and the intracellular folate co-factor distribution was analysed using the discontinuous gradient HPLC method described in section 3.2.12. The radiolabelled folate co-factors were identified by their co-elution with a spike containing *p*ABG, 5-CHO-THF, 5,10-CH_2-THF, FA and 5-CH_3-THF. The folate co-factor distribution for each cell line was carried out in duplicate by performing each HPLC analysis on two separate flasks of cells. The reason for this was to establish if the results were reproducible for each cell line, see table 5.3 for an example of duplicate results for an individual cell line.

	17180 CC	15970 CC	15041 CC	15931 CC	17219 CC	17242 CC
Cell Pellet 1	87358	118763	136950	178306	92208	87023
Cell Pellet 2	82828	131871	129660	156762	78417	90185
Cell Pellet 3	88283	138418	139421	155980	92031	94410
Mean	**86166**	**129684**	**135344**	**163683**	**87552**	**90539**
SD	**2919**	**10008**	**5075**	**12670**	**7912**	**3706**
%CV	**3.4**	**7.7**	**3.7**	**7.7**	**9.0**	**4.1**

Table 5.1: **The intracellular incorporation of [^{3}H]5-CHO-THF in MTHFR 677 CC immortalised lymphocyte cell lines.** MTHFR CC immortalised lymphocyte cell lines (n=6) were incubated for 72hr in folate free medium RPMI 1640 supplemented with 1.19nmol [^{3}H]5CHO-THF and the [^{3}H]5-CHO-THF incorporated was recorded as dpm counts. Each experiment was carried out in triplicate for each cell line.

	15083 (TT)	17243 (TT)	17259 (TT)	17219 (TT)	17265 (TT)	17274 (TT)
Cell Pellet 1	99592	103465	92117	95583	105281	76064
Cell Pellet 2	86715	94577	101843	99925	92237	77672
Cell Pellet 3	96762	93828	103284	93156	105714	88456
Mean	**94356**	**97290**	**99081**	**96221**	**101077**	**80831**
SD	**6767**	**5361**	**6074**	**3429**	**7659**	**6672**
%CV	**7.2**	**5.5**	**6.1**	**3.6**	**7.6**	**8.3**

Table 5.2: **The intracellular incorporation of [^{3}H]5-CHO-THF in MTHFR 677 TT immortalised lymphocyte cell lines.** MTHFR TT immortalised lymphocyte cell lines (n=6) were incubated for 72hr in folate free medium RPMI 1640 supplemented with 1.19nmol [^{3}H]5CHO-THF and the [^{3}H]5-CHO-THF incorporated was recorded as dpm counts. Each experiment was carried out in triplicate for each cell line.

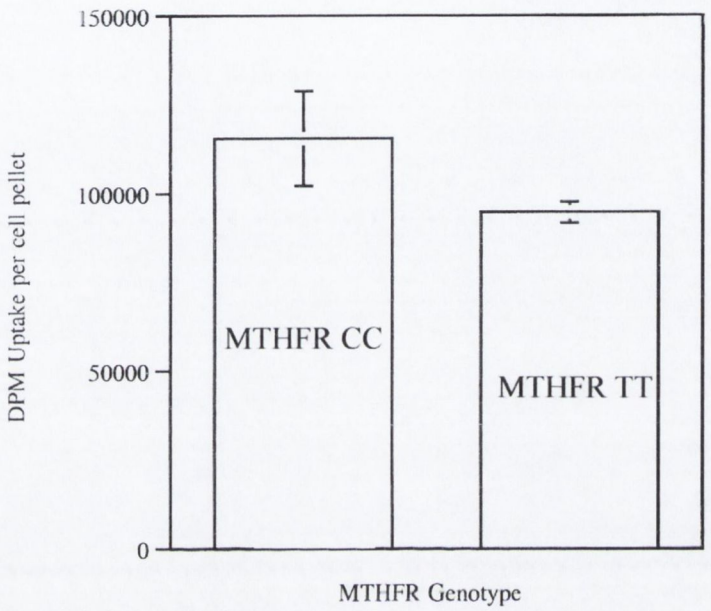


Figure 5.3: The effect of MTHFR C677T genotype on [^{3}H]5-CHO-THF incorporation in human immortalised lymphocyte cell lines.

Cells depleted of folate for one passage were seeded 2x105cells/ml and grown in 10ml of folate free medium 1640 supplemented with 1.19nmol [3H]5-CHO-THF for 72hr. The cells were harvested and resuspended in 100µl of 1X PBS. 10µl of each cell suspension was counted in a scintillation counter to determine the amount of [3H]5-CHO-THF dpm counts incorporated within the cells. The results were multiplied by 10 to determine the amount of radiolabel in the 100µl of cell suspension. Each MTHFR C677T genotype consisted of 6 cell lines (n=6). Error bars represent the SEM of 6 cell lines.

☐ = dpm uptake/cell pellet

The reproducibility of each individual folate co-factor was determined based on the results of two experiments for each of the twelve cell lines. The calculation of SD from duplicate measurements made on a number of different subjects or materials was used to determine the reproducibility of the measurement. The assumption of the calculation was that the duplicates measurements are samples from populations with the same variance but not necessarily the same mean (Jones and Payne, 1997). The variance was calculated, as the sum of the squares of the differences between the duplicates divided by twice the number of pairs and the SD was its square root. Using this method allowed a CV to be calculated for each folate co-factor. The percentage coefficient for each individual folate co-factor was consistently low, see table 5.4 indicating that the results were reliable and reproducible.

The folate co-factor distribution in the cells of the MTHFR CC genotype were compared with the cells of the MTHFR TT genotype, see figure 5.4. The results showed very little difference in the pABG levels between the two MTHFR genotypes, MTHFR CC cell line had 11.3% (±2.3) and the MTHFR TT having 8.6% (±1.4). The MTHFR TT cell lines had higher levels of 5,10-CH^{+}-THF/10-CHO-THF at 22.7%(±3.4) and 5,10-CH_2-THF at 22.5% (±3.5) when compared with MTHFR CC levels of 5,10-CH^{+}-THF/10-CHO-THF at 15.4% (±2.6) and 5,10-CH_2-THF at 15.7% (±5.2). The MTHFR CC cell lines had higher level of 5-CH_3-THF at 13.1% (±SEM0.7) when compared with MTHFR TT levels of 5-CH_3-THF at 9.0% (±1.6). The folate co-factor peaks of 5,10-CH^{+}-THF/10-CHO-THF, 5-CHO-THF and 5,10-CH_2-THF were combined and called formyl folates and when the two genotypes were compared it was clear that the MTHFR TT genotype had a higher overall percentage of intracellular formylated folates present.

Folate co-factors	Analysis 1	Analysis 2	Mean
Unknown	4	5	4.5
*p*ABG	11	14	15.5
5,10-CH^+-THF/10CHO-THF	41	34	38
THF	28	27	27.5
5-CHO-THF	9	6	7.5
DHF	ND	ND	ND
5,10-CH_2-THF	4	8	6
FA	ND	ND	ND
5-CH_3-THF	4	6	5

Table 5.3: Folate co-factor distribution in immortalised lymphocyte cell line 15083.

An immortalised human lymphocyte cell line 15083 was sub-cultured and grown in folate free RPM1 1640 medium for 96hr. Subsequently the cells were sub-cultured and grown in folate free RPM1 1640 medium supplemented with 1.19nmol [^{3}H]5CHO-THF for 72hr. The intracellular folate co-factors were extracted and separated using the HPLC method described in section 2.7. The experiment was carried out in duplicate with the radioactivity corresponding to each folate co-factor was expressed as a percentage of the total radiolabelled folate. The mean result was calculated for the duplicate runs.

Folate co-factors	Mean (n=12)	%CV
*p*ABG	9.6	5.6
5,10-CH^+-THF/10-CHO-THF	19.2	5.7
THF	29.9	5.1
5-CHO-THF	7.3	3.18
5,10-CH_2-THF	19.7	4.9
5-CH_3-THF	11.2	4.5

Table 5.4: The reproducibility of folate co-factors based on duplicate experiments using 12 cell lines.

The reproducibility of folate co-factors was determined based on duplicate experiments carried out on 12 cell lines. The result for each folate co-factor is the radioactivity corresponding to each folate co-factor expressed as a percentage of the total radiolabelled folate The variance was calculated as the sum of the squares of the differences between the duplicates divided by twice the number of pairs and the SD was its square root. The percentage coefficient for each individual folate co-factor was consistently low.

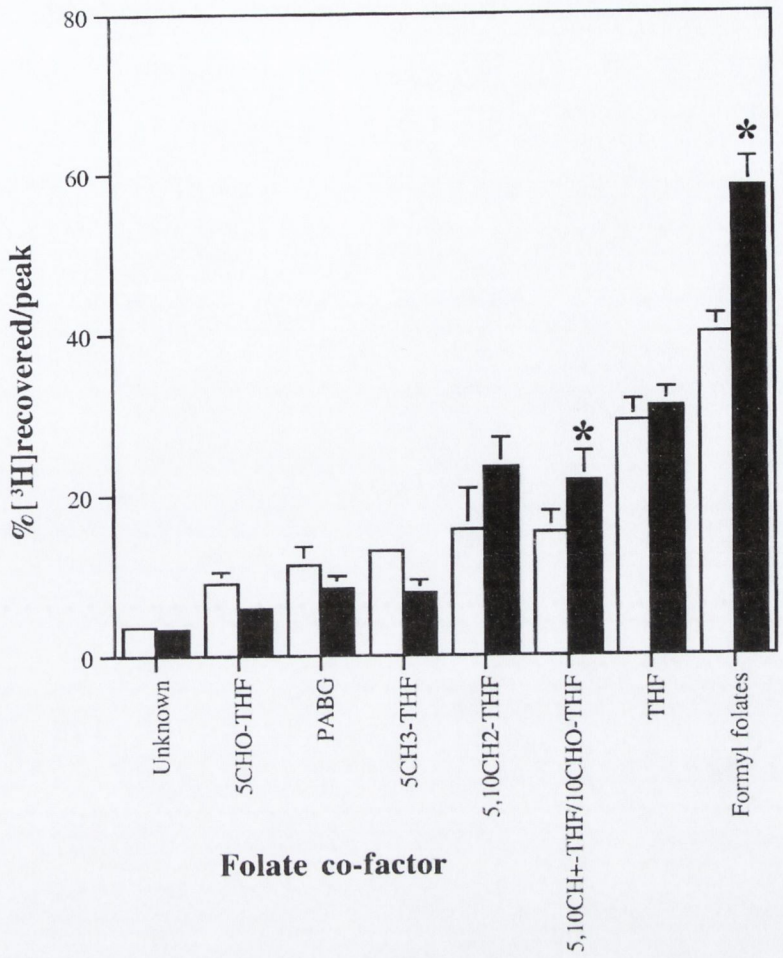


Figure 5.4: The effect of MTHFR C677T genotype on the intracellular folate co-factor distribution in human immortalised lymphocyte cell lines.

Cells depleted of folate for 1 passage were seeded $2x10^5$ cells/ml and fed with 10ml of folate free medium 1640 supplemented with 1.19nmol [³H]5-CHO-THF and re- grown for 72hr. The cells were harvested and re-suspended in 100µl of 1X PBS. The intracellular folate co-factors were deconjugated using rat serum and incubating for 1hr at 37° C. The cell suspension was spiked with a standard mix containing pABG, 5-CHO-THF, 5,10-CH_2 -THF, FA and 5-CH_3 -THF and the intracellular folate co-factors distribution was analysed using the discontinuous gradient HPLC method described in section 3.2.12. The radiolabelled folate co-factors were identified by their co-elution with a spike containing pABG, 5-CHO-THF, 5,10-CH_2 -THF, FA and 5-CH_3 -THF. The folate co-factor distribution for each cell line was carried out in duplicate by performing each HPLC analysis on two separate flasks of cells. Error bars represent the SEM of 6 duplicate cell lines for each MTHF C677T genotype (n=6). Genotype differences were compared using a student t-test. A difference is considered significant when p(value)<0.05 with the asterix representing a significant difference.

□ = MTHFR 677 CC genotype
■ = MTHFR 677 TT genotype

Genotype differences between each folate co-factor were compared using a student t-test. Using this statistical test a difference is significant when $p<0.05$, see table 5.5 for results. A significant difference was observed between genotypes for the 5,10-CH^+-THF/10-CHO-THF peak ($p=0.0031$), see and table 5.5. A p(value) of 0.056 was reported for the 5-CH_3-THF peak, which was considered borderline significant. The percentage of 5,10-CH_2-THF was higher in MTHFR TT cells lines when compared with MTHFR CC cell lines but this difference was not considered significant ($p=0.32$).

The unidentified folate co-factor peak which eluted from the column in the void volume had an identical elution time with that of the co-factor 5,10-CH^+-THF but, as discussed in chapter 3, any 5,10-CH^+-THF present in the cells is converted to 10-CHO-THF due to the pH of the extraction buffer and mobile phase. The unidentified peak may perhaps be pterins or other degradation products of folates that are positively charged and therefore do not bind to the column.

Anguera *et al.,* (2003) demonstrated that MTHFS activity regulated intracellular folate concentrations by accelerated rates of catabolism, and that newly imported folate, which is in the monoglutamate form and therefore not protein-bound, was more susceptible to catabolism. They used a HPLC method very similar to that developed in chapter 3 and the presence of *p*ABG in the cell culture medium had indicated folate catabolism. The culture medium from the lymphocyte cell lines labelled with 1.19nmol [^{3}H]5-CHO-THF was analysed using the method developed in chapter 3 to determine if increased levels of extracellular *p*ABG were present in the

Folate co-factors	MTHFR 677CC Mean (SEM),(n=6)	MTHFR 677 TT Mean (SEM), (n=6)	p(value)
Unknown	3.6(0.6)	3.3(0.2)	0.57
*p*ABG	11.3(2.3)	8.6(1.4)	0.34
5,10-CH^+-THF/10-CHO-THF	15.4(2.6)	22.7(3.4)	0.0031
THF	29.2(2.7)	29.3(2.3)	0.96
5-CHO-THF	9.0(1.4)	6.3(0.7)	0.13
DHF	ND	ND	ND
5,10-CH_2-THF	15.7(5.2)	22.5(3.5)	0.31
FA	ND	ND	ND
5-CH_3-THF	13.1(0.7)	9.0(1.6)	0.057
Formyl folates	40.1(2.3)	58.2(3.7)	0.0032

Table 5.5: Folate co-factor distribution in MTHFR CC and MTHFR TT human immortalised lymphocyte cell lines.

