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Biochemical investigations of hyperhomocysteinemia

By

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Presented to the University of Dublin (Trinity College)
in fulfilment of the thesis requirement for the degree of Ph.D

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DECLARATION

I hereby certify that this thesis, submitted to the University of Dublin for examination for the degree of Doctor of Philosophy, has not been submitted as an exercise for any other degree at any other university. I also certify that this thesis is entirely my own work.

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Vicki Betts.

Mary Victoria (Vicki) Betts

June, 2000.

*For my Mam and Dad
and
all the gang at number 23.*

Like Snail

Intrepid snail, spiralled like a volute,
At my doorstep, imperceptibly paced,
Blended with earth, like solvent and solute,
How have you been so magically placed?
Just as my step has slowed, my brain grown cold,
My pen's fluency stalled, do you appear,
With steady, snail-like progress, brave and bold,
Gliding dauntless through the autumn of the year,
Spurning the flurry of the harried world,
Resolute in your small unerring course,
Your pace defies armies with flags unfurled
Or potentate with chariot and horse.
Like snail, I'd persevere and soldier on
Till brain and heart and fluency are gone.

George N. Braman

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Table of Contents.

Summary	i
Abbreviations	iv
Chapter 1. Introduction.	1
1.1. General introduction.	1
1.2. Methionine Metabolism	3
1.2.1. Transamination of methionine.	3
1.2.2. Biosynthesis and role of S-adenosyl methionine (SAM).	4
1.2.3. Control of methionine metabolic pathways.	6
1.2.4. Remethylation of homocysteine.	6
1.2.5. Transsulphuration of homocysteine.	7
1.3. Cofactors and coenzymes in methionine metabolism.	8
1.3.1. Folic acid.	8
1.3.2. Vitamin B ₁₂ (cobalamin).	10
1.3.3. Vitamin B ₆ .	12
1.4. Defects in Methionine Metabolism.	13
1.4.1. Classical Homocystinuria.	12
1.4.1.1. Homocystinuria due to CBS defects.	14
1.4.1.2. Homocystinuria due to MTHFR defects.	16
1.4.1.3 Homocystinuria due to disorders in folate or cobalamin metabolism.	17
1.4.2. Hyperhomocysteinemia	18
1.4.2.1. Genetic lesions in hyperhomocysteinemia.	19
1.4.2.2. Nutritional deficiencies in hyperhomocysteinemia.	24
1.4.2.3. Other causes of hyperhomocysteinemia.	27
1.5. Epidemiological studies relating homocysteine to vascular disease.	27
1.6. Homocysteine theory of atherosclerosis.	31
1.6.1. Homocysteine and endothelial cell function.	33
1.6.2. Homocysteine and smooth muscle cells.	34
1.6.3 Homocysteine and oxidation.	35
1.6.4 Homocysteine and thrombosis.	36
1.7. Tumour Necrosis Factor.	37
1.7.1. TNF α production.	38

1.7.2. TNF receptors.	40
1.7.3. Role of TNF in atherosclerosis.	41
1.8. Aims	44

Chapter 2. Materials and Methods. 46

2.1. Material suppliers.	46
2.2. Methods.	51
2.2.1. Peripheral Blood Mononuclear Cell (PBMC) Isolation.	51
2.2.2. Counting and assessing viability of cells.	52
2.2.3. Culture of U-937 and THP-1 human monocytic cell-lines.	52
2.2.4. Homocysteine Estimation.	52
2.2.5. Vitamin B ₁₂ Estimation.	53
2.2.6. Folic Acid Estimation.	53
2.2.7. Total RNA isolation from cultured cells.	54
2.2.8. Assessment of RNA quantity, purity and integrity.	54
2.2.8.1. Absorbance.	54
2.2.8.2. Agarose gel electrophoresis of RNA.	55
2.2.9. Reverse Transcription (RT) of total RNA .	55
2.2.10. Northern blotting.	56
2.2.11. Lactate Dehydrogenase (LDH) Estimation.	59

Chapter 3. A method to study cellular methionine metabolism. 61

3.1. Introduction	61
3.2. Strategy.	62
3.3. Materials and methods.	62
3.3.1. Human peripheral blood mononuclear cell culture.	62
3.3.2. Cell harvesting and extract preparation.	63
3.3.3. Separation of radiolabelled methionine and its principal derivatives.	63
3.3.4. Quantitation of [³⁵ S] label in methionine derivatives.	64
3.3.5. Methionine profiles.	65
3.4. Validation of methodology.	65
3.4.1. Purity of labelled methionine.	65

3.4.2. Two-dimensional thin layer chromatography.	66
3.5. Results.	67
3.5.1. Uptake of [³⁵ S]-methionine by cultured PBMC in response to phytohaemagglutinin.	67
3.5.2. Labelling of cellular methionine derivatives.	67
3.5.2. Separation of sulphur-containing amino acids by 2-D TLC.	68
3.6. Discussion.	70

Chapter 4. Cellular methionine metabolism in hyperhomocysteinemic subjects and its relationship

to the MTHFR TT and other genotypes.	75
4.1. Introduction.	75
4.2. Subject recruitment.	77
4.3. Methods.	77
4.3.1. Blood Sampling	77
4.3.2. Cellular methionine metabolism.	77
4.3.3. Measurement of biochemical parameters.	78
4.3.4. Identification of MTHFR TT genotype.	78
4.3.5. Identification of CBS I278T genotype.	79
4.3.6. Identification of MS D919G genotype.	79
4.3.7. Verification of genotyping	80
4.3.7.1. Purification of gene fragments amplified by PCR.	80
4.3.7.2. Ethanol precipitation of purified PCR products.	80
4.3.7.3. Cloning of purified gene fragments.	81
4.3.7.4. Purification of plasmid DNA.	81
4.3.7.5. Sequencing of cloned gene fragments.	82
4.3.7.6. BLAST homology search.	82
4.4. Results.	84
4.4.1. Study subject details.	84
4.4.2. Methionine cycle enzyme genotypes.	85
4.4.3. Methionine metabolic profiles of the control subjects.	86

4.4.4. Methionine metabolic profiles of the vascular disease subjects.	89
4.5. Discussion.	93
Chapter 5. TNF alpha expression by monocyte/macrophages in response to homocysteine.	106
5.1. Introduction.	106
5.2. Strategy.	107
5.3. Materials and methods.	107
5.3.1 Investigation of the induction of TNF α by PMA in the U-937 and THP- cell lines.	107
5.3.1.1 Estimation of TNF α concentrations in cell culture media by ELISA.	108
5.3.1.2. Investigation of the effect of homocysteine on the ELISA.	108
5.3.2 Investigation of TNF α production by U-937 and THP-1 monocytes, in response to homocysteine.	109
5.3.3 Investigation of the effects of <i>pre-exposure to homocysteine</i> on PMA-induced TNF α production by the U-937 cell-line.	109
5.3.4 Investigation of TNF α production by the U-937 cell-line, in response to <i>simultaneous exposure to PMA and homocysteine</i> .	110
5.3.5 Investigation of TNF α production by <i>pre-PMA-stimulated</i> U-937 and THP 1 macrophages, in response to homocysteine.	110
5.3.6 Investigation of TNF α gene expression by PMA-stimulated U-937 macrophages, in response to homocysteine.	110
5.3.7. Investigation of homocysteine cytotoxicity.	111
5.3.8. Investigation of TNF α production by PMA-stimulated U-937 macrophages, in response to <i>cysteine</i> .	112
5.4. Results.	113

5.4.1. Assessment of the ELISA.	113
5.4.2. PMA-induced differentiation and TNF α production of monocytic cells.	113
5.4.3. Investigation of TNF α production by U-937 and THP-1 monocytes, in response to homocysteine.	115
5.4.4. Investigation of the effects of <i>pre-exposure to homocysteine</i> on PMA-induced TNF α production by U-937 cells.	116
5.4.5. Investigation of TNF α production by the U-937 cell-line in response <i>simultaneous exposure</i> to PMA and homocysteine.	116
5.4.6. Investigation of TNF α production by U-937 and THP-1 macrophages (<i>pre-PMA-stimulated</i>), in response to homocysteine.	117
5.4.7. Effect of homocysteine on TNF α gene expression by U-937 macrophages (<i>pre-PMA-stimulated</i> U-937 cells).	118
5.4.8. Homocysteine cytotoxicity.	120
5.4.9 Effect of cysteine on TNF α production by U-937 macrophages (<i>ie</i> PMA-stimulated U-937 cells).	121
5.5. Discussion.	122

Chapter 6. The Effect of Homocysteine on Platelet Function.

6.1. Introduction.	131
6.2. Materials and methods.	133
6.2.1. Platelet aggregation in response to homocysteine.	133
6.2.1.1 Platelet preparation.	133
6.2.1.2. Platelet aggregation assay.	133
6.2.2. Platelet thromboxane synthesis in response to homocysteine.	134
6.2.2.1. Thromboxane estimation.	134
6.2.3. Fibrinogen binding in response to homocysteine.	135
6.2.3.1. Fibrinogen binding assay.	135

6.3. Results	137
6.3.1. Platelet aggregation in response to homocysteine.	137
6.3.2. Effect of homocysteine on platelet thromboxane synthesis.	139
6.3.3. Effect of homocysteine on platelet fibrinogen binding.	139
6.4. Discussion.	141
Chapter 7. Discussion.	148
7.1. General discussion.	148
Bibliography	155

Summary.

Hyperhomocysteinemia, a condition where plasma homocysteine occurs at an abnormally elevated level, is a common finding in vascular disease subjects. Several genetic and nutritional factors have been implicated.

The first part of this thesis describes attempts to investigate the cellular basis for the accumulation of homocysteine seen in mild hyperhomocysteinemia. A method involving radiolabelling of methionine and its derivatives was designed to determine the fate of homocysteine in cells. It was hoped that such a system might provide insight into the factors that contribute to hyperhomocysteinemia. This study was carried out in nine control (non-hyperhomocysteinemic) individuals and in seven vascular disease subjects, four of whom had mild hyperhomocysteinemia.

The control subjects demonstrated sufficient activity in both the remethylation and transsulphuration pathways to prevent the accumulation of the label in homocysteine. In contrast, the hyperhomocysteinemic vascular disease subjects showed a trend towards increased cellular labelling of homocysteine. Altered profiles in two of these subjects could be attributed to nutritional deficiencies in folate/B₁₂.

In addition, the work also looked at the contribution of common mutations in the enzymes of the methionine cycle to the disposal of homocysteine. These included the MTHFR C677T, CBS T833C and MS A2756G polymorphisms. Five subjects presented with the MTHFR TT genotype. This genotype is associated with an elevated plasma homocysteine due to reduced enzyme activity, particularly in the presence of low folate. From the results of this small study it appears that this genotype, of itself, is not sufficient to induce accumulation of homocysteine at the cellular level. One heterozygote and one homozygote for the MS A2756G

polymorphism was identified. Their profiles indicated that the polymorphism had no substantial effect on their handling of homocysteine. The CBS T833C polymorphism was not found in any of the subjects studied.

Plasma homocysteine correlates positively with age and is inversely proportional to serum folate and B₁₂. Although a small study (n=17), the general trends seen between homocysteine and age, folate and B₁₂ corroborate those reported in the literature. However, the only variable to demonstrate a statistically significant effect on plasma homocysteine was serum folate.

While epidemiological evidence points to a role for homocysteine in atherothrombosis, the exact mechanisms by which homocysteine contributes to the process remain unclear. The second part of this thesis deals with the effects of homocysteine on macrophage and platelet function. Specifically, the effect of homocysteine on the production of the potentially pro-atherogenic cytokine TNF alpha by monocytes/macrophages was investigated.

Using the human monocytic cell-lines U-937 and THP-1, it was found that homocysteine had differential effects on TNF α production depending on the differentiation state of the cell. Phorbol-ester-induced TNF α production was enhanced 2-3fold in monocytes pre-exposed to homocysteine. However, macrophage TNF α production was reduced by 50-70% in the presence of homocysteine. Northern blotting analysis suggested that this reduction was not due to a downregulation of transcription of the TNF α gene, suggesting that homocysteine may exert its effects at the post-transcriptional stage. In addition, no changes to TNF α production were found in the presence of cysteine, implying that the effects of homocysteine are specific. Thus, the data suggest that homocysteine plays a role in regulating cytokine expression and in doing so may promote atherogenesis.

Finally, the potential pro-aggregatory role of homocysteine was investigated. Agonist-induced platelet aggregation, platelet thromboxane production in the presence of homocysteine were investigated. The response to homocysteine was not consistent. Of eight healthy individuals investigated, homocysteine was associated with an increased aggregation and concurrent increase in thromboxane synthesis in four subjects, while the remaining four subjects showed little or no enhancement of aggregation in the presence of homocysteine.

Fibrinogen binding, a necessary step in platelet aggregation, was also investigated in the presence of homocysteine. No homocysteine-mediated increase in fibrinogen-binding was noted, though this may have been a result of the constraints of the assay. Although inconclusive, a possible pro-aggregatory role for homocysteine cannot be ruled out.

ABBREVIATIONS

1-D	One-dimensional
2-D TLC	Two-dimensional thin layer chromatography
Abs	Absorbance
ADP	Adenosine diphosphate
AMV	Avian Myeloblastosis Virus
AP-1	Activating protein 1
ATP	Adenosine triphosphate
BHT	Beta hydroxy toluene
BLAST	Basic local alignment search tool
bp	base-pair
BSA	Bovine serum albumin
CAD	Coronary artery disease
cAMP	cyclic Adenosine monophosphate
CBS	Cystathionine beta synthase
CD ⁴⁺	Cluster determinant
cDNA	Complementary deoxyribonucleic acid
CHD	Coronary heart disease
cm ²	square centimetre
CO ₂	Carbon dioxide
CoA	Coenzyme A
CPM	Counts per minute
CuSO ₄	Copper Sulphate
CV	Coefficient of variation
Cys	Cysteine
Cysta	Cystathionine
dATP	Deoxyadenosine tri-phosphates
dCTP	Deoxycytosine tri-phosphates
DEPC	Diethyl pyrocarbonate
dGTP	Deoxyguanine tri-phosphates
DMF	Dimethyl formamide

DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide tri-phosphates
DTT	Dithiothreitol
dTTP	Deoxythymidine tri-phosphates
ECAM	Endothelial cell adhesion molecule
EDTA	Ethylene diamine tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence activated cell sorter
FSC	Forward scatter
FCS	Foetal calf serum
Fig.	Figure
GAPDH	Glyceraldehyde 3 phosphate dehydrogenase
H ₂ O ₂	hydrogen peroxide
HBSS	Hanks balanced salts solution
HCl	Hydrochloric acid
Hcy	Homocysteine
HRP	Horse radish peroxidase
hrs	Hours
Hsulf	Homocysteine-sulfinic acid
Htine	Homocystine
ICAM	Intracellular cell adhesion molecule
IFN γ	Interferon gamma
Il-1	Interleukin 1
IPTG	Isopropyl-1-thio- β -D-galactopyranoside
kDa	kilodalton
L - agar	Lennox agar
L - broth	Lennox broth
LDH	Lactate dehydrogenase
LDL	Low density lipoprotein
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide

MAT	Methionine adenosyl transferase
MCP-1	Monocyte chemotactic protein 1
Met	Methionine
mg	Milligram
MgCl ₂	Magnesium chloride
MI	Myocardial infarction
mins	Minutes
MMP	Matrix metalloproteinase
MOPS	3-(N-Morpholino)propanesulfonic acid
mRNA	Messenger RNA
MS	Methionine synthase
MTHFR	Methylenetetrahydrofolate reductase
Na ₂ CO ₃	Sodium carbonate
NaCl	Sodium chloride
NADH	Nicotinamide adenine dinucleotide
NaH ₂ PO ₄	Sodium phosphate
NaOH	Sodium hydroxide
NF-κB	nuclear factor kappa B
NO	Nitric oxide
NS	Not significant
oxLDL	oxidised Low density lipoprotein
p	Probability
PABA	para-amino benzoic acid
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PHA	Phytohaemagglutinin
P _i	Inorganic orthophosphate
PMA	Phorbol 13- myristate acid
PP _i	Inorganic pyrophosphate

PRP	Platelet rich plasma
ps	Penicillin streptomycin
PteGlu	Pteroyl glutamic acid
PVP	Polyvinylpyrrolidone
RBC	Red blood cell
RDA	Recommended daily allowance
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
rRNA	ribosomal RNA
RT	Reverse transcriptase
RT-PCR	Reverse transcription polymerase chain reaction
SAH	S-adenosyl homocysteine
SAM	S-adenosyl methionine
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SMC	Smooth muscle cell
SSC	Side scatter
SSC	Sodium saline citrate
TAE	Tris Acetate EDTA
TBE	Tris Borate EDTA
TGF	Transforming growth factor
TMB	Tetramethylbenzidine
TNF α	Tumour necrosis factor alpha
TXA ₂	Thromboxane A ₂
TXB ₂	Thromboxane B ₂
UV	Ultra violet
VCAM	Vascular cell adhesion molecule
VSMC	Vascular smooth muscle cell
X-gal	5-bromo-4-chloro-3-indoyl- β -D-galactopyranoside

Chapter 1

Introduction

1.1 Genome Introduction

Homozygosity has attracted considerable attention of researchers in the field of human genetics. The study of the levels of the homozygosity in the human genome is of great importance for the understanding of the risk of various diseases (Hartley, 1962; Neill, 1962). The homozygosity is a result of the inheritance of the same allele by both parents of an individual and has a high frequency in the human genome.

Chapter 1

Introduction

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Chapter 1

Introduction

1.1. General introduction.

Homocysteine has attracted considerable attention in recent years as elevated plasma levels of this non-protein forming sulphur amino acid are associated with an increased risk of vascular disease (Boushey *et al*, 1995). This amino acid is made intracellularly as a by-product of methionine metabolism and has no known biological function (Fig. 1.1). It is estimated that homocysteine production in man is approximately 20,000 μ moles/day with only 10 μ mol/day being exported to the plasma (Fortin & Genest, 1995). Homocysteine lies at the junction of two pathways, the transsulphuration pathway and the remethylation pathway. When cellular demands for methionine are high, homocysteine is remethylated by the B₁₂ (cobalamin)-dependent enzyme methionine synthase, which requires folate as co-substrate, to regenerate methionine. Alternatively, homocysteine is irreversibly sulfoconjugated to serine by the B₆-dependent enzyme, cystathionine beta synthase (CBS). Subsequently cystathionine is converted to cysteine by the action of γ cystathionase. This series of reactions, by which the four-carbon dietary amino acid methionine is converted to the three-carbon amino acid cysteine, is called the transsulphuration pathway. Deficiencies in B₆, B₁₂ or folate can therefore lead to elevated homocysteine levels, as can genetic defects in the enzymes of the pathway (Mudd, 1995a).