Immortalised human lymphocyte cell line were sub-cultured and grown in folate free RPM1 1640 medium for 96hr. Subsequently the cells and grown in folate free RPM1 1640 medium supplemented with 1.19nmol [^{3}H]5-CHO-THF for 72hr. The intracellular folate co-factors were extracted and separated using the HPLC method described in section 2.7. The experiment was carried out in duplicate with the radioactivity corresponding to each folate co-factor was expressed as a percentage of the total radiolabelled folate. The mean of six cell lines analysed in duplicate for each MTHFR C677T genotype were calculated and reported. The formyl folate group represents a combination of 5-CHO-THF, 10-CH0-THF and 5,10-CH_2-THF peaks. Genotype differences for each folate co-factor were compared using a student t-test. There was a significant difference observed between the 5,10-CH^+-THF/10-CHO-THF and formyl folate groups (differences were considered significant if the p(value) <0.05).

culture medium of the MTHFR TT genotype cell lines when compared with MTHFR CC cell lines. An increased level of *p*ABG in the extracellular medium of the MTHFR TT cells would suggest increased folate catabolism. It was necessary to set up a suitable method to concentrate folates from the extracellular medium before HPLC analysis could be preformed.

5.2.5 Development and validation of the Sep-Pak method to concentrate the folate co-factors present in the extracellular medium of immortalised human lymphocyte cell lines.

A sep-pak C18 column was conditioned as per method 2.8. A 1ml sample of media was spiked with a [^{3}H]5-CHO-THF standard. The medium sample was injected through the column and then flushed from the column by injecting 1ml methanol slowly through the column and collecting the eluting fraction. This was carried out four times and each fraction was collected to determine which fractions contained the eluted sample, see table 5.6(a) and (b). As shown the [^{3}H] folate eluted in the first and second methanol wash fractions. As result two methanol wash fractions were combined, the methanol was dried under a stream of nitrogen gas from the sample and resuspended in mobile phase and analysed on the HPLC, see method 2.8. There was a full recovery of the [^{3}H]5-CHO-THF standard from a 1ml medium sample run through a sep-pak column, see table 5.6(a), however, the [^{3}H] in the culture medium from the cells is in 10ml media and so the next step was to determine the recovery of counts from a 10ml sample. The procedure was carried out as before using a 10ml sample. As shown in table 5.6(b) there was a full recovery of counts from the sep-pak column in the first and second methanol wash fractions.

	Total radioactivity in 1ml sample (dpm)	Radioactivity in fraction collected following Sample loaded (dpm)	Radioactivity in fraction following 1st MeOH wash (dpm)	Radioactivity in fraction following 2nd MeOH wash (dpm)	Radioactibity in fraction following 3rd MeOH wash (dpm)	Radioactivity in fraction following 4th MeOHwash (dpm)
	16633.5	29.0	13888.1	4199	22.86	22.85
	18093.8	26.0	15819.7	4493.7	45.8	43.44
	19429.2	20.0	14114.5	5058	25.71	14.24
Mean	18052.2	25.0	14607.4	4583.6	31.5	26.8

Table 5.6(a): The recovery of radioactivity from a 1ml sample using a Sep-Pak column. A 1ml medium sample was loaded onto a Sep-Pak column as per method 5.2.5. The recovery of radioactivity from each fraction collected from the sep-pak column following 1ml MeOH washes.

	Total radioactivity in 10ml sample (dpm)	Radioactivity in fraction collected following Sample loaded (dpm)	Radioactivity in fraction following 1st MeOH wash (dpm)	Radioactivity in fraction following 2nd MeOH wash (dpm)	Radioactibity in fraction following 3rd MeOH wash (dpm)	Radioactivity in fraction following 4th MeOHwash (dpm)
	12568	18.2	9764.9	2329.8	42.96	34.37
	15174	46.7	11246.8	1658.8	62.54	48
	9461	49.6	10951.3	1869.6	34.37	28.72
Mean	12401.0	38.2	10654.3	1952.7	46.6	37.0

Table 5.6(b): The recovery of radioactivity from a 10ml sample using a Sep-Pak column. A 10ml medium sample was loaded onto a Sep-Pak column as per method 5.2.5. The recovery of radioactivity from each fraction collected from the Sep-Pak column following 10ml MeOH washes.

The medium containing 1.19nmol [^{3}H]5-CHO-THF which had been used to radiolabel the intracellular folate co-factors was collected and stored at –20°C for future analysis. The medium was run through a sep-pak column as per method 2.8. The collected fractions were dried under a stream of nitrogen gas and resuspended in 100µl of mobile phase. A spike containing *p*ABG, 5-CHO-THF and 5-CH_3-THF was added to each of the samples and the samples were analysed using the HPLC method described in section 3.2.12. The radiolabelled folates were identified by their co-elution with a spike containing *p*ABG, 5-CHO-THF, and 5-CH_3-THF. The radioactive folate co-factors present in the media were expressed as a percentage of the total radioactivity present in the media, see table 5.7.

An unidentified peak eluted in the void volume. The elution time of this peak was identical to that of the 5,10-CH^+-THF but as discussed previously any 5,10-CH^+-THF present would be recovered as 10-CHO-THF due to the pH of the mobile phase. A second unidentified peak eluted between the pABG and 5-CHO-THF peak. In order to try and identify this peak standards of 5-CH_3-DHF, 10-CHO-FA and *p*ABA were prepared and run on the HPLC. The unidentified peak proved to be none of the above degradation products of folates as, the pABA eluted just before the *p*ABG peak, the 5-CH_3-DHF peak eluted just after the 5-CH_3-THF peak and 10-CHO-FA peak co-eluted with the DHF peak following the 5-CHO-THF peak. When the [^{3}H]5-CHO-THF standard was run separately on the HPLC the same peak was reported and therefore it would appear to be a breakdown product present in the [^{3}H]5-CHO-THF standard. No 5-CH_3-THF was detected in the medium of either genotype and there was no

Folate co-factors	MTHFR 677CC Mean (SEM),(n=6)	MTHFR 677 TT Mean (SEM), (n=6)
Unknown	3.7(0.6)	4.2(0.5)
*p*ABG	14.3(0.4)	14.3(0.6)
Unknown	28.3(1.7)	29.9(0.6)
5-CHO-THF	53.3(1.8)	52.5(1.7)
5-CH_3-THF	ND	ND

Table 5.7: HPLC analysis of the growth medium from human immortalised lymphocyte cell lines radiolabelled with 1.19nmol [^{3}H]5CHO-THF for 72hr.
Immortalised human lymphocyte cell line were grown in folate free RPM1 1640 medium supplemented with 1.19nmol [^{3}H]5CHO-THF for 72hr. The cells were separated from the medium and the medium was stored at -20°C. The folate co-factors distribution was analysed in the medium by firstly, extracting the folate co-factors using a sep-pak column as per method 5.2.5 and secondly, analysing the purified sample using the HPLC method as described in section 2.7. The mean of six cell lines for each MTHFR C677T genotype were calculated and reported. The radioactivity corresponding to each folate co-factor was expressed as a percentage of the total radiolabelled folate in the medium.

difference between the two genotypes for the recovered peaks of 5-CHO-THF and *p*ABG.

5.3 Discussion

The aim of this chapter was investigate the folate co-factor distribution in human immortalised lymphocytes with respect to the MTHFR C677T polymorphism. In order to do this the intracellular folate co-factors in human immortalised lymphocyte cell lines were analysed using the HPLC method previously developed in chapter 3. The first step was to establish the optimal growth conditions of the immortalised human lymphocyte cell lines to ensure maximum [^{3}H]5-CHO-THF incorporation. The normal growth response for these cells was established as per method 2.6.4 using full RPMI 1640 medium, which contained 22.66nM folic acid, see figure 5.1.

The effect folic acid had on the growth response of the immortalized human lymphocyte cells was determined as per method 2.6.4 using folate free RPMI 1640 medium. There appeared to be no difference in the growth rate of the cells grown in folate free RPMI 1640 medium for one passage, see figure 5.1. Therefore the immortalized lymphocyte cells were passaged once in folate free RPMI 1640 medium in an attempt to deplete the cells intracellular folate store and re-grown in folate free RPMI 1640 medium in order to determine if there was a difference in the growth response of cells following two passages in folate free medium, see figure 5.2. As can be seen in figure 5.2 normal cell proliferation was maintained with only a slight decrease at the 96hr time-point. However, the cells grown in folate free medium supplemented with 1.19nmol5-CHO-THF appeared to have a normal growth response following a 96hr incubation. As a result, the cells were to be passaged once in folate free RPMI 1640 medium and then labeled with 0.5μCi[^{3}H]5-CHO-THF which is the

equivalent of 1.19nmol 5-CHO-THF before their intracellular folate co-factors were analyzed. This step ensured the cell proliferation rate remained normal, see figure 5.2.

A comparison of the [^{3}H]5-CHO-THF incorporation between the two MTHFR genotypes was carried out, see figure 5.3. The MTHFR CC genotype cell lines appeared to incorporate higher concentrations of [^{3}H]5-CHO-THF when compared with MTHFR TT cell lines. However, this difference was not considered significant using a student t-test.

The intracellular folate co-factor distribution was analysed in MTHFR CC and MTHFR TT cell lines using the discontinuous gradient HPLC method described in 3.2.12. The radiolabelled folate co-factors were identified by their co-elution with a spike containing pABG, 5-CHO-THF, 5,10-CH_2-THF, FA and 5-CH_3-THF. The folate co-factor distribution for each cell line was carried out in duplicate by performing each HPLC analysis on two separate flasks of cells. The mean result for the duplicate runs for each cell line were calculated and these results were then grouped according to their MTHFR C677T genotype, see table 5.3. The reproducibility of each individual folate co-factor was determined based on the results of two experiments for each of the twelve cell lines (n=24). The percentage coefficient of variation for each individual folate co-factor was consistently low <10%, see table 5.4. This proved that each folate co-factor result was reliable and reproducible.

Lathrop-Stern *et al.,* (2003) reported that under all conditions, immortalised lymphoblasts with the MTHFR 677 TT genotype had significantly lower *in vitro* MTHFR activity than cells with the MTHFR 677 CC genotype. They also reported that the folate co-factor distribution differed in relation to the MTHFR C677T genotype regardless of the nutrient conditions (Lathrop-Stern *et al.,* 2003). They compared the MTHFR activity in cells exposed to adequate nutrient conditions with cells exposed to either reduced riboflavin or combined low folate and low riboflavin status. The MTHFR activity of the TT genotype cells was significantly lower when they were exposed to inadequate nutrient conditions when compared with adequate nutrient conditions. However, the MTHFR activity in the wild type cells was not lower than in control medium under any of the treatments (Lathrop-Stern *et al.,* 2003).

The intracellular folate co-factor distribution results obtained from the human immortalised lymphocyte cell lines in this study were compared in relation to their MTHFR C677T mutation, see figure 5.4. A significant difference was observed for the 5,10-CH^{+}-THF/10-CHO-THF peak ($p=0.0031$) and for the formyl folate group ($p=0.0032$), see figure 5.4 and table 5.5. A p(value) of 0.06 was reported for the 5-CH_3-THF peak which is considered borderline significant. This pattern was similar to that demonstrated in red blood cells (Bagley and Selhub, 1998) and also in immortalised lymphocytes (Lathrop-Stern *et al.,* 2003).

Results obtained from immortalised lymphocyte cell lines in this study have provided further evidence that firstly, the MTHFR TT genotype has reduced enzyme activity as was reported by lathrop-Stern *et al.,* (2003). The reduced MTHFR activity was evident by the significantly higher proportion of formylated folates in the MTHFR TT

cell lines. Secondly, the results showed the reduced enzyme activity manifests itself as a phenotype *in vivo* by a significant increase in the percentage of intracellular formylated folates present in the immortalised lymphocyte MTHFR TT cell lines. Lathrop-Stern *et al.,* (2003) also reported a significant reduction in the methylated folates, however, in the present study the reduction was considered borderline significant ($p=0.06$). Lathrop-Stern *et al.,* (2003) reported cells with the TT genotype had approximately half the concentration of 5-CH_3-THF polyglutamates and nearly double the concentration of 5-CHO-THF polyglutamates than the CC genotype cells. In this present study, the formylated folates appeared to be mostly in the 10-CHO-THF and 5,10-CH_2-THF form with only a small percentage in the 5-CHO-THF form. The differences reported in the distribution of the formylated folate co-factors between the two studies may be due to the different extraction and method of analysis between the two studies or perhaps that in the present method the intracellular folate co-factors were analysed in cells in log phase growth. Lathrop-Stern *et al.,* (2003) reported that despite the lower enzyme activity, the mutant enzyme provided sufficient 5-CH_3-THF to meet the demands for methionine synthesis and homocysteine remethylation and that no difference was reported between genotypes in the ability to synthesise methionine under adequate, low folate or low riboflavin conditions (Lathrop-Stern *et al.,* 2003). The results from this present study also demonstrated that there was an adequate supply of 5-CH_3-THF in both genotypes.

Anguera *et al.,* (2003) suggested that in relation to the MTHFS enzyme that, the over-expression of MTHFS in a cell may accelerate folate catabolism by altering the distribution of folate co-factors resulting, in the accumulation of more labile folate co-factors (Anguera *et al.,* 2003). Therefore they suggested, MTHFS-mediated

catabolism may modulate tissue or cellular folate concentrations independent of whole-body folate status, and thereby regulate folate metabolic pathways. Increased rates of catabolism may be linked with the MTHFR C677T mutation also as the increased levels of formylated folate which are more labile and easily degraded compared with methylated folate co-factors may in fact lead to an increase in folate catabolism in MTHFR TT cell lines. There was no difference reported in the intracellular *p*ABG levels in this study. The culture medium in which the cells were grown was analysed to see if a difference could be identified in the percentage of radiolabelled *p*ABG between the MTHFR C677T genotypes. No difference was detected in the radiolabelled *p*ABG present in media or in the radiolabelled 5-CHO-THF, see table 5.7. However, the question still remains that if the cells were riboflavin or folate depleted would a difference be detected. As discussed in chapter 4 the assay required the intracellular folate co-factor to be radiolabelled with [^{3}H]5-CHO-THF prior to HPLC analysis and therefore using this method the cells would never be depleted of folates entirely.

Epidemiological data suggests that there is an interaction between the folate status and the MTHFR C677T mutation. This interaction is manifested by a higher level of plasma homocysteine and a lower level of genomic DNA methylation, but only when the folate status is low in subjects with the MTHFR TT compared with the wild type CC (Lathrop-Stern *et al.,* 2000; Friso *et al.,* 2002). It has been suggested that under conditions of adequate folate status, the MTHFR C677T mutation protects against the development of certain cancers, particularly colorectal cancer (Chen *et al.,* 1996; Ma *et al.,* 1997). The reason for this protection has been attributed to the greater availability of formylated folates, which serve as substrates for the synthesis of the

purines and pyrimidines that are necessary for DNA synthesis and repair (Ma *et al.,* 1997). It has been reported that during human GI carcinogenesis, MTHFR is highly polymorphic, and the variant genotypes result in decreased MTHFR enzyme activity and lower plasma folate level, as well as aberrant methylation (Jing and Shu, 2004). Kawakami *et al.,* (2003) reported significantly higher levels of THF in tumours from the proximal colon compared with those originating in the distal colon and rectum. Tumours with aberrant DNA methylation of the CpG islands within the promoter regions of hMLH1, TIMP3 and ARF genes also contained higher levels of 5,10-CH_2-THF and THF. In contrast, patients who were homozygous for the C677T polymorphism of the MTHFR gene had significantly lower concentrations of both theses folate co-factors in their tumor tissue. They concluded that concentrations of folate co-factors in colorectal tumours are directly related to the presence of frequent DNA hypermethylation and inversely related to the presence of a common polymorphism in the MTHFR gene. They suggest that folate co-factors could serve as biochemical markers for the risk of developing this disease (Kawakami *et al.,* 2003). The findings in this present study are consistent with this theory that in folate adequate cells there is a significant increase in the amount of formylated folates present and a decrease in the methylated folates present.

Chapter 6:

To investigate the effect of MTX on folate metabolism in relation to the MTHFR C677T polymorphism.

6.1 Introduction

In 1965, Burchall and Hitchings postulated that the diaminopyrimidines were inhibitors of DHFR. Their selectivity had been attributed to the fact that they were structural analogs of only part of the folic acid molecule and not all of it, as is the case with MTX and aminopterins (Brumfitt *et al.,* 1980).

Trimethoprim (TMP) is a selective inhibitor of bacterial DHFR with no notable toxicity against mammalian forms of the enzyme (Brumfitt *et al.,* 1980). It is a broad spectrum antibiotic and is used on its own or in combination with sulfamethoxazole (SMX) as the first line of defence against urinary tract infections and acute respiratory infections. Upon treatment with TMP the intracellular THF co-enzyme pool in bacterial cells are depleted (Quinlivan *et al.,* 2000). Quinlivan *et al.,* (2000) suggested that the depletion of the folate pool upon anti-folate treatment could in part be explained by folate catabolism. Following the treatment of *E.coli* cells with TMP they reported an accumulation of DHF, which diminished to a very low level over-time while the level of *p*ABG increased (Quinlivan *et al.,* 2000). This suggested that the DHF was being catabolised to *p*ABG and pteridines. An increase in FA was also reported over-time, which suggested that the DHF could either be catabolised to *p*ABG and pteridines or re-oxidised to FA. Following the removal of the TMP the FA which accumulated was shown to be metabolically active, which may in part explain the bactertiostatic effect of TMP on bacterial cells (Quinlivan *et al.,* 2000). Quinlivan *et al.,*(2000) hypothesised that the parallel effects of antifolates *i.e.* disruption of the cell redox state and a decrease in the intracellular pH may contribute

to the spontaneous cleavage of labile folates such as DHF and would increase the efficacy of TMP against bacterial infections (Quinlivan *et al.,* 2000).

In order to identify the intracellular folate co-factors of *E.coli,* Quinlivan *et al.,* (2000) developed an ion-pair reverse phase HPLC method. This method was further developed in this thesis (see chapter 3) in order to separate all the intracellular folate co-factors present in cells. The experiments of Quinlivan *et al.,* (2000) were repeated to ensure the method could give reproducible results. The results reported in chapter 3 verified that the consequences of TMP treatment were; 1) the depletion of THF co-enzymes 2) the acute accumulation of DHF following treatment which diminished to low levels over-time 3) an increase in *p*ABG levels over-time 4) an increase in folic acid levels over-time and 4) following the removal of TMP the folic acid re-entered the folate cycle. The re-cycling of the FA back into the folate cycle may in part explain it the bacteriostactic effect of TMP on bacterial cells.

As a result of reproducing the results previously demonstrated by Quinlivan *et al.,* (2000) further investigations were undertaken to investigate if phenotypic traits in the folate co-factor distribution could be identified for the MTHFR C677T mutation in mammalian cell lines, see chapter 4 and 5. The results from chapter 5 demonstrated that in human immortalised lymphocyte cell lines there was a significantly higher proportion of formylated folates present in the MTHFR TT cell lines and a reduction in methylated folates, which was borderline significant when compared with MTHFR CC cell lines. The aim of this final chapter was to investigate the effect MTX would have on the folate co-factor distribution in mammalian cells in relation to their

MTHFR C677T genotype and to determine if the MTHFR C677T mutation may affect the efficacy of drug treatment.

As discussed previously MTX is a folic acid analogue and has been used in high doses to treat leukaemia and in low doses to treat inflammatory disorders such as inflammatory bowel disease, psoriasis and rheumatoid arthritis. The primary mechanism of action of MTX was thought to be through the depletion of reduced folates resulting in the cessation of purine, thymidylate and protein synthesis (Jolivet, 1982). However, more recent studies demonstrated that the antifolate suppression of these biosynthesis processes occurred in MCF-7 human breast cancer cells and L1210 murine leukaemia cells despite the preservation of the major portion of the intracellular THF co-factors (Matherly *et al.,* 1987; Baram *et al.,* 1987). These results suggested the possibility of direct inhibitory effects of antifolates and/or DHF polyglutamates that accumulate in their presence, on purine and pyrimidine synthesis.

The intracellular folates in mammalian cells exist in their polyglutamated forms with up to seven glutamate moieties, which allows for the retention of the folate co-factors by the cells. Polyglutamted folates also confer enhanced affinity for several folate-requiring enzymes including AICAR transformylase and TS. MTX is also a substrate for FPGS and therefore becomes polyglutamated once inside the cell. Parent MTX and polyglutamated MTX metabolites are all folic acid analogues, which exert inhibitory effects on many of the enzymes in the folate metabolic pathway. These enzymes include thymidylate synthase (TS), 10-CHO-THF synthase, 10-CHO-THF cyclohydrolase, 5,10-CH_2-THF dehydrogenase, MTHFR and AICAR transformylase (Baggott *et al.,* 1986). DHF polyglutamates have also been reported to be potent

inhibitors of several folate dependent enzymes including MTHFR, TS, AICAR transformylase and MS. However, it is unclear if the degree of this inhibition and the levels of DHF polyglutamates that accumulate are of sufficient magnitude to produce important effects in cells beyond those attributed to antifolate-induced THF co-enzyme depletion. Seither *et al.,* (1989) had demonstrated in L1210 leukaemia cells that the direct effect of DHF polyglutamtes was unimportant however, Allegra *et al.,* (1987) demonstrated in MCF-7 breast cancer cells the DHF polyglutamtes inhibited TS (Allegra *et al.,* 1987). Polyglutamate derivatives of another MTX metabolite, 7-hydroxymethotrexate can also inhibit AICAR transformylase and other folate dependent enzymes (Baggott *et al.,* 1994). In summary the net effects of MTX include inhibition of folate dependent reactions involving the transfer of one-carbon units used in the production of thymidylate and purines, inhibition of production of methionine and local accumulation of adenosine.

MTX treatment can cause folate deficiency and the folate levels in patients taking even low dose MTX declines precipitously without folic acid supplementation. MTX treatment has also been associated with increased levels of plasma homocysteine (Van Ede *et al.,* 2002). Mildly elevated plasma homocysteine levels have been associated with an increased risk of coronary artery disease (Boushey *et al.,*1995; Graham *et al.,* 1997) and as a result coronary artery disease may be one of the adverse side effects of long-term low dose MTX treatment. Therefore due to the low folate status, increased plasma homocysteine levels and the inhibition of MTHFR caused by MTX treatment it has been hypothesised that toxicity to MTX may perhaps be aggravated in patients that have MTHFR TT and possibly CT genotype because of their reduced MTHFR

activity, increased plasma homocysteine levels and imbalances in the folate co-factor pools, which had also been associated with this polymorphism.

The first study illustrating a relationship between a folate-related variant and MTX toxicity was carried out by Ulrich *et al.,* (2001) on patients being treated with MTX to prevent graft-versus-host disease (GVHD) in patients that had undergone marrow transplantations. Toxicities included mucositis and myelosuppression. All the patients had received four doses of MTX intravenously, day 1, at $15mg/m^2$, and days 3, 6 and 11, at $15mg/m^2$. As hypothesised, patients homozygous for MTHFR TT experienced increased toxicity, evidenced by increased oral mucositis and a trend toward delayed platelet recovery.

Urano *et al.,* (2002) carried out a study using 106 MTX treated RA patients to examine the correlation between genotype and haplotype polymorphisms associated with the MTHFR gene, and the efficacy or toxicity of MTX. Single locus analysis examining each locus separately demonstrated that patients with the MTHFR 1298 CC were receiving lower doses of MTX, while a higher rate of overall MTX toxicity was demonstrated in patients with MTHFR 677 TT. Patients with the MTHFR 677C-1298C haplotype were also receiving lower doses of MTX than those without, while subjects with 677T-1298A had a higher frequency of side effects from MTX. They concluded that both single locus and haplotype analyses suggested that polymorphisms within the MTHFR gene were associated with both the efficacy and toxicity of MTX in RA patients. However, Kumagai *et al.,* (2003) reported no association with MTX related toxicity and efficacy in relation the MTHFR C677T and A1298C polymorphism but they suggest polymorphisms in TS may be a more useful indicator in determining the appropriate dose of MTX in patients with RA. It seems

likely that polymorphisms in folate metabolising enzymes will in part influence the efficacy and toxicity of MTX in the treatment of RA and other disease however, further study is required to establish which polymorphisms and which enzymes will influence the mechanism of action of MTX the most.

The aim of this chapter was firstly to investigate the effect low dose MTX treatment would have on the folate pools in human immortalised lymphocyte cell lines and in particular to examine the catabolic products and oxidised folates if present following treatment. The MTX concentration used in this study was 10μM, which is the equivalent of 4.54mg/l. This concentration fell within the low dose MTX treatment range used to treat patients with rheumatoid arthritis. Therefore, this study may give an insight into the intracellular mechanism of action of low dose MTX. The second aim of this chapter was to compare the folate co-factor distribution and pABG levels in both MTHFR C677T wild and mutant cell lines to determine if a difference could be observed after MTX treatment.

6.2 Results

6.2.1 The effect of MTX on the viability of immortalised lymphocyte cells

The first step was to determine the effects MTX had on the cell viability in human immortalised lymphocyte cell lines. Cells were grown for 1 passage in folate free medium RPMI 1640 and re-seeded at $2x10^5$cells/ml into 10ml of medium containing 1.19nmol 5-CHO-THF, which is equivalent to 0..5μCi[^{3}H]5-CHO-THF and grown for 72hr. Varying concentrations of MTX ranging from 0-20μm were added to each flask in triplicate. The cell viability was checked using a trypan blue stain at 24hr and 48hr intervals as per method 2.6.2, see figure 6.1. Each concentration and time-point was carried out on triplicate flasks. As can be seen there was very little difference in viability when the MTX treated cell viability was compared with the untreated control cell viability at each time-point. However, since the medium contained 1.19nmol 5-CHO-THF this was presumably acting as a rescue agent to the cells following the exposure of MTX.

A similar experiment was set up but following the initial 72hr incubation in folate free RPMI 1640 medium supplemented with 1.19nmol 5-CHO-THF, the cells were washed and transferred to folate free media containing varying concentrations of MTX ranging from 0-50μM. Each concentration and time-point was carried out on triplicate flasks. The cell viability was checked using a trypan blue stain at 24hr and 48hr intervals as per method 2.6.2, see figure 6.2. The results demonstrated that a working concentration of 10μM MTX covered the LD_{50} value for the drug, following 24hr MTX treatment the cell viability was 58% and following 48hr MTX treatment

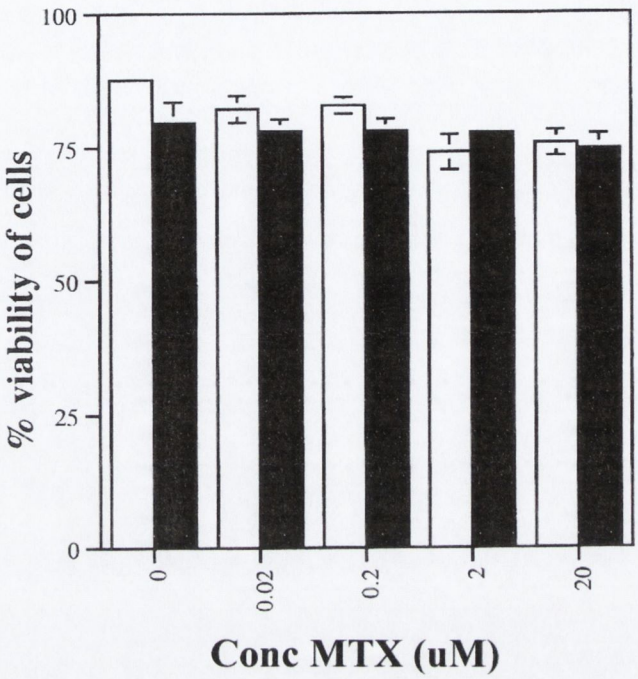


Figure 6.1: The effect of varying MTX concentrations on human immortalised lymphocyte cell viability following 24hr and 48hr treatment.