When the intracellular production of homocysteine exceeds the metabolic capacity, homocysteine is efficiently exported from the cell and accumulates in plasma (hyperhomocysteinemia) or in the case of a severe methionine metabolic disorder, is also found in urine (homocystinuria), secondary to hyperhomocysteinemia (Carson & Neill, 1962). The export mechanism complements the catabolism of homocysteine to

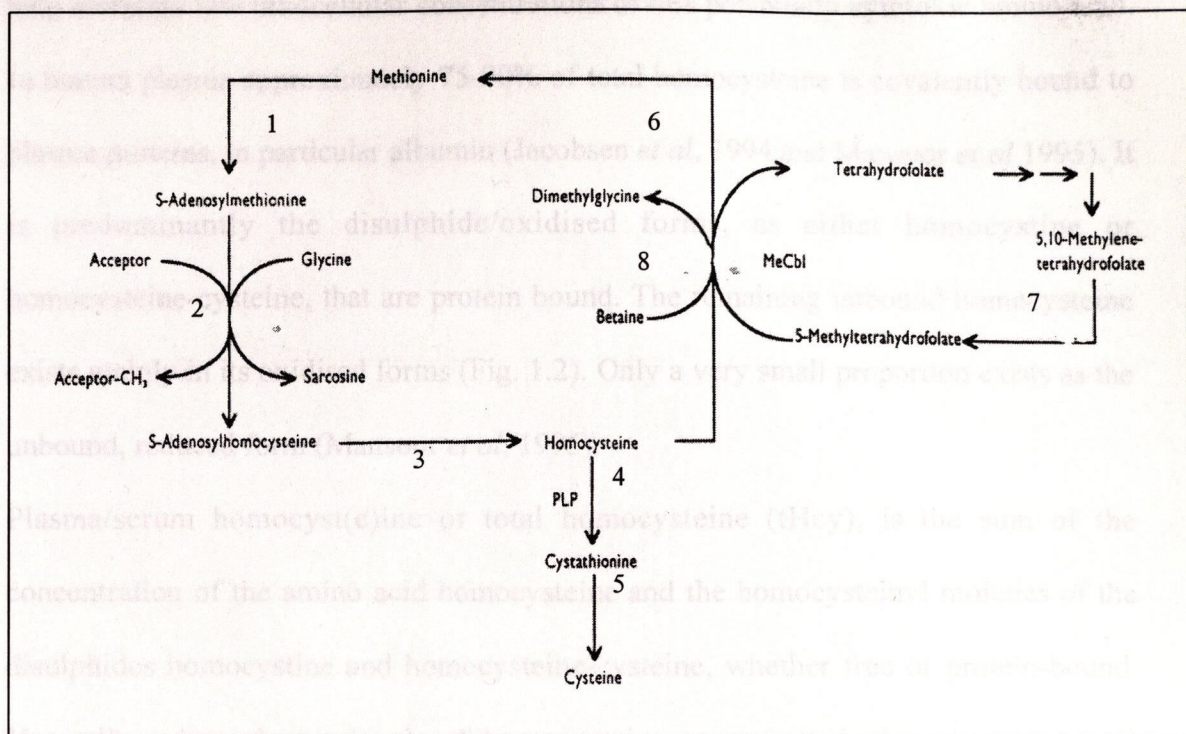


Fig. 1.1 The methionine metabolic pathway.

1: Methionine adenosyl transferase (MAT).

2: Methylases.

3. SAH hydrolase.

4: Cystathionine beta synthase (CBS).

5: Gamma cystathionase.

6. Methionine synthase (MS).

7. Methylenetetrahydrofolate reductase (MTHFR).

8. Betaine-homocysteine methyltransferase.

MeCbl: Methylcobalamin, PLP: Pyridoxal 5'-phosphate.

(Adapted from Loehrer *et al*, 1996).

help maintain low intracellular concentrations of this potentially cytotoxic amino acid. In human plasma approximately 75-90% of total homocysteine is covalently bound to plasma proteins, in particular albumin (Jacobsen *et al*, 1994 and Mansoor *et al* 1995). It is predominantly the disulphide/oxidised forms, as either homocystine or homocysteine-cysteine, that are protein bound. The remaining unbound homocysteine exists mainly in its oxidised forms (Fig. 1.2). Only a very small proportion exists as the unbound, reduced form (Mansoor *et al*, 1995).

Plasma/serum homocyst(e)ine or total homocysteine (tHcy), is the sum of the concentration of the amino acid homocysteine and the homocysteinyl moieties of the disulphides homocystine and homocysteine-cysteine, whether free or protein-bound. Normally, when plasma levels of homocysteine are reported, they represent total homocysteine concentrations. Normal fasting plasma homocysteine is estimated to lie between 6 and 15 μ M and varies depending on detection method, age, sex and genetic background (Jacobsen *et al*, 1989; Ueland *et al*, 1993 and Selhub *et al*, 1999).

In hyperhomocysteinemia plasma levels of homocysteine rise above normal to between 15 and 100 μ M. In the case of homocystinuria, levels are much higher – in excess of 100 μ M and up to 800 μ M in some cases. Often the increase in total plasma homocysteine is associated with an increase in the levels of the unusual homocysteine species such as the mixed disulphides (Jacobsen *et al*, 1994).

Genetic, nutritional and other factors are involved in the development of hyperhomocysteinemia. Up to 80% of patients are thought to have reduced CBS activity, while a significant proportion (9-17%) are thought to have reduced MTHFR activity, due to a common polymorphism which gives rise to a thermolabile variant of the enzyme and is associated with reduced activity. Nutritional deficiencies in B₆, B₁₂ and particularly folate can give rise to hyperhomocysteinemia directly or may compound a genetic lesion (Harjai, 1999).

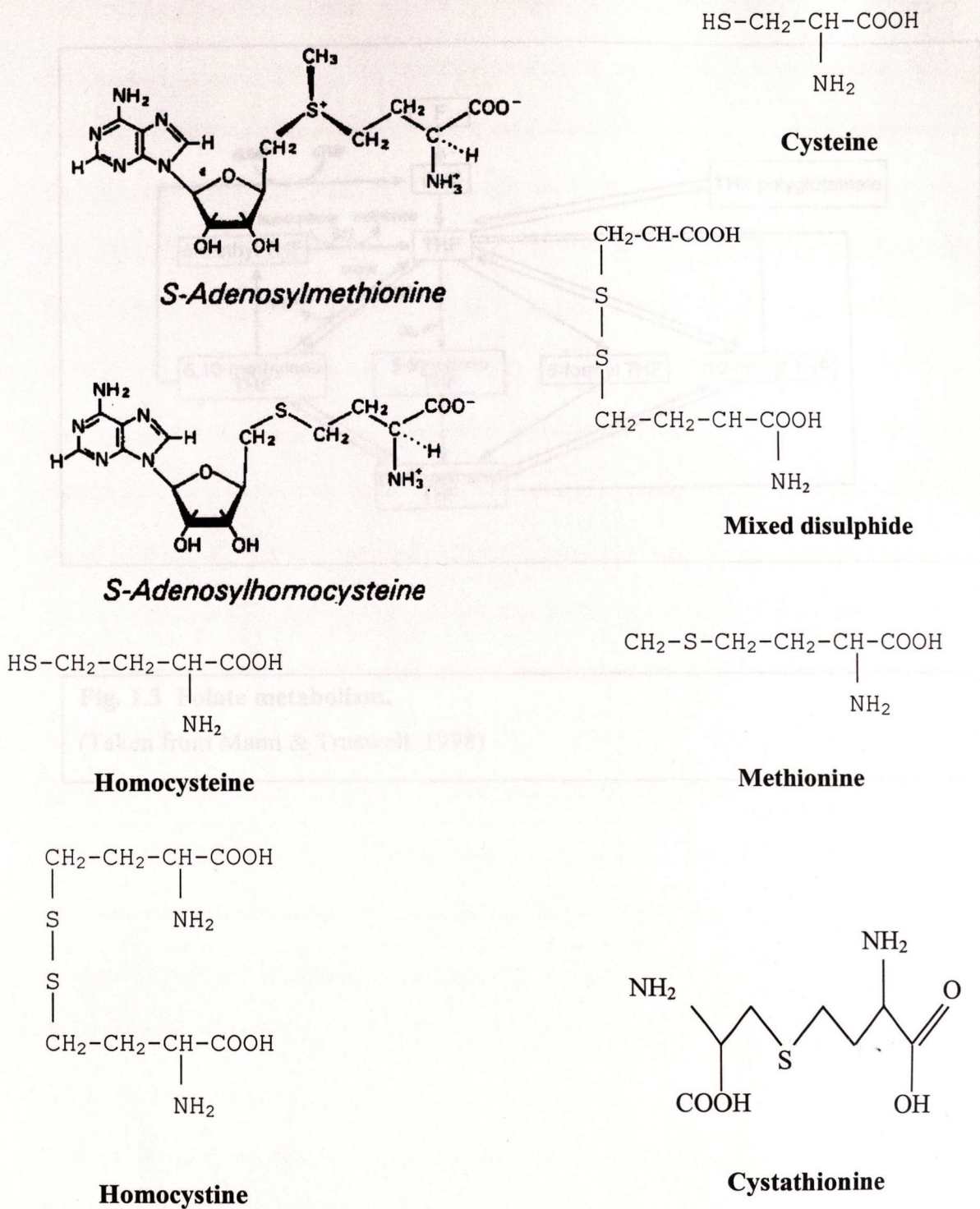


Fig. 1.2 Chemical structures of methionine, its metabolic derivatives and the oxidised species of homocysteine.

(Taken from Mudd *et al*, 1995).

1.2. Methylenetetrahydrofolate

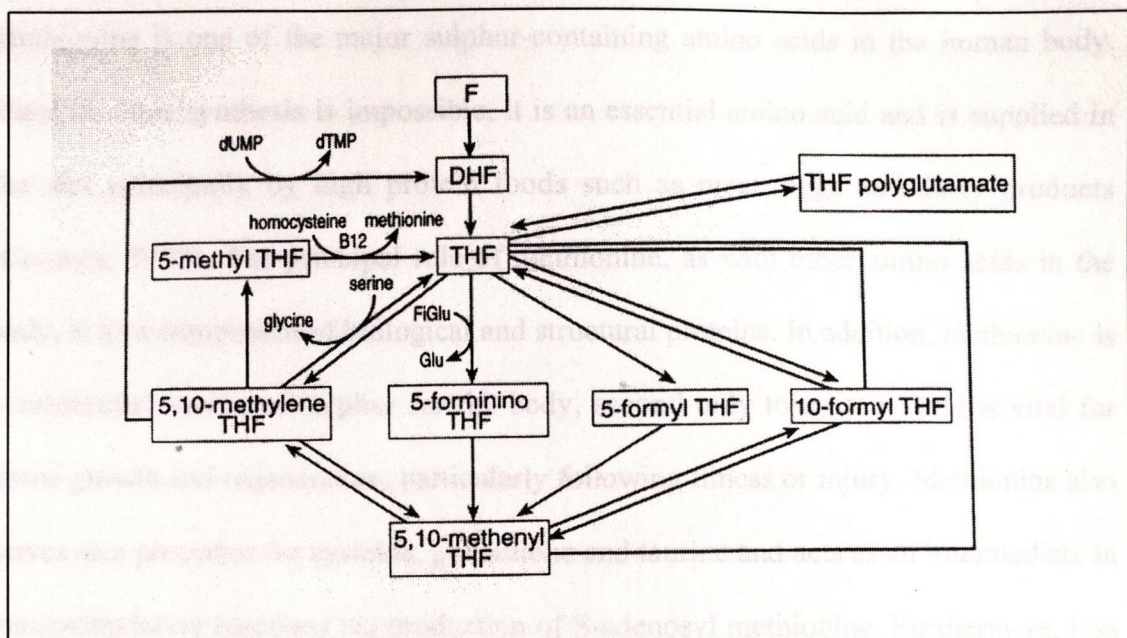


Fig. 1.3 Folate metabolism.

(Taken from Mann & Truswell, 1998)

(Mayes, 1996). Methionine, an essential amino acid, is primarily synthesized from dietary proteins by the action of methionine synthase, an enzyme that uses 5-methyltetrahydrofolate as a methyl donor. To a lesser extent, the transamination pathway

1.2.1. Transamination of methionine

Amino acids in excess of those needed for the synthesis of proteins and other biomolecules cannot be stored and are not excreted but rather are converted to keto acids via transamination reactions (Stryer, 1988). In man, the transamination of methionine is thought to play a minor role in normal methionine metabolism. It is thought to compensate for defective transsulphuration (Blom, 1990). Transamination involves the transfer of an α -amino group from an amino acid to the γ -keto portion of an α -keto acid. The amino donor becomes the α -keto acid which is then used to synthesize

1.2. Methionine Metabolism.

Methionine is one of the major sulphur-containing amino acids in the human body. Since *de novo* synthesis is impossible, it is an essential amino acid and is supplied in the diet principally by high protein foods such as meat, eggs and dairy products (Coomes, 1997). The principal role of methionine, as with other amino acids in the body, is as a component of biological and structural proteins. In addition, methionine is a substantial source of sulphur for the body, second only to cysteine and is vital for tissue growth and regeneration, particularly following illness or injury. Methionine also serves as a precursor for cysteine, glutathione and taurine and acts as an intermediate in transmethylation reactions *via* production of S-adenosyl methionine. Furthermore, loss of the methyl moiety followed by decarboxylation leaves a 3-carbon chain which is involved in the synthesis of the polyamines, spermine and spermidine (Mudd, 1995a). Once methionine has been released from dietary proteins by the action of aminopeptidases in the intestine, it is transported to the liver via the blood stream (Mayes, 1996). Methionine not used in protein synthesis is primarily metabolised here via the transsulphuration or remethylation pathway (methionine cycle) (Fig 1.1) and to a lesser extent the transamination pathway.

1.2.1. Transamination of methionine.

Amino acids in excess of those needed for the synthesis of proteins and other biomolecules cannot be stored and are not excreted but rather are used for metabolic fuel *via* transamination reactions (Stryer, 1988). In man, the transamination pathway for methionine is thought to play a minor role in normal methionine metabolism and is not thought to compensate for defective transsulphuration (Blom, 1989). Transamination involves the transfer of an α -amino group from an amino acid to the α -keto position of an α -keto acid. The amino donor becomes the α -keto acid while the keto acid becomes

an α - amino acid (Stryer, 1988). Occupying the active site of all transaminases is the biologically active form of vitamin B₆ – pyridoxal 5' phosphate (PLP) (Rodwell, 1996). In the case of methionine, transamination results in the production of 4 methylthio-2-oxobutyrate. Oxidative decarboxylation then yields 3-methylthio propionate. From this methanethiol is subsequently formed and degraded to carbon dioxide, sulphate and dimethylsulphide (Blom, 1989).

1.2.2. Biosynthesis and role of S-adenosyl methionine (SAM).

The first step of the methionine cycle, catalysed by MAT (ATP:L-Methionine S-adenosyl transferase: EC 2.5.1.6), is the transfer of an adenosine group from adenosine triphosphate (ATP) to the sulphur atom of methionine (Met) generating S-adenosyl methionine (SAM) and tripolyphosphate. The triphosphate portion of the ATP is simultaneously hydrolysed to inorganic phosphate (P_i) and inorganic orthophosphate (PP_i) by the intrinsic triphosphatase activity of the enzyme. The removal of the triphosphate makes the synthesis of SAM essentially irreversible under physiological conditions. MAT, a multisubunit enzyme of 185kDa has been partially purified from several mammalian tissues and has demonstrated a dependence on divalent and monovalent cations for activity and shows a strict specificity for ATP and a broader specificity for methionine. Non-hepatic MAT is allosterically regulated by one of its products, namely SAM, at concentrations that may occur physiologically (Kotb *et al*, 1990).

SAM plays a pivotal role as a methyl donor in a myriad of biological and biochemical reactions. This is due to the highly unstable and therefore reactive sulfonium bond, which makes the methyl a good leaving group. It exists as two stereoisomers, (S,S) - SAM and (S, R) -SAM - the chirality of the sulfonium centre being an important determinant for biological activity with the (S,S) enantiomer the predominant and

biologically active form (Chiang *et al*, 1996). When incubated with rat liver extracts, more than 99% of SAM is demethylated while about 1% is decarboxylated for polyamine biosynthesis which leads to the production of spermine (Eloranta *et al*, 1984). Quantitatively, creatine synthesis, which occurs primarily in the pancreas, kidney and liver, represents the major consumer of SAM (Fortin & Genest, 1995). Protein amino- or carboxy-terminal methylation (mediated by SAM) can lead to alterations in steric orientation, charge or hydrophobicity thereby affecting protein structure and function, *eg* activation of heat-shock proteins. Methylation of phospholipids provides an alternative route for phosphatidyl-choline synthesis. Methylation of both DNA (particularly cytosine) and RNA modulate transcriptional and translational processes. Ribosomal assembly and processing, export of mRNA from the nucleus, initiation of transcription, correct splicing and efficient translation are all dependent on methylation. SAM also plays an important role in the methylation of noradrenaline to form adrenaline (Chiang *et al*, 1996).

Loss of the methyl group from SAM results in the production of the short-lived S-adenosyl homocysteine (SAH). It is cleaved by S-adenosyl homocysteinase (EC 3.3.1.1) to generate homocysteine and adenosine. The mammalian hydrolase is a homotetramer of 190kDa. Each subunit contains a tightly bound NAD^+ molecule that is reduced to NADH when homocysteine and adenosine are released. Although the equilibrium of the reaction favours SAH accumulation, both homocysteine and adenosine are normally quickly removed *in vivo* so that the enzyme functions in the cleavage direction overall (Mudd, 1995a).

1.2.3. Control of methionine metabolic pathways.

Homocysteine lies at the junction of two pathways and depending on the methionine status of the cells it may undergo remethylation or transsulphuration. Regulation of both these pathways is dependent on several conditions, such as availability of cofactors. SAM is thought to play a vital role in the co-ordination of these pathways (Finkelstein *et al*, 1975 and Jencks *et al*, 1987). *In vitro* studies have demonstrated that SAM acts as an allosteric inhibitor of 5,10 methylenetetrahydrofolate reductase and as an activator of CBS at micromolar concentrations. It has been proposed that conditions affecting remethylation will directly result in homocysteine elevation. A disturbance of the transsulphuration pathway, due to a decreased synthesis of SAM, is then thought to further contribute to homocysteine accumulation (Miller *et al*, 1992).

1.2.4. Remethylation of homocysteine.

When demands for methionine/SAM are high remethylation of homocysteine to regenerate methionine occurs. The methyl group is supplied by 5' methyltetrahydrofolate, or to a lesser extent, in the liver, by betaine. In the case of betaine, the methyl transferase is catalysed by dimethylthetin-homocysteine methyltransferase (EC: 2.1.1.3) and also betaine-homocysteine methyltransferase (EC: 2.1.1.5) (Skiba *et al*, 1982). Following methyl transfer, betaine is converted to dimethyl glycine (Fig. 1.1). This is further demethylated by dimethylglycine dehydrogenase to sarcosine and then converted to glycine by sarcosine dehydrogenase. Glycine may be catabolised to CO₂ and ammonia by the glycine-cleavage system (Allen *et al*, 1993). No mammalian cases of betaine-homocysteine methyltransferase deficiency have yet been described and the importance of this enzyme in methionine synthesis is thus unknown.