Cells were grown for 1 passage in folate free medium RPMI 1640 and re-seeded at $2x10^5$ cells/ml into 10ml of medium containing 1.19nmol 5-CHO-THF, which is equivalent to 0.5μCi[3H]5-CHO-THF and grown for 72hr. Varying concentrations of MTX ranging from 0-20μM were added to each flask in triplicate. The cell viability was checked using a trypan blue stain at 24hr and 48hr. Each time-point was carried out in triplicate (n=3) and the error bars represent the SEM of three experiments.

□ = 24hr MTX treatment
■ = 48hr MTX treatment

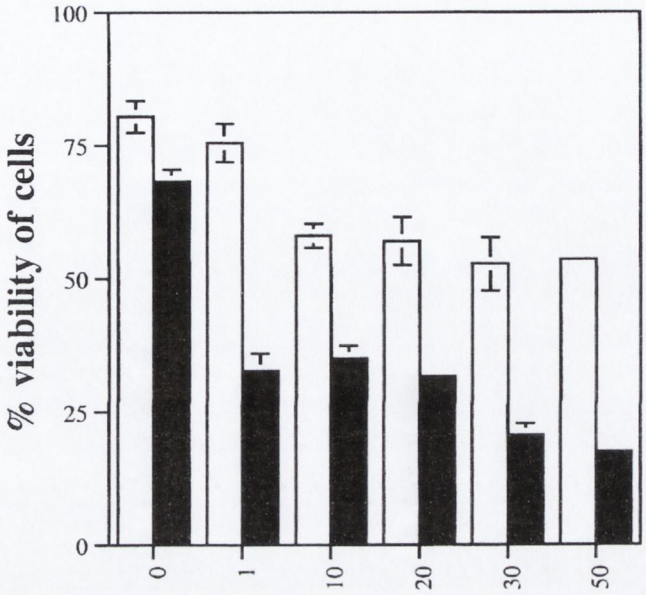


Figure 6.2: The effect of varying MTX concentrations on human immortalised lymphocyte cell viability in folate free medium following 24hr and 48hr treatment.

Following an initial 72hr incubation in folate free RPMI 1640 medium supplemented with 1.19nmol 5-CHO-THF, the cells were washed and transferred to folate free media containing varying concentrations of MTX ranging from 0-50μM. Each concentration and time-point was carried out on triplicate flasks. The cell viability was checked using a trypan blue stain at 24hr and 48hr. Error bars represent SEM of three experiments.

□ = 24hr MTX treatment
■ = 48hr MTX treatment

Treatment	MTHFR 677 CC Mean (SEM), [n=3]	MTHFR 677 TT Mean (SEM), [n=3]
Time 0hr MTX	100	100
Control 24hr	43.7(5.3)	68.8(3.6)
MTX 24hr	39.0(4.4)	51.7(1.6)
Control 48hr	38.1(7.0)	58.8(4.6)
MTX 48hr	33.1(3.7)	40.1(2.5)

Table 6.1: The effect of MTX on the percentage of intracellular folate co-factor. Human immortalised lymphocyte cell lines were grown for 72hr incubation in folate free medium RPMI 1640 supplemented with 1.19nmol[^{3}H]5CHO-THF and re-grown in folate free medium with 10μM MTX and without MTX (control) for 24hr and 48hr. The percentage of intracellular radioactivity remaining was calculated at each time-point. Each cell line was carried out in triplicate and the mean result of three cell lines was calculated for each MTHFR C677T genotype.

the cell viability was 35%. This concentration also fell within the range of treatment for patients suffering from rheumatoid arthritis (RA) therefore using this treatment regime may provide some insight into the effect low dose MTX treatment would have on folate metabolism in cells of RA sufferers.

6.2.2 To investigate the effect of culturing radiolabelled lymphocyte cells in folate free medium containing 10μm MTX on the intra-cellular [^{3}H] concentration.

Having established a suitable MTX treatment regime, the cells were grown for 1 passage in folate free medium RPMI 1640 and then seeded at a density of $2x10^5$cells/ml into 10ml of folate free medium RPMI 1640 containing 1.19nmol[^{3}H]5-CHO-THF and grown for 72hr. Following 72hr incubation, the cells were washed three times in 20ml sterile PBS and transferred to new flasks. This was designated as time 0. The cells were incubated in folate free medium supplemented 10μM MTX for 24hr and 48hr intervals. Control flasks were set up for each time-point using folate free medium RPMI 1640 minus MTX. The cell lines were grouped by MTHFR C677T genotype and the intracellular radioactivity present in the cells following 24hr and 48hr in folate free medium The percentage of radioactivity remaining in the cells treated with 10μm MTX were compared with the radioactivity remaining in cells that were not treated with the MTX, see table 6.1.

The effect of time on the total radioactivity present in control cells

The MTHFR TT cell lines had only 68.8% radioactivity remaining in the cell pellet after 24hr incubation in folate free medium and 58.8% radioactivity remaining in the cell pellet after 48hr incubation in folate free medium. This was considerably higher

than the radioactivity present in the MTHFR CC cell lines, which had 43.7% radioactivity present in the cell pellet after 24hr incubation in folate free medium and 38.1% remaining in the cell pellet after 48hr incubation. Therefore it appeared that the radiolabelled folates had a higher turnover rate in MTHFR CC cell lines. However, over-time the MTHFR TT cell lines appeared to retain a higher proportion of the radiolabelled folates. Therefore the results suggest that the MTHFR TT genotype was protecting the cells in some way from loosing radiolabelled folates.

The effect of treatment on the total radioactivity present in the cells

The cells treated with 10μ, MTX lost more radiolabelled folates when compared with untreated control cells, with this difference being more pronounced in the MTHFR TT genotype. There was approximately a 5% difference between the intracellular radioactivity present in MTX treated cells when compared with controls cells in MTHFR CC genotype group following MTX treatment for both 24hr and 48hr incubation time-points, whereas a difference of approximately 17% was found between MTX treated cells and controls for both 24hr and 48hr incubations in MTHFR TT cell lines.

In conclusion, the MTHFR CC control cells initially lost more radiolabelled folate than the MTHFR TT control cells for both 24hr and 48hr incubation time-points, but upon treatment with 10μm MTX for both 24hr and 48hr time-points the MTHFR CC cell lines did not lose as high a percentage of radiolabelled folates from their cells when compared with controls for the same time-point. Therefore treating the MTHFR TT cells with MTX accelerated the loss of radiolabelled folate when compared with MTHFR CC cell lines. The percentage difference between the

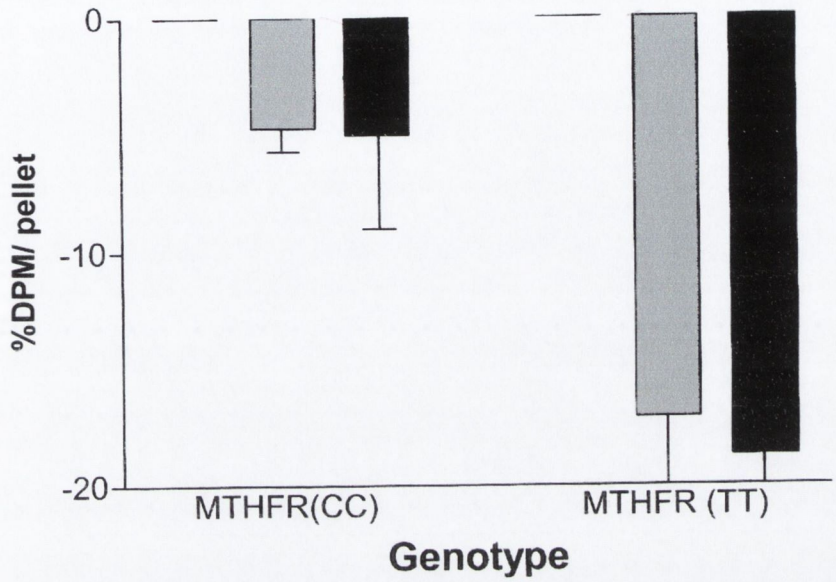


Figure 6.3: The effect of time and MTHFR C677T genotype on the total radioactivity present in control cells following MTX treatment. A two-way ANOVA analysis was carried out to determine the relationship between the difference in radiolabel remaining in the cell pellet following MTX treatment (control dpm counts-MTX treatment dpm counts) and MTHFR C677T genotype (n=3). As shown there was a significant difference in the loss of radioactivity following MTX treatment overtime ($p=0.011$) in both genotypes. There was also a significant difference between genotype and loss of radioactivity ($p=0.0119$). The MTHFR TT cell lines lost significantly more radioactivity following treatment when compared the MTHFR CC cell lines. There appeared to be an interaction between genotype and time but this interaction proved not to be significant ($p=0.152$).

□ = 0hr
▒ = 24hr MTX treatment
■ = 48hr MTX treatment

control and MTX cells were calculated for both genotypes and a two-way ANOVA was carried out on the results to investigate the interaction between the loss of radioactivity overtime following MTX treatment and the cells MTHFR genotype. As shown in figure 6.3, there was a significant difference in the loss of radioactivity following MTX treatment overtime ($p=0.011$) in both genotypes. There was also a significant difference between genotype and loss of radioactivity ($p=0.0119$). The MTHFR TT cell lines lost significantly more radioactivity following treatment when compared the MTHFR CC cell lines. There appeared to be an interaction between genotype and time but this interaction proved not to be significant ($p=0.152$), see figure 6.3.

6.2.3 To investigate the intracellular folate co-factor distribution of cells following treatment with MTX

Cells were grown for 1 passage in folate free media 1640, they were then seeded $2x10^5$cells/ml into 10ml of media containing 1.19nmol[^{3}H]5-CHO-THF and re-grown for 72hr. The intracellular folate co-factor distribution for each of the radiolabelled cell line was analysed as per method 2.7. The remaining flasks of cells were washed three times in 20ml sterile PBS and transferred to a new flask and re-grown in either folate free medium or folate free medium containing 10μM MTX for 24hr and 48hr intervals. The intra-cellular folate co-factor distribution was analysed for each cell line, as per method 2.7. Three flasks of cells were combined for analysis to ensure the final cell sample for injection contained approximately 100,000dpm counts. This ensured that each of the folate co-factor analysed gave clear distinguishable peaks. The results from each of the cell lines were grouped according to genotype and

Folate co-factors	MTHFR 677 CC Mean (SEM), [n=3]	MTHFR 677 TT Mean (SEM), [n=3]
Unknown	2.7(0.6)	3.7(0.7)
*p*ABG	12.7(0.7)	7.0(1.8)
5,10CH^{+}-THF/10-CHO-THF	11.0(1.0)	18.3(9.7)
THF	24.0(1.0)	11.7(5.4)
5-CHO-THF	1.3(0.7)	6.0(3.9)
DHF	1.3(1.4)	3.5(2.9)
5,10-CH_2-THF	28.7(1.4)	27.0(14.8)
FA	ND	ND
5-CH_3-THF	18.3(0.3)	24(3.1)

Table 6.2(a): Folate co-factor distribution of human immortalised lymphocyte cell lines following growth in folate free RPM1 1640 medium supplemented with 1.19nmol [^{3}H]5CHO-THF for 72hr and re-grown in folate free medium for 24hr. Immortalised human lymphocyte cell lines were sub-cultured and grown in folate free RPM1 1640 medium for 96hr. Subsequently, the cells were sub-cultured at a density of $2x10^5$cells/ml and grown in folate free RPM1 1640 medium supplemented with 1.19nmol [^{3}H]5CHO-THF for 72hr. Following this the cells were re-grown in folate free medium for 24hr. The intracellular folate co-factors were extracted and separated using the HPLC method described in section 2.7. The radioactivity corresponding to each folate co-factor was expressed as a percentage of the total radiolabelled folate. Each cell line was analysed once and the results grouped according to MTHFR C677T, the mean result of three cell lines was calculated for each MTHFR C677T genotype.

Folate co-factors	MTHFR 677 CC Mean (SEM), [n=3]	MTHFR 677 TT Mean (SEM), [n=3]
Unknown	2.7(0.7)	3.0(0)
*p*ABG	6.7(1.5)	9.3(1.7)
5,10CH^{+}-THF/10-CHO-THF	4.7(1.7)	5.3(2.8)
THF	9.0(3.6)	8.3(4.5)
5-CHO-THF	ND	3.0(3.1)
DHF	17.7(2.4)	21.7(6.3)
5,10-CH_2-THF	31.0(4.6)	19.0(8.3)
FA	16.3(5.8)	22.3(6.6)
5-CH_3-THF	12.0(0)	8.0(4.4)

Table 6.2(b): Folate co-factor distribution of human immortalised lymphocyte cell lines following growth in folate free RPM1 1640 medium supplemented with 1.19nmol [^{3}H]5CHO-THF for 72hr and re-grown in folate free medium for 24hr supplemented with 10μM MTX.

Immortalised human lymphocyte cell lines were sub-cultured and grown in folate free RPM1 1640 medium for 96hr. Subsequently, the cells were sub-cultured at a density of 2x10^5cells/ml and grown in folate free RPM1 1640 medium supplemented with 1.19nmol [^{3}H]5CHO-THF for 72hr. Following this the cells were re-grown in folate free medium containing 10μM MTX for 24hr. The intracellular folate co-factors were extracted and separated using the HPLC method described in section 2.7. The radioactivity corresponding to each folate co-factor was expressed as a percentage of the total radiolabelled folate. Each cell line was analysed once and the results grouped according to MTHFR C677T, the mean result of three cell lines was calculated for each MTHFR C677T genotype.

Folate co-factors	MTHFR 677 CC Mean (SEM), [n=3]	MTHFR 677 TT Mean (SEM), [n=3]
Unknown	3.3(0.9)	3.3(0.3)
*p*ABG	9.7(4.3)	9.0(1.6)
5,10CH^{+}-THF/10-CHO-THF	16.7(5.7)	27.3(5.3)
THF	17.0(3.1)	23.0(0)
5-CHO-THF	ND	1.3(1.4)
DHF	5.3(0.7)	10.0(3.3)
5,10-CH_2-THF	25.3(4.2)	10.7(0.7)
FA	ND	ND
5-CH_3-THF	22.7(5.1)	15.3(1.8)

Table 6.3(a): Folate co-factor distribution of human immortalised lymphocyte cell lines following growth in folate free RPM1 1640 medium supplemented with 1.19nmol [^{3}H]5CHO-THF for 72hr and re-grown in folate free medium for 48hr. Immortalised human lymphocyte cell lines were sub-cultured and grown in folate free RPM1 1640 medium for 96hr. Subsequently, the cells were sub-cultured at a density of $2x10^5$cells/ml and grown in folate free RPM1 1640 medium supplemented with 1.19nmol [^{3}H]5CHO-THF for 72hr. Following this the cells were re-grown in folate free medium for 48hr. The intracellular folate co-factors were extracted and separated using the HPLC method described in section 2.7. The radioactivity corresponding to each folate co-factor was expressed as a percentage of the total radiolabelled folate. Each cell line was analysed once and the results grouped according to MTHFR C677T, the mean result of three cell lines was calculated for each MTHFR C677T genotype.

Folate co-factors	MTHFR 677 CC Mean (SEM), [n=3]	MTHFR 677 TT Mean (SEM), [n=3]
Unknown	1.7(0.7)	4.3(1.8)
*p*ABG	11.3(1.9)	5.3(1.2)
5,10-CH^{+}-THF/10-CHO-THF	2.7(1.2)	6.7(3.5)
THF	9.7(0.7)	12.3(7.7)
5-CHO-THF	ND	ND
DHF	19.0(1.8)	21.3(2.1)
5,10-CH_2-THF	21.3(4.4)	10.3(3.3)
FA	21.0(7.1)	33.7(11.6)
5-CH_3-THF	13.3(5.5)	6.0(3.1)

Table 6.3(b): Folate co-factor distribution of human immortalised lymphocyte cell lines following growth in folate free RPM1 1640 medium supplemented with 1.19nmol [^{3}H]5CHO-THF for 72hr and re-grown in folate free medium for 48hr supplemented with 10μM MTX.

Immortalised human lymphocyte cell lines were sub-cultured and grown in folate free RPM1 1640 medium for 96hr. Subsequently, the cells were sub-cultured at a density of 2x10^5cells/ml and grown in folate free RPM1 1640 medium supplemented with 1.19nmol [^{3}H]5CHO-THF for 72hr. Following this the cells were re-grown in folate free medium containing 10μM MTX for 48hr. The intracellular folate co-factors were extracted and separated using the HPLC method described in section 2.7. The radioactivity corresponding to each folate co-factor was expressed as a percentage of the total radiolabelled folate. Each cell line was analysed once and the results grouped according to MTHFR C677T, the mean result of three cell lines was calculated for each MTHFR C677T genotype.

Folate Co-factors	Control Time 0 % radioactivity Mean (SEM), [n=3]	Control 24hr % radioactivity Mean (SEM), [n=3]	Control 48hr % radioactivity Mean (SEM), [n=3]
*p*ABG	14.2(3.9)	13.7(0.9)	9.7 (4.3)
DHF	0	2.7 (1.4)	5.3 (0.7)
THF co-enzymes	80.2 (3.1)	80 (1.8)	81.7(3.4)

Table 6.4(a): The effect of time on the intracellular radiolabelled folate co-factor distribution in MTHFR CC cell lines. A comparison of the percentage folate co-factor distribution in control MTHFR CC cell lines after 0, 24hr and 48hr incubation in folate free medium post labelling (n=3).

Folate Co-factors	Control Time 0 % radioactivity Mean (SEM), [n=3]	Control 24hr % radioactivity Mean (SEM), [n=3]	Control 48hr % radioactivity Mean (SEM), [n=3]
*p*ABG	10.5 (2.6)	7.0 (1.8)	9.0 (1.6)
DHF	0	2.3(2.4)	10.0 (3.3)
THF co-enzymes	89.1(3.1)	86.7 (3.5)	77.7 (1.8)

Table 6.4(b): The effect of time on the intracellular radiolabelled folate co-factor distribution in MTHFR TT cell lines. A comparison of the percentage folate co-factor distribution in control MTHFR TT cell lines after 0, 24hr and 48hr incubation in folate free medium post labelling (n=3).

Folate Co-factors	MTX Time 0 % radioactivity Mean (SEM), [n=3]	MTX 24hr % radioactivity Mean (SEM), [n=3]	MTX 48hr % radioactivity Mean (SEM), [n=3]
*p*ABG	14.2(3.9)	6.7 (1.5)	11.3 (1.9)
DHF	0	17.7 (2.4)	20.7 (0.9)
FA	0	16.3 (5.8)	21.0 (7.1)
THF co-enzymes	80.2 (3.1)	56.7 (4.2)	47.0 (8.2)

Table 6.4(c): The effect of time and MTX treatment on the intracellular radiolabelled folate co-factor distribution in MTHFR CC cell lines. A comparison of the percentage folate co-factor distribution in MTX treated MTHFR CC cell lines after 0, 24hr and 48hr incubation in folate free medium post labelling (n=3).

Folate Co-factors	MTX Time 0 % radioactivity Mean (SEM), [n=3]	MTX 24hr % radioactivity Mean (SEM), [n=3]	MTX 48hr % radioactivity Mean (SEM), [n=3]
*p*ABG	10.5 (2.6)	9.3 (1.7)	5.3 (1.2)
DHF	0	21.7 (6.3)	21.3 (2.1)
FA	0	22.3 (6.6)	33.7 (11.6)
THF co-enzymes	89.1(3.1)	43.7 (11.1)	36.3 (10.3)

Table 6.4(d): The effect of time and MTX treatment on the intracellular radiolabelled folate co-factor distribution in MTHFR TT cell lines. Comparison of the percentage folate co-factor distribution in MTX treated MTHFR TT cell lines after 0, 24hr and 48hr incubation in folate free medium post labelling (n=3).

treatment, see table 6.2(a) and (b) and 6.3 (a) and (b). The MTX treated cells were compared to control cells, which were grown in folate free medium for the same time-points. This comparison was essential because the folate co-factor distribution in the control cells differed over 48hr after the end of labelling, see table 6.2(a) and 6.3(a). In order to determine the overall effect of MTX treatment on the intracellular folate co-factor pools the THF co-enzyme results of 10-CHO-THF, THF, 5-CHO-THF, 5,10-CH_2-THF and 5-CH_3-THF co-factors were combined. Therefore, the total THF co-enzyme control results were also compared see table 6.4(a) and (b).

On examination of the control results shown in table 6.4(a) and 6.4(b) the following was observed; 1) the level of *p*ABG stayed steady throughout the incubation time-points in folate free media, 2) the DHF level increased slightly in the two genotypes over-time and 3) there was a slight decrease in the level of THF co-enzymes in MTHFR TT cells at the 48hr time point when compared with the MTHFR CC cell lines which remained steady throughout the incubation time-points.

The folate co-factor distribution in cells following MTX treatment at each time point were compared to control cells at the same time-point, see table 6.4(a-d). Three main differences between control cells and MTX treated cells were observed in both genotypes were as follows; 1) a folic acid peak was obtained in the MTX treated cells which was not present in the control cells and this peak appeared to increase in MTX treated cells over-time. After 24hr treatment with 10μM MTX the FA peak was 16.3%(5.8) for the MTHFR CC genotype and 22.3%(6.6) for the MTHFR TT genotype. Following 48hr MTX treatment the FA peak was 21.0%(7.1) for the MTHFR CC genotype and 33.7%(6.6) for the MTHFR TT genotype. The MTHFR

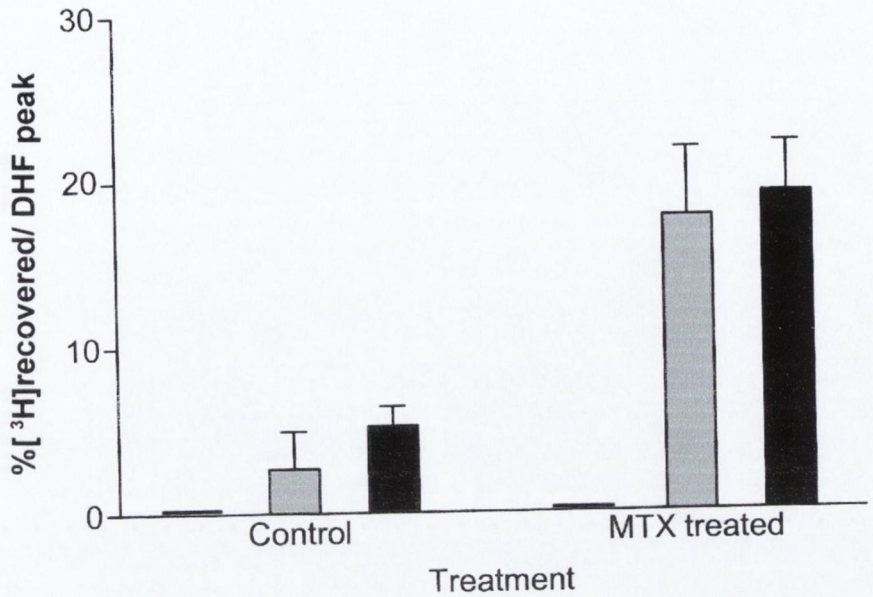


Figure 6.4(a): To determine the relationship between radiolabelled DHF and MTX treatment over-time in MTHFR 677 CC human immortalised lymphocyte cell lines.

Cells were grown for 1 passage in folate free media 1640, they were then seeded $2x10^5$ cells/ml into 10ml of media containing 1.19nmol[^{3}H]5-CHO-THF and re-grown for 72hr. The intracellular folate co-factor distribution for each of the radiolabelled cell line was analysed as per method 2.7. The remaining flasks of cells were washed three times in 20ml sterile PBS and transferred to a new flask and re-grown in either folate free medium or folate free medium containing 10μM MTX for 24hr and 48hr intervals. The intra-cellular folate co-factor distribution was analysed for each cell line, as per method 2.7. Three flasks of cells were combined for analysis to ensure the final cell sample for injection contained approximately 100,000dpm counts. A 2-way ANOVA was carried out in order to determine if there was a significant difference in the folate co-factor distribution after treatment over-time in MTHFR CC cell lines (n=3). A significant increase in radiolabelled DHF levels was reported over-time ($p<0.0001$) and there was also a significant increase after MTX treatment when compared with controls ($p<0.0001$). There was also a significant interaction between time and MTX treatment in relation to the percentage of DHF ($p=0.0002$).

□ = 0hr
▒ = 24hr MTX treatment
■ = 48hr MTX treatment

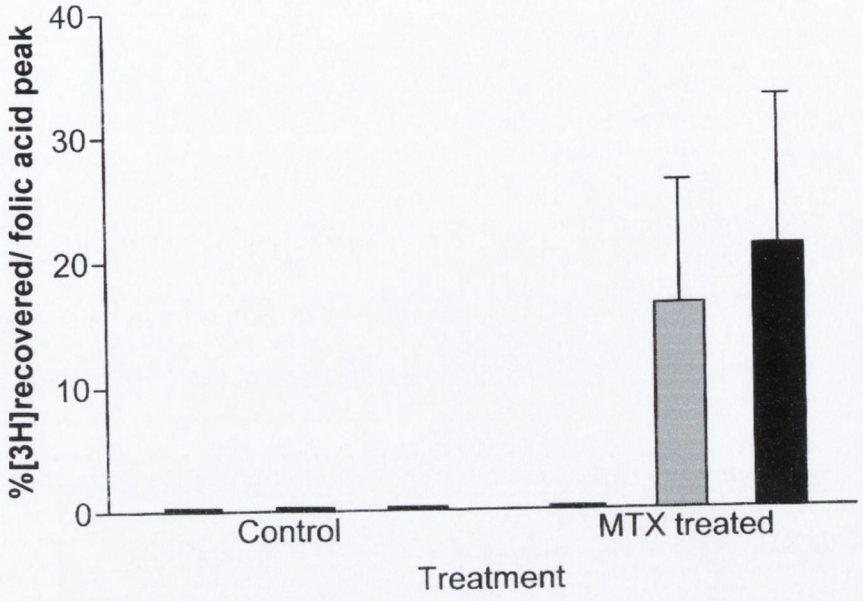


Figure 6.4(b): To determine the relationship between radiolabelled FA and MTX treatment over-time in MTHFR 677 CC human immortalised lymphocyte cell lines.

Cells were grown for 1 passage in folate free media 1640, they were then seeded 2x105cells/ml into 10ml of media containing 1.19nmol[^{3}H]5-CHO-THF and re-grown for 72hr. The intracellular folate co-factor distribution for each of the radiolabelled cell line was analysed as per method 2.7. The remaining flasks of cells were washed three times in 20ml sterile PBS and transferred to a new flask and re-grown in either folate free medium or folate free medium containing 10μM MTX for 24hr and 48hr intervals. The intra-cellular folate co-factor distribution was analysed for each cell line, as per method 2.7. Three flasks of cells were combined for analysis to ensure the final cell sample for injection contained approximately 100,000dpm counts.
A 2-way ANOVA was carried out in order to determine if there was a significant difference in the folate co-factor distribution after treatment over-time in MTHFR CC cell lines (n=3). A significant increase was reported in the percentage of radiolabelled folic acid over-time ($p=0.0352$) and a significant increase after MTX treatment when compared with controls ($p=0.0014$) was also reported.