The primary methyl donor in methionine regeneration is 5' methyltetrahydrofolate (5'MTHF). Folate supplied in the diet is reduced to tetrahydrofolate and then methylated by reaction with serine, to give 5,10 methylenetetrahydrofolate and glycine. In a step resulting in the oxidation of NADH, this 5,10 methylenetetrahydrofolate is reduced by the cytoplasmic flavoprotein, 5' methyltetrahydrofolate reductase (MTHFR) (EC: 1.1.99.15) to give the only form of folate which may be utilised in the methylation process, *ie* 5' methyltetrahydrofolate (Fig 1.3). The biosynthesis of methionine using this methyl source is catalysed by the B₁₂-dependent methionine synthase (MS) (EC: 2.1.1.13) (Mangum *et al*, 1969). This reaction is thought to be of importance in both the maintenance of methionine levels and in the production of tetrahydrofolate for other "one-carbon" unit transfer reactions, *eg* synthesis of thymidylate (Selhub & Rosenberg, 1996). This is one of only two reactions in the human body known to require the presence of B₁₂/cobalamin. The other reaction is the conversion of L-methylmalonyl CoA to succinyl CoA (Stryer, 1988). Studies have shown that MS mRNA is in highest abundance in the pancreas, skeletal muscle and heart and is reported to be present at much lower levels in the liver (Chen *et al*, 1997).

1.2.5. Transsulphuration of homocysteine.

The alternative route for homocysteine disposal is the transsulphuration pathway where homocysteine is converted to cysteine. The first step sees the condensation of homocysteine with serine to produce the thioether cystathionine. This reaction is catalysed by the vitamin B₆ / pyridoxal phosphate and heme dependent enzyme, cystathionine β synthase (CBS: EC 4.2.1.22) (Fig. 1.1).

As previously mentioned, the activity of CBS is enhanced in the presence of SAM, while activity of MTHFR is inhibited (Finkelstein *et al*, 1975 and Jencks *et al*, 1987). The net result of an elevated SAM therefore is the decreased remethylation of

homocysteine to methionine and the increased clearance of homocysteine to cystathionine *via* transsulphuration.

Cystathionine is deaminated and cleaved by another pyridoxal phosphate dependent enzyme - γ -cystathionase (EC 4.4.1.1) to yield cysteine and α -ketobutyrate. Cysteine may be metabolised in several ways, depending on the cells needs and is an important amino acid in protein structure. Cysteine is also a component of glutathione, the tripeptide γ -glutamylcysteinylglycine. The biological functions of this tripeptide are manifold. It acts as an essential cell reductant, is involved in amino acid transport across cell membranes and detoxification and may act as a cofactor in some enzymatic reactions.

1.3. Cofactors and coenzymes in methionine metabolism.

1.3.1. Folic acid.

An important aspect of methionine metabolism is its dependence on coenzymes derived from the vitamins folic acid, B₁₂ (cobalamin) and B₆ (pyridoxine).

Folic acid is the term commonly used to refer to a group of vitamins with related biological activity (Mayes, 1996). Other terms such as folacin and folates may be used interchangeably.

Folates have a pteroylglutamic acid (PteGlu) as a common structure, *ie* the base pteridine attached to one molecule each of *p*-aminobenzoic acid (PABA) and glutamic acid. They may differ in the substitution in the pyrazine ring and also in the number of glutamate residues attached to PABA. The pyrazine ring may be partially reduced at the 7, 8 positions (dihydrofolate) or, by the action of dihydrofolate reductase (EC 1.5.1.3), may be fully reduced (tetrahydrofolate) (Selhub & Rosenberg, 1996).

The main circulatory forms of folates are the monoglutamyl derivatives, mostly in the form of 5' methyltetrahydrofolate. Within the cell, folates are trapped in the

polyglutamyl forms, which are incapable of cellular exit. It is these polyglutamated forms that are probably utilised by tissues. The assimilation of circulating folate by the cell necessitates demethylation. Such demethylation may only occur through homocysteine methylation to methionine by the B₁₂-dependent methyltransferase, methionine synthase. Thus, cellular folate depletion is one consequence of vitamin B₁₂ deficiency (Selhub & Rosenberg, 1996).

The RDA for folate (200µg/day) has recently been reviewed in light of its close inverse relationship with homocysteine, a potentially reversible risk factor for premature vascular disease and neural tube defects (Daly *et al*, 1995; Molloy *et al*, 1997 and Jacques *et al*, 1999). Folate deficiency may arise in a variety of settings, such as alcoholism, low dietary intake, malabsorption or conditions of increased cellular turnover *eg* pregnancy. In addition, a variety of drugs may also induce deficiency, *eg* sulfasalazine. Folate status is typically assessed by measuring serum folate levels. Measurement of red blood cell folate may sometimes present a more accurate indication of cellular folate status as serum folate is more sensitive to intake and temporary changes in metabolism. It is now well recognised that homocysteine levels are inversely related to folate levels in a non-linear fashion and thus it has been suggested that homocysteine may be used as an indicator of folate status and *vice versa* (Hall & Chu, 1990).

The involvement of folate in the synthesis of purines, methionine and in serine-glycine interconversion lies in its ability to serve both as a methyl donor and as a methyl acceptor (Fig. 1.3). The one-carbon units carried by tetrahydrofolate represent a series in various states of oxidation, namely, methyl, methylene, methenyl, formyl and formimino, all of which are metabolically interconvertible. Serine acts as the major source of one-carbon units, which are transferred to tetrahydrofolate to form N⁵, N¹⁰ -methylenetetrahydrofolate and glycine. N⁵, N¹⁰ -methylenetetrahydrofolate may serve

as the methyl donor for thymidylate synthesis or it may be reduced to N⁵-methyltetrahydrofolate, which is involved in the remethylation of homocysteine to methionine, a reaction which is essentially irreversible at physiological pH. N⁵-methyltetrahydrofolate may also undergo oxidation to N⁵, N¹⁰-methenyltetrahydrofolate which can be hydrated to either N¹⁰-formyltetrahydrofolate or N⁵-formyltetrahydrofolate - substrates for purine synthesis. The latter is known as folinic acid, a stable form that may be used for administration of reduced folate (Rosenblatt, 1995).

The involvement of folates in a large number of diverse reactions suggests the existence of some regulatory mechanisms. It has been suggested that overall, folate metabolism may be regarded as two crucial groups of reactions competing for available cellular folate, *ie* 1) *de novo* synthesis of methionine and 2) the reactions that lead to the synthesis of nucleic acids. It is argued that of the two, methionine synthesis would take priority, as it is a precursor of SAM, the universal methyl donor, essential for survival of the cell. The theory proposes that when folate or methionine is limited in the cell, folate coenzymes are directed towards methionine synthesis at the expense of nucleic acid synthesis (Scott & Weir, 1981 and Selhub & Rosenberg, 1996).

1.3.2. Vitamin B₁₂ (cobalamin).

The vitamin B₁₂ or cobalamin molecule is composed of four reduced pyrrole rings linked together to form a macrocyclic ring structure called "corrin". This ring structure is stabilised by the presence of a cobalt cation (Co⁺) at its centre (Herbert, 1996). Different R groups may be attached to the cobalt, forming for example *cyanocobalamin* or *methylcobalamin*. Coenzymatically active forms of cobalamin are highly unstable and thus it is the stable but physiologically inactive form - cyanocobalamin, that is used

pharmacologically. Man has no circulating cyanocobalamin, except in the case of smokers, where cyanide is supplied by tobacco.

Cobalamins are water-soluble vitamins known to be required in only two reactions in the human body. Methylcobalamin is required for the conversion of homocysteine to methionine, which also results in the simultaneous conversion of methyltetrahydrofolate to tetrahydrofolate. Cobalamin metabolism is therefore tightly interlinked with that of folate. 5' Deoxyadenosyl cobalamin (coenzyme B₁₂) is involved in methylmalonic acid-succinic acid isomerisation (Fenton & Rosenberg, 1995).

To sustain normal B₁₂-related metabolism, the minimum daily absorbed requirement is just 0.1µg/day. The Recommended Daily Allowance (RDA) for B₁₂ at 2µg/day is therefore more than adequate. There are however certain groups in the population who require higher intake of the vitamin, such as pregnant or lactating women and the elderly. Because most diets naturally contain excessive B₁₂ (3-9µg/day), deficiencies are rare (Herbert, 1996).

The "methyl folate trap" hypothesis proposes that in B₁₂ deficiency, the combination of impaired B₁₂-dependent methyltransferase and irreversibility of the methylenetetrahydrofolate reductase reaction will lead to the trapping of folate in the methylated form, thus making it unavailable for other folate-dependent syntheses such as those of thymidylate and purines. This hypothesis explains the common clinical symptoms *eg* megaloblastic anemia, seen in folate and B₁₂ deficiency (Allen *et al*, 1993).

Vitamin B₁₂ deficiency results in several pathologies. One of the most serious of these is neuropathy, resulting from progressive demyelination. This is thought to be due to reduced synthesis of SAM *via* the methionine metabolic pathway and accumulation of the neurotoxic amino acid, homocysteine, due to decreased levels of the essential coenzyme methylcobalamin (Allen *et al*, 1993)

Hyperhomocysteinemia is a common finding in those deficient in cobalamin and/or folate. Reduced flux through the remethylation pathway, as a consequence of B₁₂ deficiency leads to a toxic build-up of homocysteine. However, homocysteine levels may be reduced by RDA amounts of B₁₂ or folic acid, if the accumulation is due to one of these deficiencies (Stabler *et al*, 1996 and Ward *et al*, 1997).

1.3.3. Vitamin B₆.

Vitamin B₆ occurs naturally in three forms: pyridoxine, pyridoxal and pyridoxamine. The liver is capable of converting pyridoxine and pyridoxamine to pyridoxal. Each of these forms may be phosphorylated - it is this phosphorylated form of pyridoxal (pyridoxal 5'-phosphate or PLP) that is used as a coenzyme by CBS. PLP is an especially reactive species of B₆ and readily reacts with amino groups.

The reactivity of PLP with amino acids and nitrogen-containing compounds is responsible for the high number of biological reactions in which it is involved (>100). It acts as a cofactor for a number of enzymes involved in gluconeogenesis, lipid metabolism, niacin formation, nucleic acid metabolism and nervous and immune systems function (Leklem, 1996).

B₆ status is dependent not only on B₆ intake but also on other factors, such as protein intake, alkaline-phosphatase levels and age (Selhub *et al*, 1993). Frank deficiencies in B₆ are rare, but marginal deficiencies are not uncommon, usually in conjunction with a deficiency of some other nutrient, particularly riboflavin, which is involved in its metabolism. Genetic defects in B₆-dependent enzymes mimic B₆ deficiency, as is the case in homocystinuria and cystathioninuria. The RDA for vitamin B₆ for adults is approximately 2mg/day, with lower intakes suggested for children. Supplemental B₆ has been used to treat both homocystinuria and hyperhomocysteinemia, but it appears

to be a less effective treatment than folate or B₁₂ (Mudd *et al*, 1970 and Franken *et al*, 1994).

1.4. Defects in Methionine Metabolism.

Both genetic and nutritional factors influence methionine metabolism. The enzymes affected and the particular deficiency occurring determine both the biochemical and clinical manifestations.

Hypermethioninemia/hypermethioninuria (elevated plasma/urine levels of methionine) is a rare condition that may occur by one of two mechanisms i) when methionine is underutilised *ie* impaired conversion of methionine to SAM due to a defective MAT activity or in liver disease and ii) in conjunction with hyperhomocysteinemia due to defective CBS. It is thought that some of the excess homocysteine, which cannot be transsulphurated, undergoes remethylation (Mudd, 1995b).

Cystathioninuria/hypercystathioninemia may occur by these same mechanisms, *ie* underutilisation and overproduction. Defective activity of γ -cystathionase or vitamin B₆ deficiency can lead to underutilisation, while reduced activity of the remethylation pathway due to deficiencies in vitamin B₁₂ or aberrant MTHFR or MS activity may lead to an overproduction of cystathionine (Mudd, 1995a).

Homocystinuria/hyperhomocysteinemia are the most commonly presenting abnormalities of the methionine pathway. These conditions will be discussed in more detail in the following sections.

1.4.1. Classical Homocystinuria.

Classical homocystinuria was first described in 1962 independently by both Gerritsen *et al* and Carson and Neill. The former detected high plasma and urine levels of both methionine and homocysteine in a group of children with mental retardation and

congenital abnormalities. Soon after, Carson and Neill described homocystinuria - a new inborn error of amino acid metabolism, characterised by concentrations of homocysteine in excess of $15\mu\text{M}$ ($50\text{-}300\mu\text{M}$) in the blood and excretion of the disulphide homocystine in urine (Gerritsen *et al*, 1962 and Carson & Neill, 1962).

Homocystinuria is a rare condition, inherited in an autosomal recessive manner. Its worldwide incidence varies and is estimated at about 1: 200, 000 (Franken *et al*, 1994). In contrast to this, the frequency of the disorder is much higher in some other countries including Ireland, where it is 1 in 57, 000 (Daly *et al*, 1993). Consequently, all newborns in Ireland are now routinely screened for homocystinuria.

The clinical manifestations are severe and include neurological, vascular, skeletal and ocular complications. Premature vascular disease and thrombosis are the most common causes of mortality, often occurring in teenage years (Masser *et al*, 1994).

1.4.1.1. Homocystinuria due to CBS defects.

Classical homocystinuria is caused by defective CBS activity. This CBS deficiency results in the accumulation of homocysteine due to reduced activity of the transsulphuration pathway and a concomitant increase in methionine as the remethylation pathway endeavours to accommodate excess homocysteine (Mudd *et al*, 1964). Homocystinuria may also occur in cases of severe MTHFR or MS deficiency and where folate/ B_{12} / B_6 metabolism is compromised (Mudd *et al*, 1972; McCully *et al*, 1992; Rosenblatt, 1995 and Leklem, 1996).

In response to pyridoxine treatment approximately 50% of homocystinuric patients show a reduction in plasma and urine concentrations of homocystine and methionine and a concurrent rise in cysteine (Barber and Spaeth, 1967; Mudd, 1970 and Boers *et al* 1983). It has been proposed that pyridoxine supplements are beneficial only in cases where there is residual activity of the CBS enzyme (Uhlendorf *et al*, 1973). Further

studies have indicated that betaine (or its precursor, choline) and folate supplementation given simultaneously with pyridoxine may be useful in normalising plasma homocysteine in non-pyridoxine responsive patients, while at the same time causing an increase in plasma cysteine and methionine (Smolin *et al*, 1981 and Wilcken *et al*, 1983). Plasma methionine concentrations were in some cases increased ten-fold, but no clinical effects of this accumulation have yet been observed. Restriction of dietary methionine in conjunction with supplemental cystine have shown varying degrees of success. The most dramatic results are seen when this dietary regimen is initiated in infancy. It is found to reduce or normalise plasma homocysteine and prevent the adverse clinical manifestations seen in untreated homocystinuric patients.

There appears to be great genetic heterogeneity among homocystinuric patients, which might explain the differing reactions of these patients to pyridoxine therapy (Wilcken *et al*, 1983). Several mutations in the CBS gene have been identified, these mutations occur in the catalytic domain, the pyridoxal phosphate-binding domain and in the SAM regulatory domain (Hu *et al*, 1993; Kluijtmans *et al*, 1996 and Dawson *et al*, 1997). There is a very high prevalence of the CBS homozygous mutation G307S (which arises due to a G-A transition at base pair 919) in Irish homocystinuric patients (Gallagher *et al*, 1995). It is believed that this mutation is in the pyridoxine-binding domain as supplemental pyridoxine cannot restore enzyme function. Another common variant - I278T, arises due to a T-C change at position 833 of the cDNA sequence (Hu *et al*, 1993 and Dawson *et al*, 1997). The I278T mutation is associated with pyridoxine responsive homocystinuria and a relatively mild clinically adverse phenotype (Shih *et al*, 1995).

1.4.1.2. Homocystinuria due to MTHFR defects.

Less commonly homocystinuria may arise as a consequence of inherited disorders of folate and cobalamin metabolism. These include MTHFR deficiency, functional MS deficiency due to defects in cobalamin metabolism and hereditary folate malabsorption. Of these, severe deficiency of MTHFR activity is the most common. Severe MTHFR deficiency is inherited as an autosomal recessive trait. Several MTHFR mutations have been identified that are thought to cause alterations to splice junctions, influence co-factor binding, enzyme stability and ultimately enzyme activity (Goyette *et al*, 1994; Kluijtmans *et al*, 1998). The correlation with genotype, enzyme activity and phenotype are consistent, *ie* those with the lowest enzyme activities show the earliest onset of biochemical and clinical symptoms (Goyette *et al*, 1995). Biochemically, the patients present with moderate homocystinuria and low to low-normal plasma methionine. The extent of homocystinuria seen in MTHFR-deficient patients is much less than that of CBS-deficient patients and is one of the distinguishing factors between the two. The symptoms may sometimes present in early infancy with progressive severe neurological symptoms caused by demyelination due to hypomethioninemia (Ronge & Kjellman, 1996) but may also be asymptomatic until adolescence. The most common clinical manifestation is developmental delay. MTHFR deficiency is resistant to treatment. Long-term betaine treatment has been successfully used to lower homocysteine but clinical improvements are not observed. Homocysteine levels although lowered are not normalised and so the risk of premature vascular disease remains (Rosenblatt, 1995 and Kluijtmans *et al*, 1998). Betaine-mediated methylation does not require the presence of folate or cobalamin and is possibly the most promising therapeutic agent available to date (Ronge & Kjellman, 1996). The primary goal in this case is to alleviate the hypomethioninemia as quickly as possible before the onset of neurological abnormalities, which are essentially irreversible. Supplemental folic acid, cobalamin,

methionine and pyridoxine have been used in patients with MTHFR deficiency. Efficacy is essentially dependent upon residual activity of the enzyme.

1.4.1.3 Homocystinuria due to disorders in folate or cobalamin metabolism.

The transfer of the methyl group from MTHF to homocysteine by MS is dependent on B₁₂/cobalamin. Because of the close interaction between folate and cobalamin metabolism, disorders of methionine biosynthesis due to cobalamin deficiency are grouped as disorders of cobalamin metabolism *ie* cblA, cblB, cblC, cblD, cblE, cblF, cblG (Rosenblatt, 1995; Fenton & Rosenberg, 1995 and Gulati *et al*, 1996).

Deficiency of cobalamin may arise either by defective absorption and transport or by defective cellular utilisation and coenzyme production. Defects in any one of the many steps involved in the absorption and transport of cobalamin can lead to its deficiency. Since cobalamin is then unavailable for either the isomerisation or the remethylation reactions, both homocysteine and methylmalonyl CoA are found to accumulate but not to the extent seen in cellular defects of folate metabolism. The onset of clinical symptoms such as megaloblastic anaemia and developmental delay vary depending on the cause of the absorption/transport defect and often present in infancy and early childhood. Treatment of cobalamin deficiency is usually by injection of pharmacological doses of cyano/hydroxy-cobalamin. This treatment effectively lowers homocysteine and methylmalonic acid and corrects the haematological abnormalities but is slow to reverse neurological manifestations.

In humans, folate and cobalamin deficiency due to defective folate/B₁₂ metabolism both cause indistinguishable haematological abnormalities (*eg* megaloblastic anaemia), while neurological abnormalities are found only in cobalamin deficiency. The biochemical basis for this remains unknown.

Hereditary folate malabsorption has been described in only 13 patients. The use of folate, folinic acid or MTHF administration has been effective in correcting serum folate (Rosenblatt, 1995).