□ = 0hr
▒ = 24hr MTX treatment
■ = 48hr MTX treatment

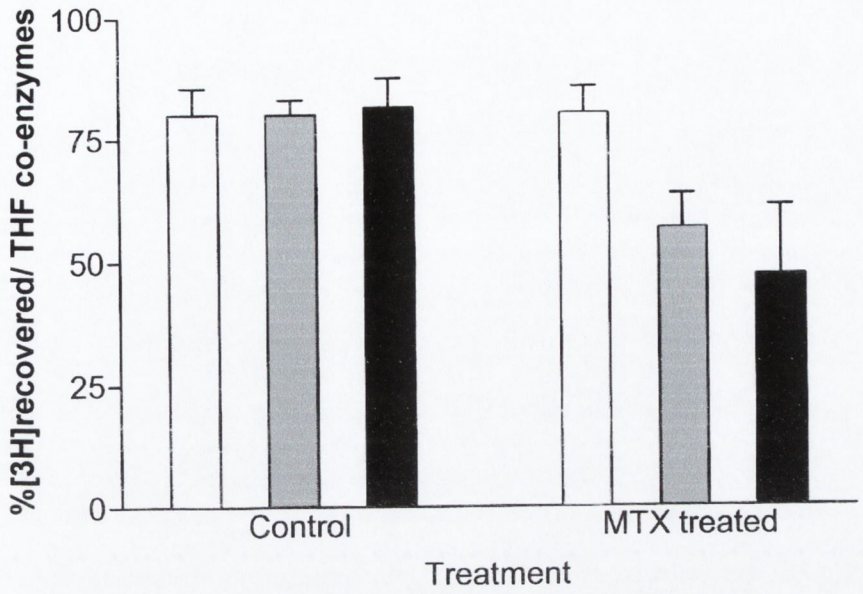


Figure 6.4(c): To determine the relationship between radiolabelled THF co-enzymes and MTX treatment over-time in MTHFR 677 CC human immortalised lymphocyte cell lines.

Cells were grown for 1 passage in folate free media 1640, they were then seeded $2x10^5$ cells/ml into 10ml of media containing 1.19nmol[^{3}H]5-CHO-THF and re-grown for 72hr. The intracellular folate co-factor distribution for each of the radiolabelled cell line was analysed as per method 2.7. The remaining flasks of cells were washed three times in 20ml sterile PBS and transferred to a new flask and re-grown in either folate free medium or folate free medium containing 10μMMTX for 24hr and 48hr intervals. The intra-cellular folate co-factor distribution was analysed for each cell line, as per method 2.7. Three flasks of cells were combined for analysis to ensure the final cell sample for injection contained approximately 100,000dpm counts.
A 2-way ANOVA was carried out in order to determine if there was a significant difference in the folate co-factor distribution after treatment over-time in MTHFR CC cell lines (n=3). A significant reduction in THF co-enzymes was observed over time ($p=0.0094$) and a significant reduction following MTX treatment ($p=0.0002$) when compared with controls. There was also a significant interaction between time and treatment in relation to the percentage reduction in THF co-enzymes ($p=0.0059$) was also observed.

□ = 0hr
▒ = 24hr MTX treatment
■ = 48hr MTX treatment

TT cell lines appeared to have a higher percentage of FA present following both 24hr and 48hr MTX treatment when compared with MTHFR CC cell lines. 2) the control levels for each time-point were subtracted from the MTX treated cell results and the results demonstrated that the DHF levels increased after 24hr MTX treatment and decreased by 48hr MTX treatment. Following 24hr treatment with 10μm MTX the DHF peak was 16.4% for the MTHFR CC genotype and 15.5% for the MTHFR TT genotype. Following 48hr treatment with 10μm MTX the DHF peak was 13.7% for the MTHFR CC genotype and 11.3% for the MTHFR TT genotype. 3) the THF co-enzymes levels were reduced following treatment with MTX for both the genotypes. When the controls results were subtracted from the MTX cell results, following 24hr treatment with 10μm MTX the THF co-enzyme reduction was 23.3% for the MTHFR CC genotype and 43.0% for the MTHFR TT genotype. Following 48hr treatment with 10μm MTX the THF co-enzyme reduction was 34.7% for the MTHFR CC genotype and 41.4%. for the MTHFR TT genotype.

Statistical analysis to determine the effect of MTX treatment over-time in MTHFR CC cell lines.

To 2-way ANOVA was carried out in order to determine if there was a significant difference in the folate co-factor distribution after treatment over-time in MTHFR CC cell lines, see figure 6.4(a-c) for results. In relation to the MTHFR CC the following was observed; 1) No significant difference was reported in the percentage of radiolabelled *p*ABG over-time ($p=0.3585$) or after MTX treatment ($p=0.4772$) and no significant interaction was observed between time and MTX treatment ($p=0.3347$) in relation to *p*ABG levels. 2) A significant increase in radiolabelled DHF levels was reported over-time ($p<0.0001$) and there was also a significant increase after MTX

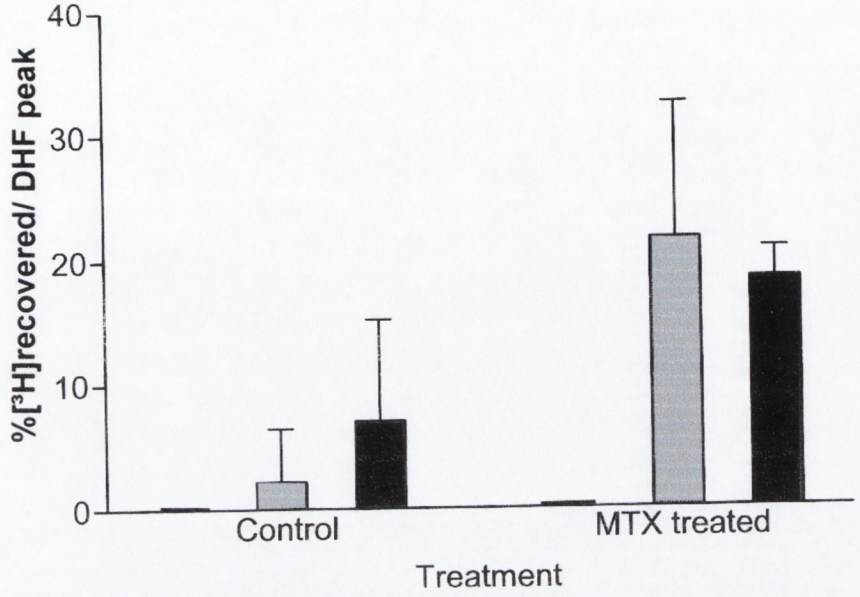


Figure 6.5(a): To determine the relationship between radiolabelled DHF and MTX treatment over-time in MTHFR 677 TT human immortalised lymphocyte cell lines.

Cells were grown for 1 passage in folate free media 1640, they were then seeded $2x10^5$ cells/ml into 10ml of media containing 1.19nmol[^{3}H]5-CHO-THF and re-grown for 72hr. The intracellular folate co-factor distribution for each of the radiolabelled cell line was analysed as per method 2.7. The remaining flasks of cells were washed three times in 20ml sterile PBS and transferred to a new flask and re-grown in either folate free medium or folate free medium containing 10μM MTX for 24hr and 48hr intervals. The intra-cellular folate co-factor distribution was analysed for each cell line, as per method 2.7. Three flasks of cells were combined for analysis to ensure the final cell sample for injection contained approximately 100,000dpm counts.
A 2-way ANOVA was carried out in order to determine if there was a significant difference in the folate co-factor distribution after treatment over-time in MTHFR TT cell lines (n=3). A significant increase in radiolabelled DHF levels was reported over time (p=0.0041) and a significant increase after MTX treatment when compared with controls (p=0.0029). There was also a significant interaction between time and MTX treatment in relation to the percentage of DHF (p=0.0423).

□ = 0hr
▒ = 24hr MTX treatment
■ = 48hr MTX treatment

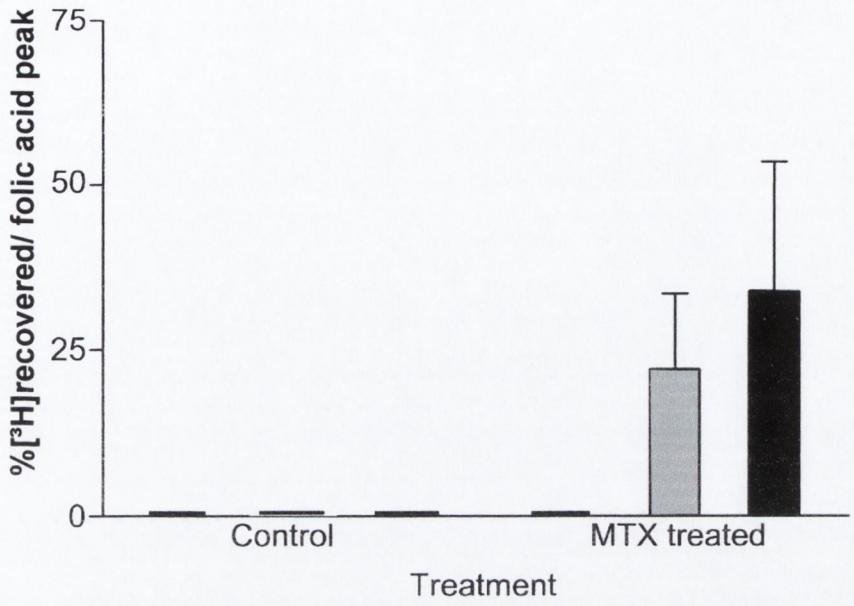


Figure 6.5(b): To determine the relationship between radiolabelled FA and MTX treatment over-time in MTHFR 677 TT human immortalised lymphocyte cell lines.

Cells were grown for 1 passage in folate free media 1640, they were then seeded $2x10^5$ cells/ml into 10ml of media containing 1.19nmol[^{3}H]5-CHO-THF and re-grown for 72hr. The intracellular folate co-factor distribution for each of the radiolabelled cell line was analysed as per method 2.7. The remaining flasks of cells were washed three times in 20ml sterile PBS and transferred to a new flask and re-grown in either folate free medium or folate free medium containing 10μM MTX for 24hr and 48hr intervals. The intra-cellular folate co-factor distribution was analysed for each cell line, as per method 2.7. Three flasks of cells were combined for analysis to ensure the final cell sample for injection contained approximately 100,000dpm counts. A 2-way ANOVA was carried out in order to determine if there was a significant difference in the folate co-factor distribution after treatment over-time in MTHFR TT cell lines (n=3). A significant increase was reported in the percentage of radiolabelled folic acid over time ($p=0.0245$) and a significant increase after MTX treatment when compared with controls ($p=0.0011$). A significant interaction was also observed between time and treatment ($p=0.0245$).

□ = 0hr
▒ = 24hr MTX treatment
■ = 48hr MTX treatment

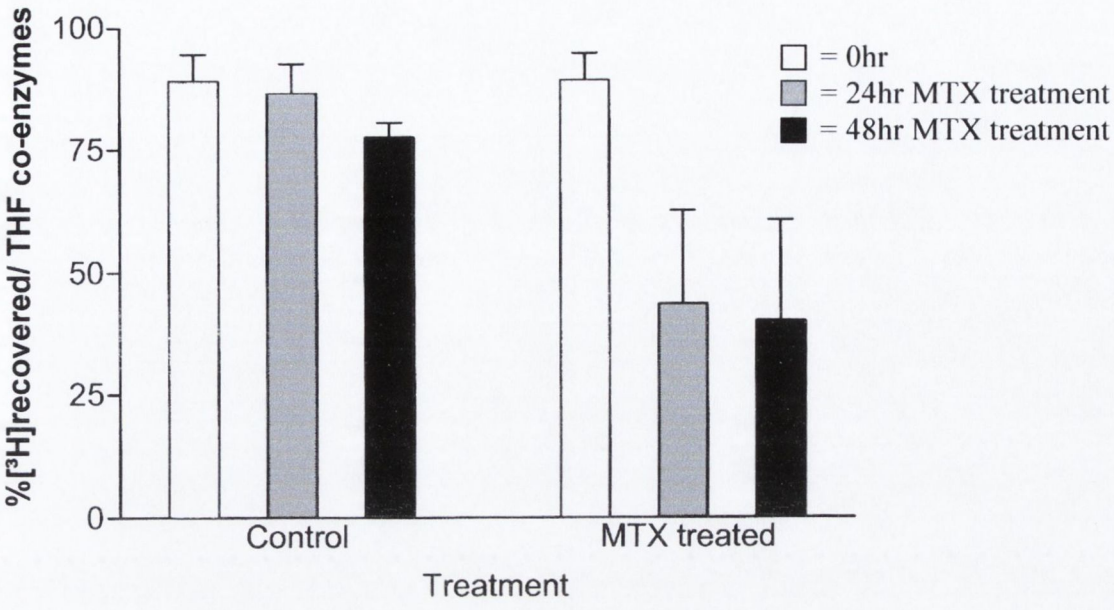


Figure 6.5(c): To determine the relationship between radiolabelled THF co-enzymes and MTX treatment over-time in MTHFR 677 TT human immortalised lymphocyte cell lines.

Cells were grown for 1 passage in folate free media 1640, they were then seeded $2x10^5$ cells/ml into 10ml of media containing 1.19nmol[^{3}H]5-CHO-THF and re-grown for 72hr. The intracellular folate co-factor distribution for each of the radiolabelled cell line was analysed as per method 2.7. The remaining flasks of cells were washed three times in 20ml sterile PBS and transferred to a new flask and re-grown in either folate free medium or folate free medium containing 10μM MTX for 24hr and 48hr intervals. The intra-cellular folate co-factor distribution was analysed for each cell line, as per method 2.7. Three flasks of cells were combined for analysis to ensure the final cell sample for injection contained approximately 100,000dpm counts.
A 2-way ANOVA was carried out in order to determine if there was a significant difference in the folate co-factor distribution after treatment over-time in MTHFR TT cell lines (n=3). A significant decrease in THF co-enzymes was observed over time ($p=0.0022$) and a significant reduction following MTX treatment ($p=0.0005$) when compared with controls. There was also a significant interaction between time and treatment in relation to the percentage reduction in THF co-enzymes ($p=0.0184$).

□ = 0hr
▒ = 24hr MTX treatment
■ = 48hr MTX treatment

treatment when compared with controls ($p<0.0001$). There was also a significant interaction between time and MTX treatment in relation to the percentage of DHF ($p=0.0002$), see figure 6.4(a). 3) A significant increase was reported in the percentage of radiolabelled folic acid over-time ($p=0.0352$) and a significant increase after MTX treatment when compared with controls ($p=0.0014$) was also reported. However, there was no significant interaction between time and treatment ($p=0.352$), see figure 6.4(b). 4) A significant reduction in THF co-enzymes was observed over time ($p=0.0094$) and a significant reduction following MTX treatment ($p=0.0002$) when compared with controls. There was also a significant interaction between time and treatment in relation to the percentage reduction in THF co-enzymes ($p=0.0059$) was also observed, see figure 6.4(c).

Statistical analysis to determine the effect of MTX treatment over-time in MTHFR TT cell lines.