In more than 90% of patients with cobalamin or folate deficiency, elevated levels of plasma homocysteine are observed (Savage *et al*, 1994). Hypomethioninaemia is not associated with these deficiencies - probably due to the use of betaine as a methyl donor. This hypothesis is supported by the findings of elevated N,N-dimethylglycine and N-methylglycine in most patients with folate deficiency (Allen *et al*, 1993). Dietary methionine content may also prevent hypomethioninaemia.

1.4.2. Hyperhomocysteinemia

Hyperhomocysteinemia is the milder more common form of homocystinuria, present in 3-4% of the general population, where total fasting plasma homocysteine levels rise above normal ($>15\mu\text{M}$). The extent of hyperhomocysteinemia may be graded into mild/moderate ($16-30\mu\text{M}$), intermediate ($31-100\mu\text{M}$) and severe ($>100\mu\text{M}$) (Malinow, 1994).

As is the case with homocystinuria, hyperhomocysteinemia occurs when the pathways involved in homocysteine catabolism (transsulphuration or remethylation) become impaired in some way, due to either genetic and/or nutritional factors (Daly *et al*, 1993). In some cases the genetic phenotype is exacerbated by a nutritional deficiency.

Recently, elevated plasma homocysteine levels have been identified as an independent risk factor for premature coronary artery disease, peripheral vascular disease, cerebrovascular disease, and thrombosis and is also associated with an increased risk of neural tube defects (Stegers-Theunissen *et al*, 1995; den Heijer *et al*, 1995; Mills *et al*, 1995; Graham *et al*, 1997; Stehouwer *et al*, 1998 and Chambers *et al*, 2000).

1.4.2.1. Genetic lesions in hyperhomocysteinemia.

Methylenetetrahydrofolate reductase.

The identification of mild hyperhomocysteinemia as a risk factor for vascular disease and the finding of hyperhomocysteinemia in families with neural tube defects has led to increased interest in the genes controlling homocysteine metabolism.

Studies by Kang *et al*, in the late 1980s revealed a significant association between a thermolabile variant of MTHFR and coronary artery disease (CAD) (Kang *et al*, 1988). In addition, this variant was found to have reduced activity (50%) compared to normal MTHFR and was associated with an elevated plasma homocysteine (13.2 μ M vs 8.5 μ M) (Kang *et al*, 1991).

The human methylenetetrahydrofolate reductase cDNA sequence was determined in 1994 (Goyette *et al*, 1994). The following year the mutation associated with this thermolabile variant was identified as a homozygous C to T transition at position 677 of the cDNA sequence, which converts an alanine to a valine residue (Frosst *et al*, 1995). The "TT" genotype is associated with thermolability of the enzyme, reduced specific activity and occasionally elevated plasma homocysteine levels (van der Put *et al*, 1995). This TT genotype is present in a high proportion of the general population. The evidence to date suggests that the frequency of the mutation differs with the population studied, *eg* 5% in a Dutch population, 12% in a French-Canadian population, 7% in an Irish population, 11% in an Australian population and 1% in African/Brazilian Blacks (Frosst *et al*, 1995; Kluijtmans *et al* 1996; Gallagher *et al*, 1996; Wilcken *et al*, 1996 and Franco *et al*, 1998).

Numerous epidemiological studies have found positive correlations between the TT genotype and plasma homocysteine levels, with CT heterozygotes demonstrating homocysteine levels intermediate between the wild-type (CC) and TT genotypes (Ma *et al*, 1996 and Kluijtmans *et al*, 1997). The TT genotype is thought to account for 25-

30% of elevated homocysteine seen in patients with premature cardiovascular disease (Engbersen *et al*, 1995). However, this relationship appears to be dependent on folate status (Christensen *et al*, 1997). Homocysteine accumulation does not occur when plasma folate levels are high, suggesting that this genetic defect can be largely compensated for by folate intake (Ma *et al*, 1996 and Schwartz *et al*, 1997). This supports the hypothesis that this part of the MTHFR gene (C677) is involved in folate binding and is consistent with the observation that homocysteine levels (especially in those with the TT genotype) may be reduced or normalised by folate supplementation (Malinow *et al*, 1997).

A meta-analysis by Brattström *et al*, found that the TT genotype was a major determinant of plasma homocysteine, with those with the TT genotype showing a 25% mean increase in plasma homocysteine (2.6µM) compared to those with the wild-type (CC) genotype (Brattström *et al*, 1998). This was demonstrated in American, Canadian, Dutch, Norwegian, Italian and Irish populations and occurred only in the presence of sub-optimal folate.

Despite the clear effect of this genetic polymorphism on increasing plasma homocysteine, especially those with low folate, there is conflicting evidence as to the relevance of this polymorphism to cardiovascular disease risk. While elevated homocysteine levels have been consistently demonstrated in a significant proportion of cardiovascular disease patients, not all studies have found a significant association between the TT MTHFR genotype and cardiovascular disease. A positive correlation between this polymorphism and coronary artery disease and/or myocardial infarction (MI) has been reported in Dutch, American and Japanese populations (Kluijtmans *et al*, 1996; Kang *et al*, 1996 and Morita *et al*, 1997). These results are in contrast to several other studies, that found equal incidence (approximately 11%) of the TT genotype in both controls and CAD patients, suggesting that the TT genotype may not serve as a

good marker for cardiovascular disease risk (Deloughery *et al*, 1996; Schmitz *et al*, 1996; Brugada & Marian, 1997 and van Bockxmeer *et al* 1997).

When Brattström *et al* carried out a meta-analysis on the incidence of the TT genotype and cardiovascular risk, involving data from 23 studies, they found no relationship between the homozygous polymorphism and incidence of cardiovascular disease or MI. The prevalence of the TT genotype varied between 5.4 and 29% but in each study almost identical distributions of the genotypes was found in both cases and controls. Overall the incidence of the TT genotype was approximately 11% (Brattström *et al*, 1998). These controversial findings suggest that homocysteine is not causally related to the pathogenesis of atherosclerosis.

Conflicting results may reflect differences in ethnic backgrounds, disease categories studied, dietary folate intake, inclusion/exclusion criteria and end points. For example, positive associations between TT and CAD were observed in Irish and Dutch populations where the fortification of foods and the routine use of vitamin supplements is uncommon. Most studies on US populations (where consumption of vitamins and fortified cereals is relatively high) found no association (Wilcken *et al*, 1996 and Verhoef *et al*, 1997a).

It has been postulated that if the remethylation pathway is impaired by the possession of the TT genotype that the transsulphuration pathway may work at a higher rate than normal to compensate for the defect. It is therefore conceivable that in this case serum B₆ levels would be reduced. A recent study showed this trend (*ie* lower serum B₆ in the presence of the TT genotype) but it was not statistically significant due to the wide spread of data (Woodside *et al*, 1997).

Methionine synthase.

The human MS gene, located on chromosome 1, was isolated, cloned and mapped in 1996 (Leclerc *et al*, 1996 and Li *et al*, 1996). Northern blot analysis indicates that it is present in a wide variety of human tissues (Chen *et al*, 1997). One relatively common polymorphism in the methionine synthase gene is an A to G transition at base pair 2756, which converts an aspartic acid residue, believed to be part of a helix involved in co-factor binding, to a glycine (Leclerc *et al*, 1996). An Irish study has found that this homozygous polymorphism (DD) is significantly associated with increased plasma homocysteine levels and unlike the MTHFR TT polymorphism, the effects of the D919G polymorphism appear to be independent of folate or B₁₂ status. However, the study did find that DD genotype was associated with higher elevations in plasma homocysteine in those with low B₆ levels (Harmon *et al*, 1999).

Cystathionine beta synthase.

The most common cause of homocystinuria is a homozygous genetic deficiency in CBS. Reduced enzyme activities in the range of obligate heterozygotes for CBS were reported in hyperhomocysteinemic patients with vascular disease. This suggested a causal role for heterozygosity for CBS deficiency in hyperhomocysteinemia and possibly vascular disease (Boers *et al*, 1985 and Clarke *et al*, 1991). The estimated heterozygote frequency for this mutation in the Irish population is approximately 1/110 (Daly *et al*, 1993). However, genetic analysis of an Irish vascular disease cohort failed to find heterozygosity for the G307S mutation, which is present in the homozygous state in approximately 50% of Irish homocystinurics (Gallagher *et al*, 1995). In addition, Kozich *et al*, could not find any mutations in the CBS cDNA of four hyperhomocysteinemic subjects with peripheral vascular disease (Kozich *et al*, 1995). Together these results seem to suggest that heterozygosity for CBS deficiency is not a

risk factor for vascular disease. However, it is believed that in some cases hyperhomocysteinemia might arise as a result of reduced expression of the CBS mRNA, in this case it is the promoter/regulatory regions of the gene that are of importance and, to date, have remained largely unanalysed (Kraus *et al*, 1998). The human CBS locus contains an unusually high number of Alu repeats, which may predispose the gene to deleterious rearrangements, which could lead to either impaired expression of CBS as seen in hyperhomocysteinemia or total loss as seen in homocystinuria (Kraus *et al*, 1998). The entire coding sequence has recently been deciphered and it is hoped that this will allow greater analysis of the untranscribed/untranslated regions of the gene (Kraus *et al*, 1998). A variant allele, a 68 base-pair insert at position 844 of the nucleotide sequence (844ins68), has been recently described (Sperandeo *et al*, 1996). A significant proportion of the populations studied (American and European) appear to be heterozygote carriers for this mutation (11.7% and 3.7% respectively) (Tsai *et al*, 1996b and Sperandeo *et al*, 1996). In addition, this insert sometimes contains a T-C mutation at position 833, which results in the conversion of an isoleucine to a threonine (I278T). It is thought that this 844ins68 mutant is a benign mutant. It is postulated that it (along with the I278T mutation if present) is not expressed, as it may be degraded in the nucleus and/or alternative splicing of the CBS mRNA results in the production of functional CBS protein, with CBS activity similar to normal controls (Sperandeo *et al*, 1996 and Tsai *et al*, 1996b). Thus the I278T mutation is pathogenic only when it occurs without the 844ins68. A significant association between the I278T mutation and premature occlusive arterial disease has been demonstrated (Orendac *et al*, 1998), highlighting the need for further investigation of this mutation.

1.4.2.2. Nutritional deficiencies in hyperhomocysteinemia.

As discussed earlier, deficiencies in the co-factors (methyl-cobalamin and pyridoxal 5'-phosphate) and co-substrate (methyltetrahydrofolate) of homocysteine metabolism may lead to homocysteine accumulation.

Selhub *et al*, studied plasma homocysteine levels and the vitamins involved in homocysteine metabolism in 1160 elderly subjects (67-96yrs), from the original Framingham Heart Study cohort (Selhub *et al*, 1993). They found that plasma homocysteine levels correlated negatively with plasma folate, B₁₂ and B₆, and with dietary intakes of both folate and B₆ but not B₁₂. Homocysteine levels also were found to increase with age. They estimated that more than 60% of hyperhomocysteinemia cases in the cohort were associated with low or moderate levels of one or more of the vitamins. Another important finding of the study was that even those reaching the RDA for folate had higher homocysteine than those whose folate intake was higher than the RDA. This suggests that the RDA is too low, at least for elderly subjects, and for other groups such as pregnant or nursing mothers, those on high protein diets and women on the oral contraceptive pill. These findings are consistent with those of Naurath *et al*, who found that vitamin supplementation (B₆, B₁₂ and folate) in an elderly study group helped reduce homocysteine (and other metabolites of enzymatic reactions involving the B group vitamins), even in those who had 'normal' levels of these vitamins before treatment (Naurath *et al*, 1995).

Sub-optimal folate status is one of the most commonly cited reasons for elevated plasma homocysteine (de Jong *et al*, 1999). Folic acid supplementation has been shown to lower plasma homocysteine, even in low doses (200µg/day) (Ward *et al*, 1997). However, factors such as initial homocysteine concentration, vitamin levels, multivitamin use, and polymorphisms in the MTHFR gene may contribute to variability in reduction. Folic acid supplementations have demonstrated the greatest effect when

initial homocysteine levels were high and initial folate levels were low (Malinow *et al*, 1997).

The dietary reference values for folate, as for other nutrients, are targeted to the general and supposedly normal population. It has been argued that in light of the high incidence of the TT genotype and its relationship to homocysteine, an established risk factor for vascular disease and neural tube defects, that the RDA for folate should be increased to accommodate this significant proportion of the Irish population (Molloy *et al*, 1997 and Shields *et al*, 1999). A recent study has found that low serum folate levels are associated with an increased risk of fatal coronary heart disease (Morrison *et al*, 1996). It is for these reasons that folate fortification of grain products (140µg/100g) was introduced in 1996 in the US. Two years after the initiation of this program it was found in a population-based sample, that plasma folate had increased by 117%, the prevalence of low serum folate had decreased by 92%, fasting homocysteine levels had fallen by 7% and the prevalence of high fasting plasma homocysteine (>13µM) was reduced by approximately 50%, thus demonstrating the effectiveness of the fortification. Greater differences were seen in those who also took multivitamin supplements that contained folic acid. Statistical analysis suggests that these differences may be a result of differing B₆ and B₁₂ status (Jacques *et al*, 1999).

Of the B group vitamins, folate appears to be the most effective at lowering plasma homocysteine in those with elevated and even normal homocysteine concentrations (den Heijer *et al*, 1998). However, there are several reasons for including both pyridoxal-phosphate and cobalamin in the treatment of hyperhomocysteinemia. This combination may have a stronger effect in subjects with low cobalamin or pyridoxine levels. Additionally, folate administration may mask vitamin B₁₂ deficiency, leading to further complications.

A normal fasting homocysteine level is not always synonymous with normal homocysteine metabolism, as a subset of patients may have normal fasting plasma homocysteine but then demonstrate an abnormal methionine load test (Miller *et al*, 1992). Methionine loading is a way of stressing the methionine metabolic pathway and may be more sensitive than measurement of fasting homocysteine levels for detecting mild disturbances in the transsulphuration pathway due to mild genetic lesions or B₆ deficiency (Wilcken & Wilcken, 1976). One study has estimated that more than 40% of subjects with clinically relevant hyperhomocysteinemia can be detected only by a methionine-loading test (Bostom *et al*, 1995b).

Ubbink *et al* found that although fasting homocysteine levels were normal in a B₆-deficient group of asthma patients, they showed an abnormal post-methionine load response, with significantly higher increases in both homocysteine and cystathionine, suggestive of impaired transsulphuration, in particular impaired activity of γ -cystathionase. Pyridoxal phosphate supplementation has been found to reduce the post-methionine load increase of both homocysteine and cystathionine, thus demonstrating the importance of B₆ in utilisation of homocysteine following an oral methionine load (Franken *et al*, 1994 and Ubbink *et al*, 1996). This is of particular importance following the results of a recent prospective study that reported that CHD risk was strongly inversely associated with vitamin B₆ levels (Folsom *et al*, 1998).

The effects of homocysteine-lowering vitamin supplementation on markers of subclinical atherosclerosis have provided some promising results (Vermeulen *et al*, 2000). However, to date, no large randomised clinical trials have evaluated the effect of lowered plasma homocysteine on cardiovascular events. Thus it remains to be seen if lowering homocysteine by increased folate/pyridoxine/cobalamin intake can improve homocysteine-related cardiovascular events and mortality rates.

1.4.2.3. Other causes of hyperhomocysteinemia.

Use of certain drugs which antagonise folate/B₁₂/B₆ metabolism may lead to hyperhomocysteinemia (*eg* theophylline, a vitamin B₆ antagonist used by asthmatics) (Ubbink *et al*, 1996). Recent experimental studies in humans and rats suggest that the kidneys play a major role in homocysteine metabolism, accounting for approximately 70% of homocysteine elimination from plasma (Bostom *et al*, 1995a). Elevated plasma homocysteine is a common finding in end stage renal disease, and to a lesser extent in dialysed patients or those who have received kidney transplants (Friedman & Dwyer 1995 and Bostom *et al*, 1997). Both the type of dialysis treatment and the use of vitamin supplementation are thought to be important in the regulation of plasma homocysteine levels. These levels correlate positively with serum creatinine, an indication that reduced excretion of homocysteine is at fault (Bostom *et al*, 1996 and Herrmann *et al*, 1998). Hyperhomocysteinemia therefore represents an additional reversible cardiovascular risk factor in this group.

Hyperhomocysteinemia is a common finding in the elderly. This is probably as a consequence of reduced vitamin status due both to reduced intake and absorption (Selhub *et al*, 1993). Generally, irrespective of age it has been found that homocysteine levels are higher in men than in women, and are higher in postmenopausal women compared to premenopausal women (Masser *et al*, 1994 and Selhub *et al*, 1999).

1.5. Epidemiological studies relating homocysteine to vascular disease.

Over the past decade, a consensus has emerged that elevated plasma homocysteine is associated with an increased risk for premature atherosclerotic disease in the coronary, cerebrovascular and peripheral vessels, independent of other classical risk factors, such as hypercholesterolemia, hypertension and smoking (Boers *et al*, 1985; Malinow *et al*, 1989; Clarke *et al*, 1991; den Heijer *et al*, 1996 and Malinow, 1996). Between 13% and

47% of patients with vascular disease have been reported to have hyperhomocysteinemia (Genest *et al*, 1991). This relationship between homocysteine and vascular disease appears to be graded and linear and holds for both fasting plasma homocysteine and post-methionine load homocysteine (Verhoef *et al*, 1997a and b). Homocysteine may also relate to the severity of atherosclerotic disease (van der Berg *et al*, 1996 and Chao *et al*, 1999).

With few exceptions, most retrospective and cross-sectional epidemiological studies have shown a positive correlation between plasma homocysteine and coronary artery disease (Wu *et al*, 1994 and Hopkins *et al*, 1995).

Boushey *et al*, carried out a meta-analysis in 1995 on 27 studies relating homocysteine and atherosclerotic vascular disease risk. The analysis revealed that homocysteine acted as graded risk factor and that for every 5 μ M increase in plasma homocysteine, men had a 1.6 fold increase in CHD risk, while women had a 1.8 fold increase - the equivalent risk that is associated with a 0.5mM increase in plasma cholesterol. This also applied to stroke and peripheral vascular disease. The study estimated that about 10% of vascular disease cases could be attributed to hyperhomocysteinemia (Boushey *et al*, 1995). Since this publication, additional retrospective (Verhoef *et al*, 1997a and de Jong *et al*, 1999) and prospective (Nygård *et al*, 1997) studies have been carried out that support these findings. The recent European Concerted Action project which involved 750 vascular disease patients and 800 controls, confirmed homocysteine as an independent graded risk factor for vascular disease and suggested that an elevated homocysteine level interacted with conventional risk factors to further increase vascular disease risk (Graham *et al*, 1997).

Selhub *et al*, studied the relationship between plasma homocysteine and the B vitamin group and the occurrence of carotid artery stenosis in the elderly. Carotid artery stenosis/atherosclerosis is used as a predictor of coronary and cerebrovascular disease

(which may lead to myocardial infarction or stroke). They found that elevated homocysteine and sub-optimal folate and B₆ were positively correlated to increased risk of stenosis (>25%) (Selhub *et al*, 1995). These results are in accordance with several prospective and retrospective studies relating homocysteine to carotid artery wall thickening and stroke (Brattström *et al*, 1984; Brattström *et al*, 1992; Clarke *et al*, 1992; Malinow *et al*, 1993; Stehouwer *et al*, 1998; Bostom *et al*, 1999 and McQuillan *et al*, 1999).