The same two-way ANOVA as described above was carried out to determine if there was a significant difference in the folate co-factor distribution after treatment over-time in MTHFR TT cell lines, see figure 6.5(a-c) for results. In relation to the MTHFR TT the following was observed; 1) No significant difference was reported in the percentage of radiolabelled *p*ABG over time ($p=0.2460$) or after MTX treatment ($p=0.7821$) and no interaction between time and MTX treatment ($p=0.3253$) in relation to *p*ABG levels was observed. 2) A significant increase in radiolabelled DHF levels was reported over time ($p=0.0041$) and a significant increase after MTX treatment when compared with controls ($p=0.0029$). There was also a significant interaction between time and MTX treatment in relation to the percentage of DHF ($p=0.0423$), see figure 6.5(a). 3) A significant increase was reported in the percentage

of radiolabelled folic acid over time (p=0.0245) and a significant increase after MTX treatment when compared with controls (p=0.0011). A significant interaction was also observed between time and treatment (p=0.0245), see figure 6.5(b). 4) A significant decrease in THF co-enzymes was observed over time (p=0.0022) and a significant reduction following MTX treatment (p=0.0005) when compared with controls. There was also a significant interaction between time and treatment in relation to the percentage reduction in THF co-enzymes (p=0.0184), see figure 6.5(c).

Statistical analysis to determine the effect of MTX treatment over-time in relation to the MTHFR C677T polymorphism.

To two-way ANOVA was carried out in order to determine if any significant differences were observed between the radiolabelled folate co-factor levels after MTX treatment in relation to the MTHFR C677T genotype. Significant differences were reported over-time in the percentage of radiolabelled THF co-enzymes, DHF and FA however, none of the differences were significant in relation to genotype and there was no significant interaction between time and genotype following MTX treatment.

In chapter 5 the growth media of the lymphocyte cell lines was analysed to identify the radiolabelled folate peaks and the folate breakdown products present. It would seem logical that the same analysis be carried out on the medium collected following the radiolabelled cells incubation in folate free media for 24hr and 48hr with and without MTX supplementation to determine if the radioactivity lost from the cells was in the form of a folate degradation product or oxidised folates. Unfortunately, the loss of radioactivity from the radiolabelled cells was not sufficient to produce identifiable peaks.

6.3 Discussion

Several lines of evidence suggest that the cellular action of low-dose MTX, which underlies its anti-inflammatory/immune modifier effect, may not be related to the inhibition of DHFR. Firstly, overt inhibition of DHFR is not required for efficacy but rather is a sign of toxicity in low-dose MTX. Co-administration with folinic acid, which by-passes the blockage of DHF, ameliorates many of the side effects of MTX but if given in excess can retard the drugs efficacy (Shiroky *et al.,* 1993; Weinblatt *et al.,* 1993). However, co-administration of folic acid while also being effective in reducing the side effects can be given in a high dose without reducing the efficacy of the MTX (Morgan *et al.,* 1994; Duhra *et al.,* 1993). Lastly trimetrexate which is an un-polyglutamated analogue of folic acid and which inhibits DHFR strongly but is inactive against other folate enzymes is ineffective in a rat model of chronic inflammation. These results suggested that inhibition of folate dependent enzymes distal to DHFR in the metabolic pathway of folic acid by MTX and its metabolites may be the events responsible for this drugs anti-inflammatory activity.

MTX is used as a chemotherapeutic agent and against rheumatoid arthritis. Chemotherapeutic doses of MTX are high (up to 30μg/m^2), however in autoimmune diseases MTX is administered in low doses of between 7-15mg/week (Herman *et al.,* 2003).

Van Ede at al (2002) investigated the effects of MTX treatment in RA patients on folate and homocysteine metabolism, the role of folinic acid and folic acid supplementation and the influence of the MTHFR C677T mutation. They reported

that low-dose MTX treatment in RA patients leads to an increased plasma homocysteine level. Concomitant folate supplementation with either folinic acid or folic acid decreased this level and they found no relationship between the presence or absence of the MTHFR C677T mutation. Homocysteine metabolism did not appear to be associated with efficacy or toxicity of MTX treatment. In contrast, a study by Ulrich *et al.,* investigated whether the MTHFR C677T mutation modified the responses to MTX in patients undergoing bone marrow transplantation (Ulrich *et al.,* 2001). MTX was administered to patients undergoing marrow transplantation to prevent graft-versus-host disease. They hypothesised that MTX sensitivity would vary with genetic variability in folate-metabolising enzymes. They reported that patients who were MTHFR TT experienced higher toxicity, evidenced by increased oral mucositis and a trend toward delayed platelet recovery. The identification of a phenotype in immortalised lymphocytes for the MTHFR C677T mutation in this thesis, see chapter 5 for results and also by Lathrop-Stern *et al.,* (2003), reinforces the case for the hypothesis that increased MTX sensitivity is likely to occur due to genetic variability in folate-metabolising enzymes. This chapter was designed to investigate the effect the MTHFR C677T polymorphism would have on the folate co-factor distribution of human immortalised lymphocyte following treatment with MTX.

The first step was to determine the effect MTX had on cell viability with varying incubation times. Varying concentrations of MTX ranging from 0-20μM were added to the folate free medium supplemented with 1.19nmol 5-CHO-THF and the cell viability was determined at 24hr and 48hr, see figure 6.1. Control cells with no MTX added were set up for each concentration and time-point (n=3). In this study there

was very little difference reported in the viability of the cells treated with MTX when compared with the untreated control cells at each time-point and concentration. It had been reported that folinic acid can by-pass the blockage of DHF and ameliorate many of the side effects of MTX but if given in excess can retard the drugs efficacy (Shiroky *et al.,* 1993; Weinblatt *et al.,* 1993). Because the medium contained 1.19nmol 5-CHO-THF it would appear to be acting as a rescue agent for the cells following exposure to MTX.

As a result a similar experiment was set up, but following the cells 72hr incubation in folate free medium containing 1.19nmol 5-CHO-THF, the cells were washed and transferred to new flasks with folate free medium containing varying concentrations of MTX ranging from 0-50μM. The cell viability was checked at 24hr and 48hr intervals, see figure 6.2 and a working concentration of 10μM MTX was chosen as the drug concentration to be used in this study because this concentration covered the LD_{50} value.

Once the treatment regime had been established the next step was to determine the effect culturing the cells in folate free medium and folate free medium supplemented with 10μM MTX had on the concentration of intracellular radiolabelled folates. The cells appeared to lose radiolabel following incubation in folate free medium post labelling, see table 6.1. The cells treated with 10μM MTX lost more radioactivity when compared with control cells untreated for both 24hr and 48hr time points with the difference being more pronounced in the MTHFR TT genotype. Overall the MTHFR TT cells retained more radiolabelled folate when compared with MTHFR

CC cells therefore it appeared that the MTHFR TT genotype was protecting the cells from loosing their radiolabelled folates. Following a two-way ANOVA a significant decrease in the percentage radiolabelled folate remaining in the cell pellets over-time was shown and there was also a significant difference in each MTHFR C677T genotype over-time following MTX treatment. However, there was no significant interaction between time and genotype following MTX treatment, see figure 6.3. The loss in radioactivity is difficult to explain as it did not appear to relate to the percentage viability of cells following MTX treatment as the percentage cell death was lower than the percentage loss of counts from the pellet over-time. The THF co-enzymes present in the cells before and after MTX treatment would be polyglutamated and protein bound and therefore would be less susceptible to breakdown. One possible explanation may be that the DHF that accumulates in the cell over-time post-labelling may be degraded and its breakdown products excreted into the extracellular medium. However, further investigation would be required to prove this.

The folate co-factor distribution results from each cell lines were grouped according to their MTHFR C677T genotype and treatment regime, see table 6.2(a) and (b) and 6.3(a) and (b). The radioactivity corresponding to each folate co-factor was expressed as a percentage of the total radiolabelled folate in the cell pellet. It was essential to set up control cells for each time-point as the flux of radiolabelled folate co-factors is constantly changing, see table 6.2(a) and (b) and 6.3(a) and (b). When the control results obtained from each time point in folate free media were compared in relation to their MTHFR C677T genotype the following results were obtained; 1) the level of *p*ABG stayed steady throughout the 48hr incubation in folate free media. 2) the DHF

level increased slightly in both genotypes. 3) a slight decrease in the THF co-enzymes in MTHFR TT cells was reported at the 48hr time point when compared with the MTHFR CC cell lines which remained steady throughout incubation, see table 6.4 (a) and (b). The THF co-enzyme result was the combined peaks of 10-CHO-THF, THF, 5-CHO-THF, 5,10-CH_2-THF and 5-CH_3-THF.

The folate pools of the 10μm MTX treated cells at each time point were compared to their control at that time point, see table 6.4(a-d). Three main differences between the controls and MTX treated cells can be observed in both genotypes as discussed below. 1) A folic acid peak was obtained in the MTX treated cells, which was not present in the control cells and increased from 24hr to 48hr incubation in folate free media. 2) The DHF peak increased following MTX treatment and decreased over-time. 3) A reduction in the THF co-enzymes levels following treatment with MTX was reported in the two genotypes. Using a two-way ANOVA no significant difference was reported in any of the folate co-factors following MTX treatment over-time in relation to their MTHFR C677T genotype. However, a difference may be present but went undetected because of the small sample size (n=3). Therefore increased sensitivity to MTX due to genetic variation in folate-metabolising enzymes cannot be ruled and more research is necessary to answer this question.

The method used in this thesis required the cells to be labelled with [^{3}H]5-CHO-THF before analysis and as a result the cells cannot be depleted of folates prior to analysis. Using a method that does not require this labelling step may perhaps be a better approach to answer the question of genetic variability and MTX action. It would allowthe cells to be depleted of both folates and riboflavin and analysed to see if

differences could be observed between the MTHFR C677T genotypes and their folate and riboflavin status. Studies carried out by Lathrop-Stern *et al.,* (2003) have reported that in human immortalised lymphocyte cell lines the MTHFR activity in the TT genotype cells was significantly lower when exposed to either reduced riboflavin or combined low folate and low riboflavin status when compared with adequate nutritional conditions. While the MTHFR activity in the wild type cells was not lower than cells grown in control medium under any of the treatments. Under conditions of folate or riboflavin depletion if the MTHFR is lower in MTHFR TT cells than MTHFR TT cells grown in adequate nutritional conditions perhaps a difference in the folate co-factor distribution following MTX treatment could be identified. This might suggest that the folate, riboflavin and MTHFR C677T genotype would all effect the efficacy of MTX treatment. If MTX had an increased toxic effect on cells with the MTHFR C677T mutation in RA patients their folate and perhaps riboflavin status would also have an effect on the efficacy of the MTX treatment and therefore the MTX treatment regime would need to take all these factors into account.

The re-oxidation of the accumulated DHF to FA was reported to occur in *E.coli* after treatment with TMP (Quinlivan *et al.,* 2000). Oxidation of folates had been proposed to occur by at least two distinct irreversible mechanisms. The first, being the sequential oxidation to DHF and then to FA through a quininoid DHF intermediate (Archer and Reed, 1980). This mechanism was similar to that elucidated for tetrahydropterin oxidation (Blakley, 1969), which proceeded through a 4a-carbinolamine intermediate. Alternatively, reduced folates can undergo oxidative scission at the C9 and N10 bond, after which the folate is no longer a viable metabolic

co-factor. Due to the fact the intracellular *p*ABG level in this study did not increase following MTX treatment and the THF co-enzymes levels reduced it would appear that the DHF which accumulated was being re-oxidised to FA. This is a very significant finding and may perhaps in part explain how MTX works as a low dose treatment for autoimmune diseases. The MTX would appear to have a cytostatic effect on the cells similar to the bacteriostatic effect TMP had on *E.coli* (Quinlivan *et al.,* 2000). Upon removal of TMP from the *E.coli* growth medium the folic acid which had accumulated re-entered the folate cycle. A similar mechanism of action may perhaps apply to the FA, which has been reported to accumulate after low dose MTX treatment in this study. From the results reported in this study folate catabolism of the accumulated DHF cannot be ruled out due to the fact that the cells were shown to lose radioactivity post labelling and that this loss was higher following MTX treatment when compared with controls in both MTHFR CC and TT cell lines. This loss may perhaps be a breakdown product of folates that have been excreted from the cells. However, further study is required to prove this hypothesis.

If time had permitted this study would have been repeated using a higher concentration of endogenous radiolabelled [^{3}H]5-CHO-THF *i.e.* 1.0-2.0μCi [^{3}H]5-CHO-THF. Previous experiments carried out in the laboratory had shown that the uptake of radiolabelled folate was proportional to the concentration present in the media. Therefore, if the turnover of folates is higher it would be expected that the percentage of counts lost by the cells following incubation in folate free media with or without MTX treatment would increase. The result would be media samples with perhaps close to 100,000dpm counts present which would enable the identification of any folate degradation peaks by HPLC analysis by the method developed in chapter 5.

Resistance to MTX is becoming an increasingly important problem associated with the efficacy of MTX treatment especially in the treatment of cancer. The major biochemical mechanisms of resistance are decreased drug uptake, amplification of the DHFR gene and thus an increase in the target enzyme, mutations in the DHF gene and decreased ability to form MTX polyglutamates inside cells (Mauritz *et al.,* 2002). As a result there is on-going research into the development of novel antifolates that are designed to inhibit crucial DHFR-independent enzymes in metabolism such as TS. The results obtained from this study have shown that although there is a reduction in THF co-enzymes after MTX treatment there is still residual 10-CHO-THF and 5,10-CH_2-THF present. These two co-factors are key folates involved in purine and pyrimidine biosynthesis and therefore it would appear that the inhibition of purine and pyrimidine biosynthesis is not solely the result of folate depletion but a result of the direct inhibition of metabolically critical folate-requiring enzymes. This mechanism of action was shown also to occur MCF-7 breast cancer cells (Allegra *et al.,* 1986). Therefore, designing novel antifolate drugs to inhibit crucial DHFR-independent enzymes in metabolism such as TS may prove very important in the treatment of certain cancers. Altering the redox state of the target cells may also improve the efficacy of MTX because under acidic conditions the DHF that has been reported to accumulate after MTX treatment in this chapter may perhaps be more inclined to breakdown to *p*ABG and pteridines instead of being re-oxidised to FA. This would eliminate the possibility of the FA being re-cycled back into the folate cycle upon removal of the MTX and therefore increase the efficacy of the MTX treatment regime.