Vascular thromboembolisms account for approximately 50% of the vascular events in homocystinuria. Studies relating hyperhomocysteinemia to incidence of both recurrent venous thrombosis and deep vein thrombosis have provided conflicting results. Den Heijer *et al* reported a positive association between homocysteine and deep vein thrombosis (den Heijer *et al*, 1995 and 1996). Amundsen *et al*, failed to confirm these findings. However, this is due possibly to the small number of patients included in the study (Amundsen *et al*, 1995).

In contrast to findings of cross-sectional and case-control retrospective studies relating homocysteine to vascular disease, results from prospective studies have been less consistent. Eight prospective studies, on European and American populations, found a statistically significant association between homocysteine and cardiovascular disease, while six other reports did not confirm these findings (reviewed by Eikelboom *et al*, 1999). Four of the latter reports however did find a trend towards cardiovascular risk with high homocysteine. It is possible that the use of less extreme end-points contributed to the lack of statistical significance.

Other reasons for weaker associations in prospective studies may be the deterioration of homocysteine in the plasma sample over time, the variation in subjects over time or the chosen end-point. Evans *et al*, for example, reported no association between plasma homocysteine and the incidence of myocardial infarction in their prospective study.

However, some of the serum samples used for homocysteine analysis were up to twenty years old. It is quite possible that loss of homocysteine had occurred over this time (Evans *et al*, 1997). Retrospective studies relating cardiovascular disease to homocysteine may be complicated by the fact that elevations in plasma homocysteine have been observed following both MI and stroke (Lindgren *et al*, 1995 and Egerton *et al*, 1996).

Eikelboom and colleagues have recently reviewed the epidemiological evidence relating homocysteine and cardiovascular disease risk (Eikelboom *et al*, 1999). These authors note that the heterogeneity of the available studies makes meta-analysis problematic. However, they conclude that the association between homocysteine and cardiovascular disease is consistent, strong, and biologically plausible, while the data from prospective studies is weaker with some conflicting results. Despite the conflicting evidence of prospective and retrospective studies and despite the lack of definitive proof regarding the mechanism of homocysteine toxicity, it may nonetheless be regarded as a risk factor for vascular disease for several reasons. First, untreated homocystinuric children develop atherosclerosis and thrombosis (Mudd *et al*, 1995a). Second, in adults with hyperhomocysteinemia, treatment to lower homocysteine levels appears to decrease the rate of progression of carotid plaque formation (Petersen & Spence, 1998). Third, methionine load-induced hyperhomocysteinemia in young healthy adults reduces flow-mediated brachial artery reactivity and finally, dietary-induced hyperhomocysteinemia has been shown to induce abnormal vascular reactivity in animal studies (Bellamy *et al*, 1998 and Lentz *et al*, 1996). These observations provide the rationale for a randomised, controlled trial of the effect of homocysteine-lowering vitamin therapy on vascular disease.

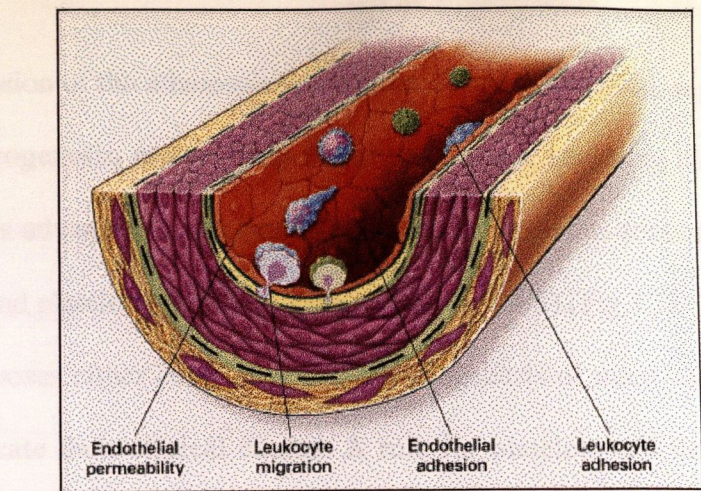
Elevated plasma cysteine has recently been proposed as a risk factor for vascular disease (Jacob *et al*, 1999). Though less reactive than homocysteine, it does share many

of the cytotoxic properties of homocysteine. It is toxic to human arterial endothelial cells and may contribute to oxidative stress, formation of oxidised LDL and therefore foam cell formation (Jacob *et al*, 1999). Elevations in cysteine have not been observed in the presence of sub-optimal vitamin status but may occur in parallel with an elevated homocysteine (Collins, this laboratory, unpublished observation). It is therefore possible that cysteine is simply a marker for hyperhomocysteinemia but is not the noxious agent in hyperhomocysteinemia-associated vascular disease.

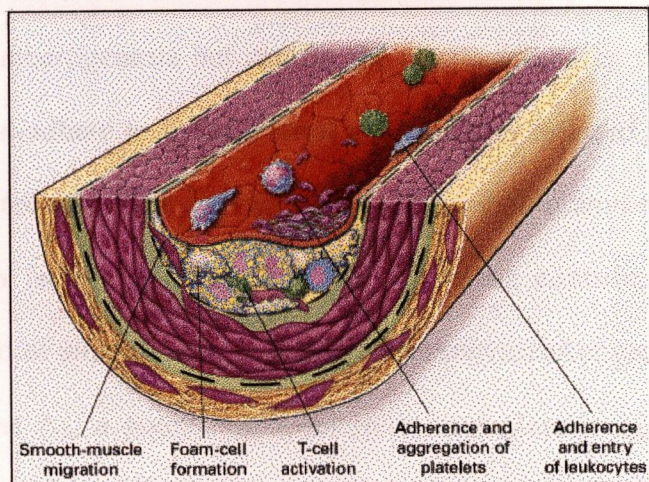
1.6. Homocysteine theory of atherosclerosis.

Atherosclerosis literally refers to a "thickening and hardening of the arteries". It results in coronary heart disease, cerebrovascular disease (stroke) and peripheral vascular disease. Atherosclerotic lesions result from an excessive pro-inflammatory response to different forms of injury (Ross, 1993). Injury to the endothelium results in an increase in leukocyte and platelet adhesion, an increase in endothelium permeability and induction of a number of cytokines and growth factors. If this inflammatory response does not remove the injurious agent, it can continue indefinitely, leading to atherosclerosis (Fig.1.4, panel A).

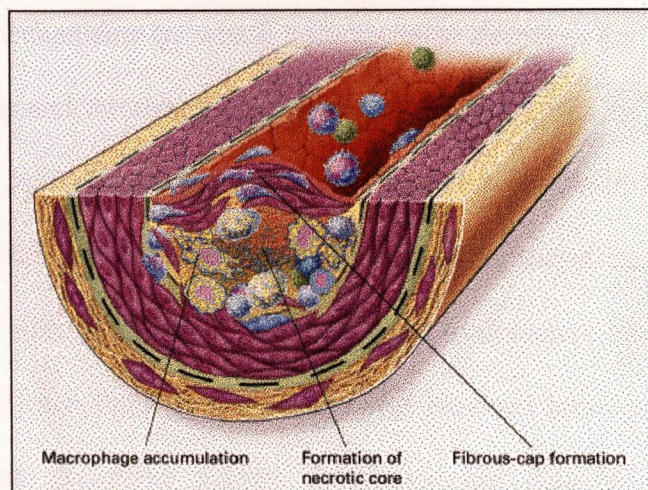
The earliest recognisable lesion of atherosclerosis is the 'fatty streak'. It is initially composed of an intimal collection of lipid filled monocyte-derived macrophages and a number of T-cells (Fig.1.4, panel B). Later, these are joined by smooth muscle cells and surrounded by a connective tissue matrix of collagen fibrils, elastic fibres and proteoglycans, to form the intermediate lesion (Ross, 1999). Monocytes/macrophages and T-cells are the primary inflammatory cells present in the atherosclerotic lesion. Continued inflammation results in their recruitment to the site and activation there. Activated macrophages release a host of enzymes *eg* matrix metalloproteinases (MMP), cytokines *eg* TNF α , and IL -1 and growth factors *eg* PDGF, which further the



A



B



C

Fig. 1.4 The atherosclerotic process.

A: Endothelial injury, the first step in atherosclerosis.

B: Formation of the 'fatty streak'.

C: The advanced atherosclerotic lesion.

See section 1.6 for a more detailed description (Taken from Ross, 1999).

progression of the atherosclerotic lesion. A complex lesion, representing the final stage of atherogenesis, projects into the arterial lumen, impeding blood flow (Fig. 1.4, panel C). This advanced lesion provides a site for thrombus formation and accumulation of fibrin and platelets (Breslow, 1996 and Ross, 1999) (Fig 1.5).

Thromboses either contribute to the pathogenesis of many cardiovascular disorders or complicate their clinical course. A role for platelets and the coagulation proteins in atherosclerosis has now been established (Ross, 1999). These play a role not only in the process of atherogenesis but also in the events following plaque rupture (Fig. 1.5). Platelets can adhere to dysfunctional endothelium, exposed collagen or macrophages (Fig 1.6, panel A). When activated, they release their granules which contain cytokines and growth factors that together with thrombin contribute to the migration and proliferation of smooth muscle cells and monocytes. Activated platelets also produce thromboxane A₂, a potent vasoconstrictor and platelet aggregation substance and leukotrienes that can amplify the inflammatory process (Fig 1.6, panel B).

The exact mechanisms by which homocysteine damages the vessel wall and supports atherogenesis and thrombosis are unknown. Kilmer McCully was the first to suggest a relationship between homocysteine and vascular disease following the post-mortem examination of vascular lesions in patients with homocystinuria due to CBS deficiency and patients with cobalamin C deficiency due to defective methionine synthase. As the only link between these two disorders was elevated homocysteine, a theory was put forward that excessive plasma homocysteine was in some way a cause of atherosclerosis (McCully, 1969).

Induction of hyperhomocysteinemia in baboons (60-190 μ M) by continuous intravenous administration of homocysteine coincided with patchy endothelial cell injury and proliferation of smooth muscle cells resulting in the formation of lesions similar to early atherosclerotic lesions found in man (Harker *et al*, 1983). These observations are

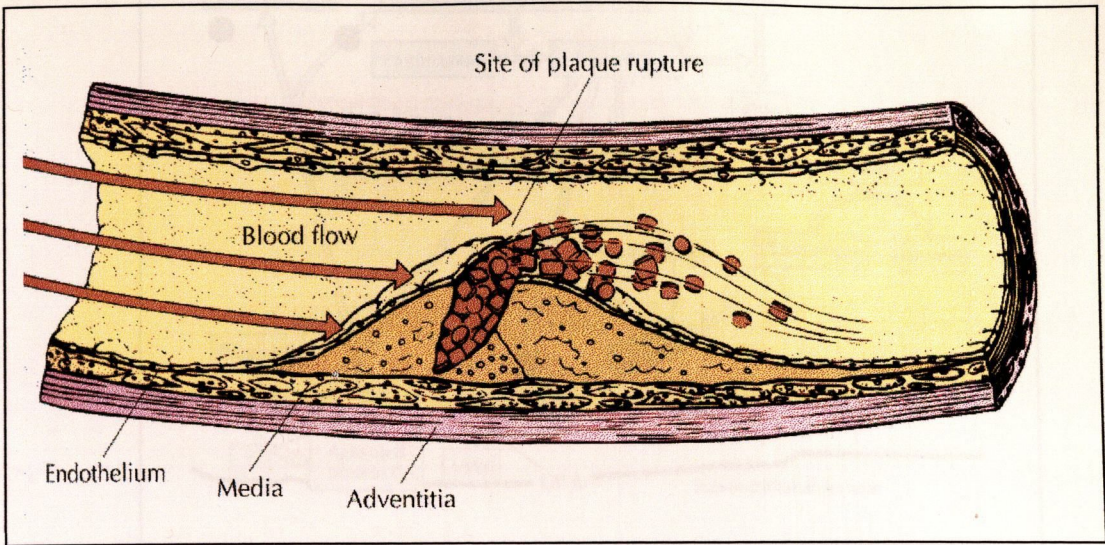


Fig. 1.5 Diagram of arterial thrombus responsible for acute myocardial infarction (MI).

Platelet adhesion and aggregation occur at the site of plaque rupture. Activated platelets exert pro-coagulant effects and the soluble coagulation cascade is activated.

(Taken from Califf, 1997).

consistent with those found in other animal models (McCully & Ragsdale, 1970 and Matthias *et al*, 1996).

1.6.1. Homocysteine and endothelial cell function.

Endothelial injury is an early event in atherogenesis. There is increasing evidence that homocysteine may affect the coagulation system and the resistance of the endothelium to thrombosis. Impairment of flow-mediated vasodilation in the presence of elevated homocysteine has been observed in a number of studies and suggests a decrease in bioavailability of nitric oxide (NO or endothelium-derived relaxing factor) (Tawakol *et al*, 1997; Bellamy *et al*, 1998; Chambers *et al*, 1998 and Chao *et al*, 2000). NO bioavailability is a result of both NO production by NO synthases and NO oxidative degradation by hydrogen peroxide and superoxide radicals (Verhaar *et al*, 1998). It is believed that the adverse effects of homocysteine on endothelial cells are mediated through oxidative stress mechanisms, most notably *via* hydrogen peroxide, formed by the oxidation of homocysteine's sulfhydryl group. Hydrogen peroxide accumulation could be exacerbated by a reduced glutathione peroxidase activity (which has been observed in homocysteine-treated bovine aortic endothelial cells) (Upchurch *et al*, 1997a and b). The net result would be an increase in hydrogen peroxide which reacts with and inactivates nitric oxide, leading to vasoconstriction, platelet aggregation and monocyte adhesion, all of which may lead to atherogenesis and/or thrombosis (Stamler *et al*, 1993). Supporting this hypothesis is the observation that homocysteine-induced impairment of vasodilation may be reversed by antioxidant treatment *eg* vitamin C (Chambers *et al*, 1999). Furthermore, genes shown to be downregulated in cultured endothelial cells by homocysteine include the antioxidant enzymes superoxide dismutase and glutathione peroxidase (Outinen *et al*, 1999).

Lee and Wang have proposed that homocysteine at physiological concentrations may induce endothelial dysfunction by inhibiting endothelial regeneration. Homocysteine at 10-50 μ M concentrations but not cysteine, inhibits endothelial cell DNA synthesis and growth. This is associated with hypomethylation of signalling factors important in cellular proliferation (Lee & Wang, 1999). Increased expression of factors associated with growth arrest, has been reported in endothelial cells exposed to high concentrations of homocysteine (5mM) (Outinen *et al*, 1999). Furthermore, endothelial cell damage may cause increased uptake of modified LDL cholesterol in the vascular wall. An earlier suggested atherogenic mechanism involves inhibition of endothelial cell growth and stimulation of proliferation of smooth muscle cells by homocysteine, leading to thickening of the artery wall (Tsai *et al*, 1994).

Using differential display and cDNA microarrays, molecular biologists have identified several endothelial cell genes whose expression is altered upon homocysteine exposure. Kokame *et al* identified an increase a stress protein associated with the presence of misfolded proteins within the cell (Kokame *et al*, 1996). Consistent with these studies, Outinen *et al* found that homocysteine alters expression of several genes known to be sensitive to ER stress (Outinen *et al*, 1999). This substantiates the theory of Lentz and Sadler, who proposed that aberrant processing and secretion of von Willebrand factor in the presence of high homocysteine is a result of altered redox potential, affecting proper disulphide bond formation in certain proteins, with subsequent retention of that protein in the endoplasmic reticulum (ER) (Lentz & Sadler, 1993).

1.6.2. Homocysteine and smooth muscle cells.

Proliferation of vascular smooth muscle cells (VSMC) is a hallmark of atherosclerosis and has been observed in an animal model and in cultured rat and human VSMC, in response to homocysteine (Harker *et al*, 1983; Tsai *et al*, 1994 and Chen *et al*, 2000).

This appears to occur *via* activation of cyclin A and D1, important regulators of the cell cycle (Tsai *et al*, 1996a). Furthermore, this upregulation is enhanced in the presence of serum, suggesting a synergistic interaction of homocysteine with an unidentified growth factor, possibly platelet derived growth factor β (PDGF β) (Tsai *et al*, 1996a). In addition, homocysteine-treated VSMC have demonstrated enhanced production and accumulation of collagen (Majors *et al*, 1997).

1.6.3 Homocysteine and oxidation.

Enhanced free radical activity, including enhanced lipid peroxidation is considered to play a major role in the pathogenesis of atherosclerosis. Lipid peroxides are not only toxic to cell membranes but are a key component in the formation of foam cells in the early stages of atherosclerosis. Autooxidation of thiols, in the presence of free transition metals, leads to the production of a wide range of reactive oxygen species (ROS), such as H_2O_2 , $OH^\cdot$, and superoxide ($O_2^\cdot$), which due to their high reactivity may be detrimental to enzymes and other biological compounds and also induce lipid peroxidation (Fig. 1.7) (Heinecke *et al*, 1987 and 1993 and Halvorsen *et al*, 1996). Enhanced lipid peroxidation has been observed *in vivo* in hyperhomocysteinemic pigs (Young *et al*, 1997). Similar findings have been reported in men, where plasma homocysteine was positively correlated with *in vivo* lipid peroxidation (Voutilainen *et al*, 1999). Furthermore, an increase in the activity of plasma anti-oxidant enzymes has been reported in patients with an elevated plasma homocysteine, supporting the theory that homocysteine represents an oxidative stress (Wang *et al*, 1999 and Moat *et al*, 2000). Earlier suggestions of such a mechanism have been inferred from the findings that atherosclerosis could be induced in rabbits fed a high-methionine diet. This coincided with a significant increase in plasma and aortic TBARS (an index of lipid peroxidation) and increase in aortic antioxidant enzyme activity (Toborek *et al*, 1995).

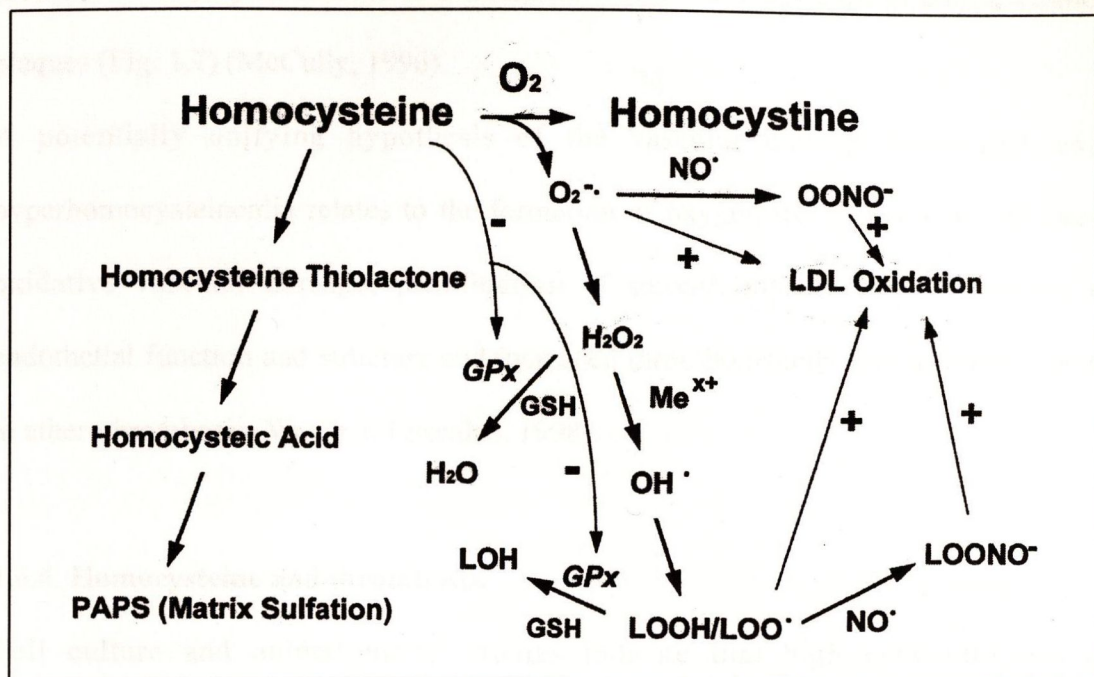


Fig. 1.7 Oxidative cytotoxic mechanisms of homocysteine.

Abbreviations:

$O_2^{\bullet -}$: superoxide anion radical, $NO^{\bullet}$: Nitric oxide, $OONO^{\bullet -}$: peroxynitrite,

H_2O_2 : Hydrogen peroxide, LOH: lipid alcohol, LOOH: Lipid peroxide,

LOO $^{\bullet}$: lipid peroxy radical, LOONO $^{\bullet -}$: lipid peroxynitrite,

$OH^{\bullet}$: Hydroxyl radical, GSH: Reduced glutathione, GPx : Glutathione peroxidase,

PAPS : Phosphoadenosine phosphosulphate.

(Taken from Welch *et al*, 1996).

In addition, the highly reactive anhydrous form of homocysteine, homocysteine thiolactone, may be oxidised and converted to phosphoadenosine phosphosulphate (PAPS), the coenzyme that forms the sulphated glycosaminoglycans of arteriosclerotic plaques (Fig. 1.7) (McCully, 1996).

A potentially unifying hypothesis of the vascular damage associated with hyperhomocysteinemia relates to the formation of oxygen free radicals, which cause oxidative vascular damage, proliferation of smooth muscle cells, alteration in endothelial function and structure and increased thrombogenicity that ultimately leads to atherothrombosis (Welch & Loscalzo, 1998).

1.6.4 Homocysteine and thrombosis.

Cell culture and animal model studies indicate that high concentrations of homocysteine alter the normally anti-thrombogenic nature of the vascular endothelium. High homocysteine has been found to: inhibit thrombomodulin activity and protein C activation (Hayashi *et al*, 1992 and Lentz *et al*, 1996), inhibit von Willebrand factor processing and secretion (Lentz & Sadler, 1993), reduce cellular binding of t-PA via annexin II (Hajjar, 1993 and 1998) and induce tissue factor, Factor V and prothrombin activity (Rodgers & Kane, 1986 and Fryer *et al*, 1993). Furthermore, homocysteine appears to increase the affinity of the atherogenic apolipoprotein (a), (Lp(a)) for fibrin. Such a complex may inhibit plasmin and favour thrombosis (Harpel *et al*, 1992). The net result of these processes is the conversion of the endothelium from an anti-thrombotic to a pro-thrombotic state (Fig. 1.6, panel A). It should be noted however that many of these observations were made in the presence of supra-physiological homocysteine concentrations (1-10mM) and may therefore not relate well to the *in vivo* situation.

Al-Obaidi *et al* have recently reported an association between plasma homocysteine, Factor VIIa and thrombin generation, supporting a role of homocysteine in enhancing thrombosis (Al-Obaidi *et al*, 2000). Induction of hyperhomocysteinemia by methionine loading or folate-deficient diet in rats has been associated with an enhancement of platelet aggregation, an increase in thromboxane synthesis and an increase in macrophage tissue factor activity (Durand *et al*, 1996b and 1997a). A decrease in the "protective" (n-3) polyunsaturated fatty acids and an increase in lipid peroxidation products was also observed in these models and suggests that the thrombogenicity of homocysteine may be due to oxidative stress. It is proposed that oxidation of homocysteine may lead to the generation of free-radicals that can alter arachidonic acid metabolism (central to platelet activation) and therefore promote platelet hyperactivity (Durand *et al*, 1996a and 1997b).

A reduction in platelet survival, which was reciprocated by a three-fold increase in platelet turnover, was observed in hyperhomocysteinemic baboons (Harker *et al*, 1983). These findings are in agreement with a similar study of platelet function in four untreated human homocystinuric patients (Harker *et al*, 1974). No significant difference was observed in platelet aggregation in either the primate model or the patients. The homocystinuric patients demonstrated a slight decrease in fibrinogen survival, which was not observed in the primate model (Harker *et al*, 1974 and 1983).

1.7. Tumour Necrosis Factor.

Tumour necrosis factor (TNF) describes a family of cytokines, composed of two members, namely TNF α and β , which share approximately 30% amino acid identity. The name itself is somewhat misleading as, although these factors can induce regression of some tumours, it is not their only action.

Tumour necrosis factor (TNF α) or cachexin was first described in 1975 as an agent that caused haemorrhagic necrosis of mouse tumours (Carswell *et al*, 1975 and Beutler *et al*, 1985). Extensive studies on this cytokine have since revealed that a multitude of processes, particularly inflammatory processes, are regulated by this factor.

Human TNF α is a non-glycosylated protein, with a monomeric MW of 17.5kDa. The biologically active form exists as a compact trimer composed of three identical subunits of 157 amino acids (aa) (Jones *et al*, 1989). Like many other cytokines, each subunit is processed from a larger precursor (233aa) which exists as an integral membrane protein.

TNF β or lymphotoxin, first identified in 1968 as a cytotoxic factor produced by lymphocytes (predominantly activated B and T cells), is the second member of the tumour necrosis factor family of cytokines (Wong & Goeddel, 1989 and Steffen *et al*, 1998). Human TNF β is a glycoprotein with a monomeric MW of 25kDa. Its primary activity is enhancement of the phagocytic activity of both macrophages and neutrophils.

1.7.1. TNF α production.

TNF α was originally thought to be exclusively produced by activated monocytes/macrophages. However, it is now clear that while in certain cases, monocytes/macrophages remain the predominant source of TNF α , they are not the sole source of this cytokine.

TNF α is not constitutively produced, but may be induced by a number of stimuli including phorbol esters, endotoxins and cytokines. Several agents are known to downregulate TNF α production, *eg* glucocorticoid hormones, protein kinase C inhibitors and agents that increase levels of cAMP (Wong & Goeddel, 1989).

TNF α gene expression is complex, being controlled at the transcriptional, post-transcriptional and translational levels. The signal transduction pathways mediating

production of TNF α appear to differ depending on the stimuli and cell type involved. PMA-induced TNF α production by monocytes/macrophages involves activation of protein kinase C and subsequent formation of AP-1/c-jun heterodimers, which activate transcription (Rhoades *et al*, 1992), while induction by lipoprotein lipase (LPL) involves the NF- κ B family of transcription factors. AP-1 sites occur in the promoter region of several other cytokines, suggesting a role for this transcription factor in the coordinate control of cytokine expression. Both AP-1 and NF- κ B are sensitive to redox-status and it is therefore possible that homocysteine, as a source of oxidant stress, may regulate TNF α expression.

The TNF α promoter contains sequences that permit augmented gene transcription, in response to various stimuli. Conversely, the 3' untranslated region of the gene contains sequences that repress translation. Induction of TNF α expression by lipopolysaccharide (LPS) in the macrophage cell line RAW 264.7 involves both activation of the promoter and 'de-repression' of the 3' region of the gene (Han *et al*, 1991).

Endotoxin/(LPS), a cell wall component of gram-negative bacteria, is one of most potent inducers of macrophage TNF α expression. It upregulates transcription of the TNF α gene and augments the stability of its mRNA (Renier *et al*, 1994). Several autocrine and paracrine stimuli are responsible for TNF α induction in monocytes/macrophages. The primary physiological inducer is interferon gamma (IFN γ), produced by activated T lymphocytes (Collart *et al*, 1986). Other cytokines such as IL-1 and IL-2, and TNF α itself, are also involved in the autocrine production of TNF α in macrophages. (Wong & Goeddel, 1989 and Renier *et al*, 1994).

Lipoprotein lipase (LPL), a key enzyme in lipoprotein metabolism, is found predominantly in tissues that have a high demand for free fatty acids but is also expressed by macrophages in atherosclerotic lesions (Yla-Herttuala *et al*, 1991). It is hypothesised that it may be pro-atherogenic, playing a role in foam cell formation.

Although constitutively produced by macrophages, it may be down-regulated by TNF α (eg in cachexia) and also by LPS (White *et al*, 1988). Conversely it appears to promote TNF α gene expression by increasing both transcription and mRNA stability in mouse peritoneal macrophages - a promotion that is further enhanced by the presence of IFN γ (Renier *et al*, 1994). Low levels of oxidised LDL have also been shown to induce TNF α release by cultured human monocytes/macrophages (Jovinge *et al*, 1996). The investigators postulated that LDL oxidation resulted in the formation of ROS, that could activate AP-1 and therefore increase TNF α expression. It is possible that homocysteine, as a source of oxidant stress, may have similar effects on gene transcription.

Peripheral blood T cells (both CD4⁺ and CD8⁺) can produce TNF α and β . As is the case with monocytes, this production is not constitutive but is a response to various signals such as phorbol myristic acid (PMA), calcium ionophores, and IL-2 (Sung *et al*, 1988 and Steffen *et al*, 1998).

Vascular smooth muscle cells (SMC), particularly medial SMC have the capacity to produce TNF α in response to injury (Jovinge *et al*, 1997). Recent studies of porcine coronary arteries (both *in vivo* and in culture) have demonstrated that this production is regulated by at least two independent pathways, namely the activation of NF- κ B and the inhibition of cAMP synthesis (Newman *et al*, 1998). These mechanisms of regulation are also found in operation in human monocytes and endothelial cells (Ollivier *et al*, 1996).

1.7.3. TNF receptors.

TNF α has widespread activity due to the prevalence of its receptors on a variety of cell types. Immune/inflammatory cells such as monocytes/macrophages, lymphocytes,

and neutrophils and non-immune cells such as fibroblasts, endothelial cells and smooth muscle cells all express cell surface TNF receptors (Kunkel *et al*, 1991). Two receptors from the TNF receptor (TNFR) superfamily, designated p55 and p75 based on their molecular weights (or TNF-RI and TNF-II respectively) bind both TNF α and β with equally high affinity (Baker & Reddy, 1998). The p55 receptor is mainly expressed by epithelial cells, while myeloid cells express the p75 variant.

Binding requires the association of one ligand trimer (TNF α or β) with three receptor molecules (Loetscher *et al*, 1991). There is no significant homology between the intracellular domains of the two TNF receptors, suggesting different signalling mechanisms.

The cell surface expression of these receptors is regulated by a variety of agents, including cytokines (interferons, IL-1,2,4,6,8, TNF and GM-CSF), protein kinase C and A activators, steroids and Ca^{2+} ionophores. Signal transduction through the TNF receptors involves several intermediates including transcriptional factors, phospholipases, protein kinases, phosphatases and G-proteins (Aggarwal & Reddy, 1994).

1.7.4. Role of TNF in atherosclerosis.

As a mediator of inflammatory and immune responses, much study has focused on the role of TNF α in several specific disease states, including those of bacterial, viral and parasitic origin. Elevated TNF α levels are also associated with the pathogenesis of allograft rejection and rheumatoid arthritis (Kunkel *et al*, 1991). Additionally, a major role for TNF α in the pathogenesis of atherosclerosis has been proposed.

The production of cytokines by the cells of and surrounding the atherosclerotic plaque is important in the initiation and amplification of inflammation at these sites. TNF α appears to be of particular importance in atherosclerosis, due to its pro-

atherogenic/thrombotic properties. Plaques are composed of approximately 80% macrophages, 5-10% lymphocytes and to a lesser extent, mast cells and smooth muscle cells.

Within the atherosclerotic plaque, TNF α is produced by macrophages (including those transformed to foam cells), intimal smooth muscle cells, proliferating endothelial cells and in rupture-prone areas, mast cells (Barath *et al*, 1990a and 1990b; Kaartinen *et al*, 1996; and Falkenberg *et al*, 1998). Enhanced TNF α production by carotid plaque macrophages, compared to blood monocytes, has been demonstrated both *in vitro* and *in situ* (Tipping & Hancock, 1993). Similar findings have been reported in patients with ischaemic heart disease (Vaddi *et al*, 1994). Lei and Buja have demonstrated increased production of TNF α by macrophages in the later stages of atherosclerotic lesion development in Watanabe Heritable Hyperlipidemic rabbits (Lei & Buja, 1996).

Increased TNF α secretion at the site of the plaque, stimulated by recent infection, has been recently recognised as one of the factors which may precipitate a coronary event (Pesonen, 1994 and Kol *et al*, 1998). It does so by inducing factors that may destabilise the atherosclerotic plaque, allowing the formation of thromboses (Libby & Aikawa, 1998).

Elastase, a product of endothelial cells and smooth muscle cells, that regulates the components of connective tissue, is proposed to play a key role in atherogenesis. Aberrant activity of this enzyme can lead to changes in elastic fibres of the vessel wall, creating sites for lipid and calcium deposits. Cultured endothelial cells demonstrate increased elastase activity in response to TNF α and linoleic acid, a response that is attenuated by vitamin E, an antioxidant (Toborek & Hennig, 1993).

Vascular smooth muscle cells, one of the primary targets of TNF α in atherogenesis, are thought to play a crucial role in the pathogenesis of intimal thickening. Barath *et al*, have shown by immunohistochemistry that one of the primary targets of TNF α within

the plaque appears to be medial smooth muscle cells, while the intimal SMC are a significant source of this cytokine (Barath *et al*, 1990 a and b). TNF α can modulate the phenotype of *medial* SMC, by inducing several *intimal* SMC characteristics (Morisaki *et al*, 1993). This property of TNF α may be of importance in the pathogenesis of atherosclerosis, as intimal SMC are more pro-atherogenic than medial SMC. TNF α is thought to enhance vascular SMC migration by altering cytoskeletal structures, such as F-actin and vinculin. In addition, TNF α appears to increase expression of the transcription factor, ets-1, that is closely linked to the expression of many proteins involved in matrix degradation (a pre-requisite for migration of cells in tissue) (Jovinge *et al*, 1997).

In proliferating endothelial cells (EC), both *in vivo* and *in vitro*, TNF α may cause oxidative stress and increases in intracellular calcium, culminating in apoptosis (Toborek *et al*, 1997 and Spyridopoulos *et al*, 1998). These processes are amplified by the presence of linoleic acid, a product of hydrolysis of triglyceride-rich lipoproteins by LPL (which is elevated in atherosclerosis). This and other unsaturated fatty acids can disrupt endothelial cell integrity (Toborek *et al*, 1996 and 1997).

However, in non-proliferating EC, TNF α induces expression of a host of proteins effecting coagulation, adhesiveness, chemotaxis and cell viability. Overall, these changes convert the endothelium from an anti- to a pro- coagulant form. Increased expression of tissue factor-like molecule, a plasminogen activator inhibitor, platelet-activating factor (PAF) and von Willebrand factor and decreased expression of tissue-type plasminogen activator (tPA) and suppression of the protein C pathway are all observed in response to TNF α (Grau & Lau, 1993).

Other TNF α -inducible endothelial cell factors include heparin-binding epidermal growth factor-like growth factor (HB-EGF, a potent SMC mitogen), endothelial leukocyte adhesion molecule 1 (ELAM-1), vascular cell adhesion molecule (VCAM-1),

intercellular adhesion molecule (ICAM -1), monocyte chemotactic factor (MCF-1) and neutrophil chemotactic factor (NCF) (Dixit *et al*, 1990 and Yoshizumi *et al*, 1992). With the exception of HB-EGF, all of these factors promote pro-inflammatory endothelium-leukocyte interactions. ICAM-1 expression is not limited to endothelial cells but is also expressed by monocytes and T lymphocytes and may be upregulated by TNF α in neutrophils (Wang *et al*, 1997 and Ahmad *et al*, 1998).

1.8. Aims.

In summary, the exact interaction between genetic factors, in particular the MTHFR TT genotype and nutritional deficiencies that culminates in elevations in plasma homocysteine are incompletely understood and therefore require further investigation. Despite considerable epidemiological evidence identifying homocysteine as a risk factor for atherosclerosis and thrombosis, the precise molecular mechanisms underlying homocysteine-induced atherosclerosis and thrombosis are unclear. The majority of studies have focussed on the effects of homocysteine on endothelial cell and smooth muscle cell, with some studies on the effects of homocysteine and platelet activity. The relationship between homocysteine and macrophage function, especially in the context of atherosclerosis, has been all but ignored.

In light of these uncertainties, discussed in this introduction, particularly with regards to the precise cellular basis for hyperhomocysteinemia and the possible effects of homocysteine on macrophage and platelet function through oxidative stress mechanisms, the project forming the basis of this thesis set out to investigate two areas:

- 1) methionine metabolism in mildly hyperhomocysteinemic subjects
- 2) the mechanisms by which homocysteine might elicit some of its pro-atherogenic/thrombotic properties.

To study methionine metabolism, a method was devised to investigate the cellular basis for altered methionine metabolism in hyperhomocysteinemic subjects. In conjunction with this, the presence of the common TT polymorphism in the MTHFR gene was investigated to determine if there was any correlation between the presence of this polymorphism and cellular methionine metabolism.

To address the role of homocysteine as a pro-atherothrombotic agent, its affect on TNF α expression in cultured human monocyte-derived macrophages, at both the transcriptional and translational level was investigated.

In addition, the effects of homocysteine on platelet function, specifically platelet aggregation, were assessed.

Chapter 2

Materials and Methods

Chapter 2

Materials and methods.

2.1. MATERIALS SUPPLIERS.

Materials were obtained from the following suppliers:

Abbott Laboratories, Illinois, USA.

IMx[®] folate assay kit, homocysteine assay kit, vitamin B₁₂ assay kit.

Air Products, Dublin, Ireland.

N₂ (gas and liquid) and CO₂ (gas).

Amersham Pharmacia Biotech UK Ltd, Buckinghamshire, England.

[α -³²P] dCTP, Hybond-N+ positively-charged nylon membranes, L-[³⁵S]-labelled methionine, Thermo Sequenase fluorescent-labelled primer cycle sequencing kit, Deoxyadenosine 5'-triphosphate (dATP), Deoxycytidine 5'-triphosphate (dCTP), Deoxyguanosine 5'-triphosphate (dGTP), Deoxythymidine 5'-triphosphate (dTTP).

American Type Culture Collection, Manassas, Virginia, USA.

THP-1, U-937 monocytic cell lines.

BDH, Laboratory Supplies, Poole, Dorset, England.