Chapter 7: Overall Discussion

7.1 Overall Discussion

The relationship between genes and disease are very broad, ranging from single gene disorders to multifactorial conditions (Khoury *et al.,* 2000). The identification of several inherited disorders in folate transport metabolism (Rosenblatt, 1989) suggests that folate status is likely to be under considerable genetic control. Genotype changes are common, but resultant phenotype changes can range from none at all to something quite subtle. The main problem associated with the identification of these genotype/phenotype relationships is that the anticipated phenotype alteration will be relatively small if present at all and our current ability to detect small changes in function is poor in most cases (Scott, 2001).

The MTHFR C677T polymorphism is a well-documented folate metabolising enzyme mutation, which expresses numerous phenotypic traits. It is believed that 5-20% of the population possesses this MTHFR thermolabile gene (Pepe *et al.,* 1998). Individuals heterozygous CT and homozygote TT for this MTHFR C677T polymorphism have an *in vitro* enzyme activity that is 65% and 30% of the normal activity, respectively (Kang and Wong, 1988). Homozygosity for the MTHFR C677T polymorphism is associated with mild hyperhomocysteinemia in humans with plasma folate concentrations below the median (Jacques *et al.,* 1996) and is the most common genetic cause of mild elevations in plasma homocysteine in humans (Schwahn *et al.,* 2001). Mildly elevated plasma homocysteine has been associated with an increased risk of coronary artery disease (Boushey *et al.,* 1995; Graham *et al.,* 1997). Hyperhomocysteinemia has also been associated with complications in pregnancy including miscarriage, pre-eclampsia and

neural tube defects in the human fetus (Molloy *et al.,* 2002). Decreased levels of red cell folates have been reported in pregnant and non-pregnant women homozygous for this thermolabile variant when compared to heterozygous or wild type groups (Molloy *et al.,* 1997). Other studies have reported that riboflavin status is associated with elevated plasma homocysteine levels, but only in persons who had both the MTHFR TT genotype and low folate status. With higher folate status, the homocysteine levels were low irrespective of the MTHFR genotype and riboflavin status (Jacques *et al.,* 2002; Lathrop-Stern *et al.,* 2003).

The reduced MTHFR enzyme activity due to the thermolabile MTHFR C677T polymorphism results in significantly higher levels of intracellular formylated folates when compared with methylated folates in human red blood cells and human immortalised lymphocyte cell lines (Bagley and Selhub, 1998; Stern *et al.,* 2003). Similar phenotypic traits were found using human immortalised lymphocyte cell lines in this thesis, see chapter 5. The results demonstrated that a significantly higher percentage of formyl folates were present in the MTHFR TT cell lines when compared with MTHFR CC cell lines and that the percentage of methylated folates present in MTHFR TT cell lines was lower when compared with MTHFR CC cell lines.

Folate catabolism *in vivo* was assumed to be a result of random, non-regulated, non-enzymatic degradation of labile folate co-factors, in particular DHF and 10-CHO-THF (McNulty *et al.,* 1995: Martinasevic *et al.,* 1999) which results in the production of a pterin, and *p*ABG. The susceptibility to oxidative cleavage is even more pronounced

with DHF, which is very readily cleaved. Cellular accumulation of DHF in prokaryotes results in increased folate catabolism. Bacteria exposed to DHFR inhibitor TMP displayed rapid accumulation of both DHF and *p*ABG, with subsequent folate deficiency (Quinlivan *et al.,* 2000). In mammals, increased rates of folate catabolism have been associated with increased rates of cell division, presumably resulting from robust thymidylate synthesis and therefore DHF formation (McNulty *et al.,* 1995). The HPLC method developed in chapter 3 of this thesis not only identified all the eight intracellular folate co-factors but also identified intracellular *p*ABG levels. It was hypothesised that the significantly higher proportions of formylated folates present in MTHFR TT cells would result in increased intracellular *p*ABG because formylated folates are more susceptible to breakdown than methylated folates. If increased levels of *p*ABG could be observed in MTHFR TT cells this may in part explain why individuals homozygous TT have decreased levels of red cell folates when compared to heterozygous or wild type groups (Molloy *et al.,* 1997). However, the results from chapter 5 reported no increase in intracellular *p*ABG levels in MTHFR TT cells. The culture medium was also analysed for *p*ABG, which may have been excreted from the cells but no difference was reported in the culture medium of the MTHFR TT cell lines analysed when compared with MTHFR CC cells.

Finding by Stern *et al.,* (2003) have shown that despite the lower enzyme activity of MTHFR TT, the mutant enzyme provided sufficient 5-CH_3-THF to meet the demands for methionine synthesis and homocysteine remethylation (Stern *et al.,* 2003). No difference

was reported between genotypes in the ability to synthesise methionine under adequate, low folate or low riboflavin conditions (Stern *et al.,* 2003). The increased levels of formylated folates present in MTHFR TT human immortalised lymphocyte cell lines when compared with MTHFR CC cell lines reported in chapter 5 are consistent with the findings of Lathrop-Stern *et al.,* (2003). No difference was reported in the folate degradation products in relation to the MTHFR C677T genotype. However, the question still remains that if the cells were folate or riboflavin deficient would a difference in pABG levels be detected, as some studies have only reported phenotypic changes in MTHFR TT individuals deficient in riboflavin or in both riboflavin and folate deficient individuals (Jacques *et al.,* 2002; McNulty *et al.,* 2002). As discussed in chapter 5, the HPLC method used to determine the intracellular folate co-factor distribution in human immortalised lymphocyte cell lines required the cells to be grown in culture medium supplemented with [^{3}H] 5-CHO-THF in order to radiolabel all the intracellular folate co-factors prior to HPLC analysis. As a result of this step it would prove very difficult to deplete the cells of folates. Methods which do not require this pre-labelling step have been developed (Bagley *et al.,* 1998; Nelson *et al.,* 2002) and perhaps these method would prove more suitable to investigate the link between the MTHFR C667T genotype and increased rates of folate turnover. However none of these methods have reported pABG levels present and if the methods could detect this folate breakdown product the rate of folate catabolism in cells following exposure to folate and riboflavin inadequate growth conditions could also be assessed. These methods could then be used to test our hypothesis that there is an increased rate of folate catabolism in MTHFR 677 TT cells as a result of the increased level of intracellular formylated folates being present.

Some studies have reported that the MTHFR C677T polymorphism significantly reduces the risk of colorectal cancer (Chen *et al.,* 1996; Slattery *et al.,* 1999) and acute lymphatic leukemia (Blount *et al.,* 1997). These studies have hypothesized that MTHFR TT homozygotes are protected against some cancers because the reduced MTHFR enzyme activity causes a diversion of 5,10-CH_2-THF toward thymidine synthesis which minimizes the intracellular dUMP:dTMP ratios and in turn reduces the chance of uracil being incorporated into DNA (Blount *et al.,* 1997). Excessive uracil mis-incorporation leads to point mutations and also results in the generation of single and double stranded DNA breaks, chromosome breakage and micronucleas formation (Blount *et al.,* ,1997; Duthie and Hawdon, 1998). However, studies by Crott *et al.,* (2001) have reported that the MTHFR C677T polymorphism did not affect the ability of the cell to resist chromosome damage induced by folic acid deficiency *in vivo* (Crott *et al.,* 2001). The increase in formylated folates in the MTHFR 677 TT human immortalized lymphocyte cell lines when compared with the MTHFR 677 CC as reported in chapter 5 provides evidence that there are more formylated folates available for purine and pyrimidine biosynthesis in MTHFR 677 TT cells. An interesting result reported in chapter 6 was that the MTHFR TT cells lines retained higher levels of intra-cellular radiolabelled folates when compared with MTHFR 677 CC cell lines post radiolabelling over-time. It would appear that the MTHFR TT genotype may in some way be protecting the cells from loosing their radiolabelled folates. The greater availability of formylated folates in TT cells which serve as substrates for the synthesis of the purines and pyrimidines which are necessary for DNA synthesis and repair and the reduced loss of radiolabelled folates from

the cells may perhaps explain why the MTHFR C677T polymorphism is protective against certain cancers.

The critical function of DHFR in restoring THF concentrations following thymidylate biosynthesis established the basis for antifolate drug therapy. TMP is an antifolates drug that inhibits bacterial DHFR and has no notable effect on mammalian DHFR. It is used on its own or in combination with SMX as a treatment for urinary tract infections and acute respiratory infections. Treating bacterial cells with TMP causes an accumulation of DHF in bacterial cells as a result of the DHFR inhibition. Quinlivan *et al.,* (2000) reported that upon treatment with TMP, DHF levels accumulated acutely but diminished over-time with a subsequent rise in the levels of pABG and FA. They demonstrated that the accumulated DHF was either being broken down to pABG and pteridines or being re-oxidised to FA with the FA becoming incorporated back into the folate cycle upon removal of the TMP. This novel mechanism of action of TMP, which was first reported by Quinlivan *et al.,* (2000) and has been verified in chapter 3 of this thesis. This mechanism of action may explain how TMP works to exert a bactericidal effect on the bacterial cells as opposed to the bacteriostatic effect, which was previously predicted. Quinlivan *et al.,* (2000) suggested that altering the redox state of the cells *i.e.* making the intracellular matrix more acidic may favour DHF catabolism instead of re-oxidation to FA. The resultant increase in DHF catabolism would improve the drugs efficacy and allow it to have a bacterostatic effect.

It is essential to determine if a similar mechanism of action occurs in mammalian cells following the treatment with DHFR inhibitors as this may determine cytostatic or cytotoxic effect of the drug on the cell. As discussed in chapter 1, MTX is an inhibitor of the mammalian form of DHFR but its mechanism of action of MTX is not fully understood. It was developed in the 1940's and along with its close analogue aminopterin were the first drugs to induce remission in acute leukaemia (Chu and Allegra, 1996) and has since been used to treat two chronic inflammatory disorders rheumatoid arthritis and psoriasis. Due to its efficacy in treating inflammatory disorders in the 1980's it was used to treat inflammatory bowel disease. The net effects of MTX include the inhibition of folate dependent reactions involving the transfer of one-carbon fragments such as the production of thymidylate and purines, folate depletion and the inhibition of production of methionine which results in an increased level of hcy and local accumulation of adenosine. Adenosine is a potent substance, which is lymphotoxic, immunosuppressive and anti-inflammatory (Barankiewicz *et al.,* 1990) and may be one of the actions which could explain the drugs anti-inflammatory response at low doses which is unclear.

The mechanism of action of low-dose MTX (10μM) had on immortalized human lymphocyte cell lines was investigated in chapter 6 in relation to the MTHFR C677T polymorphism. Upon exposure to MTX a notable increase in DHF levels was observed. Due to the fact MTX is a DHFR inhibitor this was an expected result. However, the appearance of a folic acid peak in the MTX treated cells that was not present in the control was a novel result. This study is the first to identify FA in cells treated with MTX. The identification of a FA peak following MTX treatment in mammalian cells is a

very significant finding because in theory it could be recycled into the folate cycle upon the removal of MTX. This has been reported to occur in *E.coli* cells following the removal of TMP treatment, which is also a DHFR inhibitor (Quinlivan *et al .,* 2000). If this can be demonstrated it may explain why MTX is exerting a cytostatic effect as opposed to a cytotoxic effect, which would be the preferred mechanism of action in high dose treatment against cancers.

Van Ede *et al.,* (2002) reported low-dose MTX treatment in RA patients lead to an increased plasma homocysteine level. Concomitant folate supplementation with either folinic acid or folic acid decreases the level of plasma homocysteine. They found no relationship between the presence and absence of the MTHFR C677T mutation. Homocysteine metabolism was not associated with efficacy or toxicity of MTX treatment (Van Ede *et al.,* 2002). A study carried out by Ulrich *et al.,* (2001) also investigated the MTHFR C677T mutation would modify the response to MTX in patients undergoing bone marrow transplantation (Ulrich *et al.,* 2001). MTX was administered to patients undergoing marrow transplantation to prevent graft-versus-host disease. They reported that patients who were MTHFR TT experienced higher toxicity, evidenced by increased oral mucositis and a trend toward delayed platelet recovery. Low folate (dietary or MTX induced) may further decrease availability of folate for nucleotide synthesis (Ulrich *et al.,* 1999).

Results from the study carried out in chapter 6 of this thesis have reported no significant difference in MTX toxicity and efficacy in relation to the MTHFR C677T polymorphism.

There was also no increase in intracellular pABG levels, this would have suggested an increased rate of folate catabolism as a result of the increased levels of DHF present in the cells after MTX treatment. However, increased sensitivity to MTX due to genetic variations in folate-metabolizing enzymes cannot be ruled and more research is necessary to investigate this hypothesis further. The identification of a phenotype for the MTHFR C677T polymorphism both in chapter 5 of this thesis and by Lathrop-Stern *et al.,* (2003) highlight the importance of such investigations. As discussed above the method used in this thesis requires the cells to be labeled with [^{3}H] 5-CHO-THF before analysis and so the cells are not folate depleted and may perhaps have an intracellular store of folate that they can utilize. Using a method that does not require this labeling step and could identify the intracellular folate co-factors and *p*ABG may perhaps be a better approach as cells could be depleted of both folates and riboflavin and analyzed to determine if differences in the rate of folate catabolism and intracellular folate co-factor distribution occurs between the two genotypes following MTX treatment. If intracellular differences could be identified between MTHFR C677T genotypes following MTX treatment a patients MTX treatment regime could be designed to take these differences into account. Future work investigating the link between genotypes and drug toxicity is vital to delivering a treatment which best suits each individual patient. Having a comprehensive understanding of the underlying genetic, nutrition and biochemical etiologies of disease would lead to the rational design of effective interventions and therapies. Ultimately this would lead to the development of genomically based, tailor-made diets for disease prevention and management. RA is one such example of a disease where understanding the etiology of the disease could lead to therapies being tailor made to suit each

individual sufferer. In order to achieve this, the most relevant statistical, biological and genomic approaches need to be integrated to address the interaction of risk factors (Stover, 2004).

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