Ammonium hydroxide, bromophenol-blue, chloroform, copper sulphate, ethanol, ethylenediaminetetra-acetic acid (EDTA), formic acid, isopropanol, methanol, β -mercaptoethanol, ninhydrin, perchloric acid, phenol, sodium acetate, sodium carbonate, sodium chloride, sodium citrate, sodium dihydrogen phosphate, sodium hydroxide,

sodium tartrate, sulphuric acid, NNN'N'-tetramethylethylenediamine (TEMED), Tween 20.

Becton Dickinson, Meylan, Cedex, France.

Lithium-heparin vacutainers, 23G hypodermic needles, Falcon six-well tissue culture dishes.

BioData Corp, Horsham, Pennsylvania, USA.

Siliconised flat-bottomed test-tubes, plastic-coated stir bars.

Biotech Laboratories Inc, Houston, Texas, USA.

UltraspecTM-II RNA isolation system.

Boehringer-Mannheim (Diagnostics and Biochemicals) Ltd, East Sussex, England.

Glycogen, *Hinf* I, *Hinf* I digestion buffer (50mM Tris-HCl, 100mM MgCl₂, 1mM dithioerythritol (DTT), (pH 7.5)), *Pvu* II, *Pvu* II digestion buffer (10mM Tris-HCl, 50mM NaCl, 10mM MgCl₂, 1mM DTT) 10X TAE buffer (0.4M Tris-acetate, 0.01 M EDTA), 10X TBE buffer (0.89M Tris, 0.89M Boric Acid, 0.02mM EDTA disodium salt, (pH 8.4)), 20X SSC (3M sodium chloride, 0.3M sodium citrate, (pH 7.0)).

Corning Costar, Bucks, England.

Plate sealers, 10ml sterile pipettes, cell scrapers.

Cruachem, Glasgow, Scotland.

Isolate II DNA isolation kit.

Genzyme Diagnostics, Kent, England.

Human TNF α DuoSeT™ ELISA kit.

Gibco BRL, Life Technologies Ltd, Paisley, Scotland.

Hanks balanced salts solution, RPMI 1640 cell culture medium, hepes, liquid antibiotic penicillin/streptomycin/amphotericin B, foetal calf serum (FCS), deoxynucleotide triphosphates (dNTPs), glutamine, random hexanucleotide primers.

Oxoid Ltd, Hampshire, England.

Fuji Film Co, Tokyo, Japan.

Fuji Medical X-ray film.

Pharmacia Biotech, Uppsala, Sweden.

LIP Equipment and Services, Galway, Ireland.

Liquid scintillation vials.

Polaroid Corp, Massachusetts, U.S.A.

Merck, Darmstadt, Germany.

Cellulose thin layer chromatography plates (20cm x 20cm).

Promega, Madison, WI, U.S.A.

Murex Diagnostics Ltd, Dartford, England.

Phytohaemagglutinin.

Nalge Nunc International, Denmark.

Nunc™ Maxisorp™ microtitre ELISA plates.

National Diagnostics, Atlanta, Georgia.

Ecoscint scintillation fluid.

Ecospin scintillation fluid.

Ecospin scintillation fluid.

New England Biolabs, Hertfordshire, England.

Bsr I, *Bsr* I digestion buffer (100mM NaCl, 50mM Tris-HCl, 10mM MgCl₂, 1mM DTT).

Oligonucleotide Synthesis Unit, Queens University, Belfast, N. Ireland.

CBS, MS and MTHFR primers.

Oxoid Ltd, Hampshire, England.

Tryptone, yeast extract, agar (bacteriological, No. 1)

Pharmacia Biotech, Uppsala, Sweden.

Ficoll-Paque, Cy5 labelled M13 universal primer.

Polaroid Corp, Massachusetts, USA.

Polaroid 665 camera film.

Promega, Madison, WI, USA.

Hae III, *Hae* III digestion buffer (6mM Tris-HCl, 6mM MgCl₂, 50mM NaCl, 1mM DTT), Reverse transcription kit, Taq DNA polymerase, 10X Taq polymerase buffer (100mM Tris-HCl, 500mMKCl, 1% (v/v) Triton[®] X-100), Wizard[®] PCR Preps DNA Purification System, Wizard[®] Plus SV Minipreps DNA Purification, Prime-a-gene[®] random prime labelling kit.

Randox Laboratories, Antrim, N. Ireland.

Lactate dehydrogenase (LDH) diagnostic kit (50mM phosphate buffer, 0.5mM pyruvate, 0.18mM NADH).

R & D Systems, Abingdon, England.

TXB₂ immunoassay kit.

Scotlab, Strathclyde, Scotland.

Easigel (6% (w/v) acrylamide, 0.3% (w/v) bis acrylamide, 7M urea, 1X TBE (ratio 19:1))

Sigma Chemical Company, Poole, Dorset, England.

Acridine orange, adenosine diphosphate (ADP), agarose, ammonium persulphate (APS), ampicillin, boric acid, bovine serum albumin, cystathionine, cysteine, diethyl pyrocarbonate (DEPC), ethidium bromide, ficoll, formaldehyde, formamide, glycerol, homocysteine, homocystine, homocysteine-sulfinic acid, human serum albumin, methionine, N, N dimethyl formamide (DMF), 3-[N-orpholino]propanesulfonic acid (MOPS), peroxidase, p-hydroxy phenylacetic acid, phorbol myristic acid (PMA), phosphate buffered saline (PBS) tablets, polyvinylpyrrolidone (PVP), S-adenosyl homocysteine, S-adenosyl methionine, salmon sperm DNA, Sephadex G-25, sodium bicarbonate, sodium dodecyl sulphate (SDS), 3, 3', 5, 5' -tetramethylbenzidine (TMB) one-step liquid substrate system, Tris.HCl, Triton-X-100, xylene cyanol.

Stratagene Cloning Systems, Cambridge, England.

PCR-Script™ Amp Cloning Kit, Isopropyl-1-thio-β-D-galactopyranoside (IPTG), 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-Gal).

Unigene, Oregon, USA.

Oregon green® fluorescently-labelled fibrinogen.

The following specialised equipment was also used:

Abbott IMx[®] Analyser.

Alf Express[™] Automated DNA Sequencer.

Biodata platelet aggregation profiler.

FACScan (Becton Dickinson).

Hybaid hybridisation oven.

Perkin Elmer Cetus DNA thermocycler.

PYE Unicam SP6-500 spectrophotometer.

Sysmex K-1000 haematology analyser.

Titertek Multiskan[®] MCC/340 plate reader.

Wallac 1214 Rackbeta liquid scintillation counter.

Vilber Lourmat densitometer (using Bio-1D analysis software).

2.2 METHODS.

2.2.1. Peripheral Blood Mononuclear Cell (PBMC) Isolation.

PBMC were isolated by the method of Boyum (1968).

Whole blood was collected by venepuncture into lithium-heparin vacutainers. Blood samples were centrifuged within 1hr at 3500rpm for 10mins to separate plasma from cells. The cells were mixed with an equal volume of HBSS and layered over Ficoll-Paque. Centrifugation at 3500rpm for 35mins resulted in sedimentation of the erythrocytes and granulocytes, while the PBMC were contained in the interphase ("buffy layer") between the upper and lower phases. The interphase was carefully removed and washed with HBSS and centrifuged at 3500rpm for 15mins to remove any platelet contamination. The resulting pellet was subsequently washed - once with HBSS

and once with cell culture medium (RPMI 1640/10%(v/v) FCS/1%(v/v) ps). The cells were then resuspended in medium and counted as described below.

2.2.2. Counting and assessing viability of cells.

A 20 μ l aliquot of a cell suspension was diluted 1:1 with acridine orange/ethidium bromide (1/50 dilution of following stock solution: 0.1%(w/v) ethidium bromide, 0.03%(w/v) acridine orange in 2%(v/v) ethanol). Cell numbers were estimated by microscopy using a Neubauer counter. Viable cells allow the entry of acridine orange, which displays a green fluorescence under ultraviolet light (510nm) but exclude ethidium bromide. Non-viable cells are permeable to both dyes but as ethidium bromide has a higher affinity for DNA than acridine orange, these cells fluoresce orange. Thus, the viability of cells may be assessed by identifying and counting the numbers of green and orange-stained cells.

2.2.3. Culture of U-937 and THP-1 human monocytic cell-lines.

The human monocytic cell lines U-937 and THP-1 were obtained from the American Type Culture Collection, Maryland, USA, as frozen stocks. The cells were rapidly thawed at 37°C and transferred to 75cm² culture flasks containing 90% (v/v) RPMI 1640 supplemented with 10% (v/v) foetal calf serum, 2mM glutamine and 100U/ml penicillin/streptomycin. The cells were cultured at 37°C in 95% (v/v) humidified air/5% (v/v) CO₂. Cell density was maintained between 1-10 x 10⁵ cells/ml (U-937) and 2-10 x 10⁵ cells/ml (THP 1).

2.2.4. Homocysteine Estimation.

Fasting blood samples were collected by venepuncture into lithium-heparin vacutainers. Blood samples were centrifuged within 1hr at 3500rpm for 10mins to separate plasma from cells. Plasma was removed and stored at -70°C, for not more than 3 months.

Plasma homocysteine concentrations were estimated by an Abbott IMx[®] analyser using a fluorescence polarisation immunoassay (Shipchandler & Moore, 1995). Homocysteine, mixed disulphides and protein-bound forms are reduced using dithiothreitol (DTT). Total homocysteine is then converted to SAH by SAH hydrolase and excess adenosine. SAH levels are determined by immunoassay and equated to total homocysteine concentrations.

2.2.5. Vitamin B₁₂ Estimation.

Fasting blood samples were collected by venepuncture into vacutainers containing no additive. The blood samples were then allowed to sit at room temperature for 30mins, before being centrifuged (3500rpm for 10mins). Serum was collected and stored at -70°C, for not more than 3 months. Serum vitamin B₁₂ concentrations were estimated by the Abbott IMx[®] B₁₂ microparticle enzyme intrinsic factor assay.

2.2.6. Folic Acid Estimation.

Fasting blood samples were collected by venepuncture into vacutainers containing no additive. The blood samples were then allowed to sit at room temperature for 30mins, before being centrifuged (3500rpm for 10mins). Serum was collected and stored at -70°C, for not more than 1 month.

An Abbott IMx[®] analyser was used to estimate serum folate concentrations using an ion capture assay. The folic acid, B₁₂ and homocysteine estimations were kindly carried

out by Mr. Jim Moulton of the diagnostic laboratory in the Biochemistry Department, RCSI.

2.2.7. Total RNA isolation from cultured cells.

This was carried out using Ultraspec™ -II, an RNA isolation reagent and is a modification of the method described by Chomczynski & Sacchi, (1987).

Cells were lysed directly with Ultraspec™ -II in the culture flask after the medium had been removed. 2mls of Ultraspec™ -II was used for a culture flask of 75cm² in area. The lysed cells were transferred to an RNAase-free eppendorf tube and chloroform was added (0.2ml/1ml Ultraspec™ -II). The contents were mixed vigorously and allowed to stand at 4°C for 5mins. Following centrifugation at 12,000g for 15mins at 4°C, the homogenate separated into a lower yellowish, phenol-chloroform phase and interphase, which contained proteins and DNA and an upper aqueous colourless phase which contained RNA. The RNA-containing phase was removed and transferred to a fresh eppendorf. RNA was precipitated by the addition of 0.5 vol isopropanol and 0.05 vol RNATack™ resin (or 2µg glycogen). The RNA pellet was then isolated by centrifugation (12,000g for 1min) and washed twice with 1ml 75% (v/v) ethanol by vortexing and subsequent centrifugation at 12,000g for 1min. This pellet was then dried by vacuum and the RNA was dissolved in 30-40µl of RNase-free water.

2.2.8. Assessment of RNA quantity, purity and integrity.

2.2.8.1. Absorbance.

The ultra violet (UV) absorbance spectrum of pure RNA is such that an absorbance of 1 unit at 260nm with a 1cm path length, indicates the presence of 40µg/ml of single stranded RNA. An aliquot of each sample (1-2µl) was diluted in 999-998µl of deionised

water and its UV absorbance at 260 and 280nm was measured in duplicate. The ratio of absorbance 260/280 gives an indication of purity. Pure RNA has a 260/280 ratio of 1.8-2.0. A ratio outside this range indicates protein/phenol and/or DNA contamination.

2.2.8.2. Agarose gel electrophoresis of RNA.

RNA was separated by agarose gel electrophoresis using a horizontal gel electrophoresis unit (Horizon 58™, BRL Life Technologies, Paisley, UK) to determine its integrity . Prior to electrophoresis, the RNA was denatured by heating to 70°C for 10mins in RNA-denaturing buffer* to disrupt secondary structure. Samples were electrophoresed on a 1.0-1.5% (w/v) agarose gel for 2 hrs at 30V. RNA was visualised under UV light following staining by ethidium bromide. The presence of two bands on the gel, which are the 18s and 28s ribosomal RNA (rRNA), with the intensity of the 28s band approximately twice that of the 18s, indicates that the RNA is intact. Degraded RNA appears as a smear on the gel below the 18s rRNA band.

* Denaturing Buffer: 50% (v/v) formamide, 0.5X MOPS, 2.2M formaldehyde, 0.04% (v/v) bromophenol-blue.

2.2.9. Reverse Transcription (RT) of total RNA .

Reverse transcription of sample RNA was carried out using an RT kit supplied by Promega.

The first step of the reaction contained the following:

	Volume	Final Conc.
RNA	Xµl*	1µg
Random hexamers	1µl	1µg
H ₂ O	Yµl*	

The Northern blotting process required the following solutions:

* The volume of X and Y here depend on the concentration of the RNA sample and the volume of water respectively, needed to give a final volume of 11.85 μ l.

3.0M NaCl

The reaction mixture was heated to 70°C for 10mins. This resulted in the denaturation of the RNA and allowed primer binding. The reaction mixture was then stored on ice while the following components were added, in order:

Prehybridisation Solution

0.1g BSA

	Volume	Final Conc.
rRNasin ribonuclease inhibitor	0.5 μ l	1U/ μ l
25mM MgCl ₂	4.0 μ l	5mM
10X RT buffer	2.0 μ l	1X
10mM dNTP mix	2.0 μ l	1mM each dNTP
AMV* reverse transcriptase	0.65 μ l	15U/ μ g
Total Volume	20.0 μ l	

*AMV: Avian Myeloblastosis Virus

The reaction was incubated at 37°C for 3-12hrs. The cDNA produced by this reaction was stored at -20°C.

2.2.10. Northern blotting.

All glassware used in the protocol was first treated to remove any RNase contamination. Briefly, glassware was soaked in 1M HCl for 2hrs, then rinsed in distilled water and baked at 180°C overnight. RNase-free water was prepared by DEPC treatment (0.05% v/v) overnight, followed by autoclaving (15lb/in² for 30mins) (Sambook *et al*, 1989).

The Northern blotting process required the following solutions:

20X SSC (Sodium Saline Citrate)

- 3.0M NaCl
- 0.3M Sodium citrate
- pH 7.0

Prehybridisation Solution

- 0.1g BSA
- 0.7g SDS
- 5mls 1M Phosphate buffer pH 6.8
- 20µL 0.5M EDTA
- 4mls H₂O

Wash Buffer A

- 2.5g BSA
- 25g SDS
- 20mls 1M Phosphate buffer, pH 6.8
- 1ml 0.5M EDTA
- adjust volume to 0.5L with H₂O*

Wash buffer B

- 10g SDS
- 40mls 1M Phosphate buffer, pH 6.8
- 2mls 0.5M EDTA
- adjust volume to 1L with H₂O*

RNA was prepared and its quality assessed as described in section 2.2.7. and 2.2.8. RNA samples (20µg) were evaporated to dryness in a vacuum dryer, resuspended in 8µl of RNase-free water and added to 22µL of denaturing buffer (15µl formamide, 5.5µl 37% (v/v) formaldehyde, 1.5µl 10X MOPS buffer). The RNA samples were denatured by heating to 65°C for 15mins and 3µl of loading dye was added. RNA samples were

then separated by denaturing formaldehyde gel (1g agarose, 10mls 10X MOPS buffer, 83mls water, 17mls 37% (v/v) formaldehyde) electrophoresis. After electrophoresis, the gel was rinsed briefly in 2X SSC. The RNA was then transferred to nylon (Hybond N+) membrane over 24hrs in 20X SSC. After transfer was complete, the nylon membrane was rinsed in 3X SCC, and the RNA was fixed by laying on filter paper that had been soaked in 0.05M NaOH. The membrane was then rinsed in 2X SSC for a few minutes (Sambrook *et al*, 1989). Once fixed, the membranes can be stored indefinitely, wrapped in cling-film, slightly moist (2X SSC) at 4°C.

A TNF α probe was made by PCR of cDNA using the primers described below that were kindly donated by Dr.Aideen Long (Biochemistry Dept, RCSI), which generated a 433bp product. cDNA was prepared as described above in section 2.2.9. The PCR reaction included 5 μ L of cDNA, 1X buffer, 1.7mM MgCl₂, 200 μ M dNTPs, 1 IU *Taq* polymerase, and 0.5 μ M of each primer (described below) and deionised water to a final volume of 25 μ L. The PCR reaction was overlaid with 5 μ L of mineral oil to prevent evaporation during thermocycling. The thermocycling conditions were: 95°C x 5mins (hot start), followed by 35 cycles of 95°C x 1min (denaturation), 55°C x 45secs (annealing) and 72 °C x 1min (extension). This was followed by a final extension of 72°C x 10mins.

Forward 5' GTG CTT GTT CCT CAG CCT C 3'

Reverse 5' GAT GGC AGA GAG GTT GAC C 3'

Following PCR-amplification, the TNF α PCR product was electrophoresed in an agarose gel (1% w/v) and the band of interest (433bp) was excised. The PCR-generated probe was eluted from the gel using the Wizard[®] PCR preps DNA purification system (Promega).

Levels of the house-keeping gene, GAPDH, were determined in order to ensure that the mRNA content of each RNA sample did not vary substantially. The GAPDH probe was a kind donation of Donncha Dunican, (Dept of Biochemistry, RCSI).

Both probes were [$\alpha^{32}\text{P}$] dCTP-labelled with random hexanucleotide primers and [$\alpha^{32}\text{P}$] dCTP using the Promega Prime-a-gene[®] random prime labelling kit, according to manufacturer's instructions. The pre-hybridisation and hybridisation were carried out in cylindrical hybridisation bottles in a rotary oven (Hybaid). Pre-hybridisation was carried out for 1-2hrs at 65°C, using the pre-hybridisation solution described above. Hybridisation was accomplished by adding the labelled probe to the pre-hybridisation buffer. Hybridisation was allowed to proceed for at least 12 hrs at 65°C. The membranes were then washed twice with Wash buffer A (prepared fresh) at 65°C for 10mins and then with Wash buffer B again at 65°C for 10mins, until non-hybridised probe had been removed. The nylon membranes were then exposed to Fuji X-ray film at -70°C.

The filters were probed successively for GAPDH and then TNF α . Membranes were stripped of each probe by washing in boiling 0.1X SSC/0.1% (v/v) SDS.

The relative expression of the GAPDH and TNF α mRNAs was determined by measuring the intensity of the spots on the film (corresponding to each gene) by densitometry, using Bio 1-D densitometric analysis software (Vilber Lourmat, Cedex, France).

2.2.11. Lactate Dehydrogenase (LDH) Estimation.

To assay for cytotoxicity, LDH release by cells into culture medium was examined, using LDH opt kit (Randox).

The assay is based on the following reaction:



1.0 ml of the reagent which contains 0.18mM NADH, phosphate buffer (pH 7.5) and 0.6mM pyruvate was added to 0.02ml of the standard control or 0.08 ml of sample medium which provides LDH. NADH has a maximum absorbance at 340nm, thus a decrease in absorbance at 340nm over time indicates LDH activity. The reaction was carried out in a cuvette at 37°C and the initial absorbance at 340nm was noted 0.5mins after the sample/standard had been added. Subsequently, absorbance readings were taken at 1 minute intervals for 3 minutes.

LDH activities were calculated as follows:

$$\text{LDH activity (U/L)} = 8095 \times \text{Change in } A_{340\text{nm}}/\text{min}$$

Further methodological procedures and statistical analysis are described in the subsequent chapters, as appropriate to the experiments being carried out.

A method to study cellular methionine metabolism.

3.1. INTRODUCTION.

The identification of homocysteine as a potential risk factor for cardiovascular disease has prompted significant interest in the metabolism of this amino acid. Homocysteine may contribute to the structural abnormalities of blood vessels, which may lead to atherosclerosis.

Homocysteine metabolism has been described in detail in several reviews (1, 2). Homocysteine is a sulfur-containing amino acid that is normally found in the blood. It is a risk factor for cardiovascular disease, and its levels are elevated in patients with certain genetic disorders, such as homocystinuria.

Chapter 3***A method to study cellular methionine metabolism.***

The purpose of this chapter is to describe a method to study cellular methionine metabolism. This method involves the use of a specific inhibitor of methionine metabolism, which allows for the measurement of the rate of methionine metabolism in cells.

This method is based on the principle that the rate of methionine metabolism is proportional to the concentration of the inhibitor. By measuring the rate of methionine metabolism in cells in the presence of different concentrations of the inhibitor, the rate of methionine metabolism can be determined.

This method is a simple and reliable way to study cellular methionine metabolism. It can be used to study the effects of various factors on methionine metabolism, such as the effects of different concentrations of the inhibitor, or the effects of different cell types.

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A method to study cellular methionine metabolism.

3.1. INTRODUCTION.

The identification of homocysteine as a potential risk factor for vascular disease has prompted substantial interest in the metabolism of this amino acid and the factors that may contribute to its abnormal accumulation in plasma (Daly *et al*, 1993). Homocysteine metabolism has been described in detail in section 1.2. Briefly, methionine is converted to homocysteine *via* SAM and SAH. Homocysteine is then disposed of by remethylation to methionine or by transsulphuration to cystathionine and cysteine.

The majority of studies investigating the aetiology of raised plasma homocysteine have looked at a specific *in vitro* enzyme activity, gene mutation or nutritional deficiency (Boers *et al*, 1985; Kang *et al*, 1991; Dudman *et al*, 1993 and Selhub *et al*, 1993). There is some evidence to suggest that up to a certain point, a defect in one catabolic pathway of homocysteine may be compensated for by increased activity in another (Stabler *et al*, 1993). In addition, nutritional factors play a key role in the phenotypic expression of some polymorphisms, *eg* folate and the MTHFR TT genotype. In these cases, relating the incidence of hyperhomocysteinemia to one enzyme activity, polymorphism or vitamin may be less than informative.

To our knowledge no investigations into homocysteine metabolism as a whole at the cellular level have been carried out. The objective of the following study was to design a system to investigate methionine cycle function at the cellular level, so that the fate of homocysteine in the cell could be followed. To do this, a system was devised to isolate and separate homocysteine and the other components of the methionine metabolic pathway, from a cell extract sample. It was hoped that by studying the fate of

radiolabelled methionine in a cell culture system, valuable insight into the factors that contribute to hyperhomocysteinemia could be gained.

3.2. STRATEGY.

Peripheral blood mononuclear cells were isolated and cultured for three days in the presence of the plant mitogen, phytohaemagglutinin (PHA). The cells were then exposed to [35 S]-labelled methionine for 0-4hrs. As the cells take up and metabolise the methionine, the sulphur-containing constituents of the methionine pathway (cystathionine, cysteine, homocysteine, S-adenosyl homocysteine and S-adenosyl methionine) become labelled. A cell extract was prepared at various times after the addition of the label and the amino acids and adenosylated products were separated and identified by co-chromatography with authentic standards. The relative amount of radioactive label in each of the metabolites was quantitated by scintillation counting (Fig. 3.1). The appearance and persistence of the label in cystathionine and cysteine indicates activity of the transsulphuration pathway. Likewise activity of the remethylation pathway results in the channelling of the label into SAH and SAM. Such a system could permit monitoring of homocysteine metabolism within the cell.

3.3. MATERIALS AND METHODS.

3.3.1. Human peripheral blood mononuclear cell culture.

PBMC were isolated as described in section 2.2.1 within one hour of whole blood collection. Cells were plated at a density of 5×10^5 cells/ml in RPMI 1640/ 10%(v/v) FCS/ 1%(v/v) penicillin streptomycin. PHA was added to the medium at a concentration of $10 \mu\text{g/ml}$. The cells were cultured for 3 days at 37°C , 5%(v/v) CO_2/air . After this time, [^{35}S]- methionine at a concentration of $5 \mu\text{Ci/ml}$ was included in the

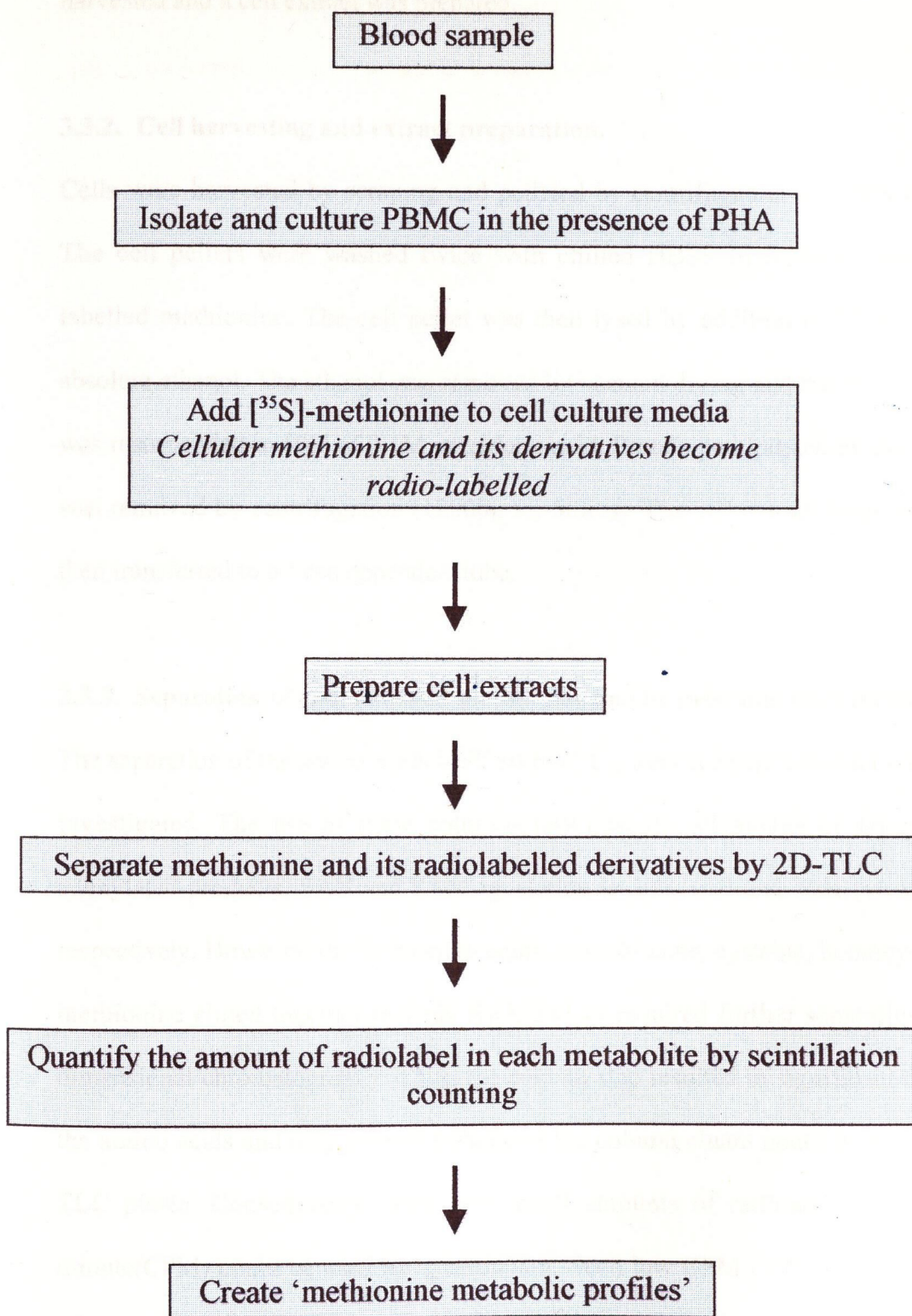


Fig. 3.1 Flow diagram showing the strategy employed to study cellular methionine metabolism.

medium. After 0.5, 1, 2 and 4 hours exposure to the labelled methionine, the cells were harvested and a cell extract was prepared.

3.3.2. Cell harvesting and extract preparation.

Cells were harvested by scraping and pelleted by centrifugation at 5000g for 5mins. The cell pellets were washed twice with chilled HBSS to remove contaminating labelled methionine. The cell pellet was then lysed by addition of 500 μ l of chilled absolute ethanol. The ethanol was removed by vacuum drying and the resultant residue was resuspended in 50 μ l of 0.1M perchloric acid. Protein precipitated by this treatment was removed by centrifugation (12000g for 5mins). The cell extract (supernatant) was then transferred to a fresh eppendorf tube.

3.3.3. Separation of radiolabelled methionine and its principal derivatives.

The separation of the amino acids by "Sep-pak" C₁₈ ion exchange columns was initially investigated. The use of these columns provided the advantage of separating the adenylated products, SAH and SAM by elution in 30%(v/v) and 70% (v/v) methanol respectively. However, the four amino acids; cystathionine, cysteine, homocysteine and methionine eluted together in 2mls H₂O, and so required further separation by two-dimensional chromatography. Thus, the column step resulted in significant dilution of the amino acids and only a small volume of the column eluant could be applied to the TLC plates. Consequently, only very small amounts of radioactivity (counts per minute/CPM) could be used for quantitation. Such low CPM (100s) had a deleterious effect on the sensitivity of the assay. The following process, whereby methionine and each of its derivatives could be separated by a single procedure, was devised.

The principal metabolites of the methionine pathway were separated by two-dimensional thin layer chromatography (2-D TLC).

A sulphur amino acid mix containing 10mg/ml of each of the following amino acids; cystathionine, cysteine, homocysteine, methionine, SAH and SAM, in 0.1% (v/v) β -mercaptoethanol, was prepared. Fifty microlitres of this carrier mix was added to the acidified cell extract for co-chromatography with the sulphur amino acids of the cell extract. Co-chromatography of the cell extract with standards allows identification of methionine and each of its derivatives.

The following solvents were used for 2-D TLC:

Solvent 1: Isopropanol: 25mM NaH_2PO_4 , pH 3.2: formic acid (75:25:6).

Solvent 2: Saturated phenol: ammonium hydroxide (10:1). (Solvent 2 was prepared immediately prior to use).

Cellulose TLC plates were activated by heating at 80°C for 10mins. Thirty microlitres of the cell extract (with carriers) was applied to the bottom left hand corner (origin) of the cellulose TLC plate. Plates were run in solvent 1 for 17hrs. Excess solvent was removed by drying at 80°C for 10mins. The plates were then run in solvent 2 in the 2nd dimension for a further 20hrs. Following separation, the plates were dried and the locations of the adenosylated products and amino acids were determined by visualisation under UV light (SAH and SAM) and by staining with ninhydrin. Both SAH and SAM fluoresce under UV light. All of the amino acids and SAH, but not SAM, could be visualised by staining with ninhydrin.

3.3.4. Quantitation of [^{35}S] label in methionine derivatives.

The spots representing methionine and its five metabolites were scraped off the plates and the amount of radioactivity in each of these derivatives was determined by scintillation counting in 5mls scintillant (Ecoscint™).

3.3.5. Methionine profiles.

Methionine profiles were constructed by plotting the percentage of the total plate radioactivity in each of the methionine metabolites vs time.

3.4. VALIDATION OF METHODOLOGY.

3.4.1. Purity of labelled methionine.

To ensure that the labelled methionine contained no significant labelled contaminant, 0.5 μ Ci was subjected to 2-D TLC (section 3.3.3), in the presence of the sulphur amino acid standards. Following separation, the plate was exposed to photographic film and after a one day exposure the film was developed to reveal the position of radiolabelled species. One predominant spot appeared on the autoradiograph (Fig. 3.2). Ninhydrin-staining of the TLC plate revealed that the radioactive spot coincided with the position of methionine. An insignificant amount of the labelled material remained at the origin of the second solvent. This may represent excess methionine or may represent some minor by-product produced during the manufacturing process. The manufacturers state that the [35 S]-methionine is >90% pure (radio-chemically) but do not allude to the identity of any contaminant. This area did not stain immediately with ninhydrin but took 2-3 days to stain up, implying that the amino acid concentration in this area is relatively low.

The entire area of the cellulose TLC plate was cut into 2cm² sections and the amount of radioactivity in each section was determined by scintillation counting. Sections demonstrating the highest concentration of radioactivity coincided with the position of methionine (by both ninhydrin staining and autoradiography), thus confirming the quality of the [35 S]-methionine. This also verified that methionine ran cleanly to the place indicated in Fig. 3.3, leaving very little of the [35 S]-label in its wake. This was

important as, if labelled methionine left a trail behind it, this would interfere with the calculation of radioactivity in each of the other metabolites, thereby giving false indications as to the activity of the remethylation and transsulphuration pathways.

3.4.2. Two-dimensional thin layer chromatography.

A two-dimensional thin layer chromatographic system that would allow the separation of methionine and its derivatives in one step was successfully devised (Fig. 3.3). In an effort to validate this separation method, each of the amino acid standards was run separately and in pairs to confirm identity and also to investigate the possibility of new sulphur amino acid species being generated by the interaction of the different amino acids in the carrier mix. No new species were generated over prolonged periods of time (up to three months) when the amino acid mix was stored at -70°C . However, the quality of the cysteine standard deteriorated to the extent that cysteine had to be prepared fresh for each run.

To confirm the accuracy of the profiles generated by counting radioactivity in the ninhydrin-staining spots of the cell extracts, duplicate samples were run and these TLC plates were exposed to photographic film for 3 days before developing (Fig 3.4). The intensities of the radiolabelled amino acids on the autoradiograph were then compared to the actual amount of radioactivity for each amino acid, as measured by scintillation counting. In all cases, the intensity of the spot on the autoradiograph correlated well with the actual concentration of radioactivity in the spot, *ie* those amino acids demonstrating the highest amount of radioactivity corresponded to the most intensely staining areas on the autoradiograph (Fig. 3.4).

3.5. RESULTS.

3.5.1. Uptake of [³⁵S]-methionine by cultured PBMC in response to phytohaemagglutinin.

A fundamental prerequisite of the strategy described in section 3.2 was the uptake of significant amounts of radiolabeled methionine by cultured PBMC. These cells are normally quiescent in cell culture and therefore have a relatively low requirement for methionine. In an effort to maximise uptake of the label, the effect of the addition of the mitogen, PHA, to the cell cultures was investigated. Without exception, all cell cultures (n=6) demonstrated increased uptake of the radiolabelled methionine (7.9 - 44.8 fold) in response to PHA, although the level of enhancement varied between cell cultures (Fig. 3.5).

There was progressive accumulation of the [³⁵S]-label in the cells over the time frame studied (4hrs) (Fig. 3.6). Although the actual amount of label taken up by different cultures varied substantially, the cells nonetheless showed continuous accumulation of the label over a four hour exposure.

The label was found in both an acid-soluble fraction, designated the cell extract (mainly low molecular weight non-protein metabolites *eg* amino acids) and an acid-insoluble fraction (presumably mainly proteins) (Fig. 3.7). Initially (after a 30mins exposure), the majority of the label was found in the cellular extract. Following this, the label appeared predominantly in the protein fraction. This is probably a reflection of the incorporation of labelled methionine and cysteine into newly synthesised proteins.

3.5.2. Labelling of cellular methionine derivatives.

It was clear that the cells were capable of accumulating the label in both non-protein and protein fractions. It was now necessary to investigate if the label could be detected in the metabolites of the methionine pathway. Cell extracts were prepared as described

in section 3.3.2 and subject to 2-D TLC as described in section 3.3.3. Following separation, the amino acids, detected by ninhydrin staining, were scraped from the plate and the amount of radiolabel in each was determined by scintillation counting. "Metabolic profiles" were constructed by plotting the percentage of total label in each metabolite over time (Fig. 3.8). The presence of the label in both cystathionine and/or cysteine was taken to indicate activity of the transsulphuration pathway, while the presence and persistence of the label in both SAH and SAM was taken to indicate activity of the remethylation pathway. For the sake of clarity of graphical presentation, the CPM for both cystathionine and cysteine were pooled, as were the CPM for SAH and SAM. As can be seen from Fig. 3.8, [^{35}S]-label appears in both cystathionine/cysteine and in SAH/SAM, indicating that both pathways are active in these PHA-stimulated cells. There was no significant accumulation of the label in homocysteine.

The data presented in Fig 3.8 is the profile generated by a healthy non-hyperhomocysteinemic individual and it is broadly representative of the profiles of other controls (see Fig 4.5 later for composite profile of control subjects). Furthermore the profile obtained with a given individuals cells would appear to be reproducible over time as a profile generated by one individual was reproduced on a further occasion six months later

From the error bars depicted in Fig 3.8, representing the range and mean percentage label in duplicate experiments it can be seen that although the actual percentages in each metabolite might vary, the overall pattern of labelling did not.

3.5.3. Separation of sulphur-containing amino acids by 2-D TLC.

The separation of the sulphur amino acids by 2-D TLC as described in section 3.3.3 is shown in Fig. 3.3. When standards alone were run, only the six spots (1-6) representing

cystathionine, cysteine, homocysteine, methionine, SAH and SAM respectively, were present (as assayed by ninhydrin staining). However, when the amino acid mix was run with a radioactive cell extract, some extra spots, which could be faintly detected by ninhydrin staining were apparent. Upon further investigation, two of these were identified as homocysteine-sulphinic acid (x) and homocystine (y) (Fig. 3.3). A third species that appeared in the area between cysteine and SAH on the TLC plates remained unidentified but was not cystine, cysteine sulphinic acid, homocysteic acid or cysteic acid. It could not be visualised under UV light and was therefore not an adenosyl-derivative. It was slow to stain with ninhydrin, suggesting that a) it was not an amino acid species or b) it was present in very low concentration or c) it has a complex structure that does not readily allow the binding of the ninhydrin dye to its amino group. Autoradiography, in conjunction with scintillation counting, demonstrated that the unidentified species represented only a small proportion of the total amount of cellular radioactivity (<3%). Thus its contribution to the "methionine metabolic profiles" is probably not of consequence.

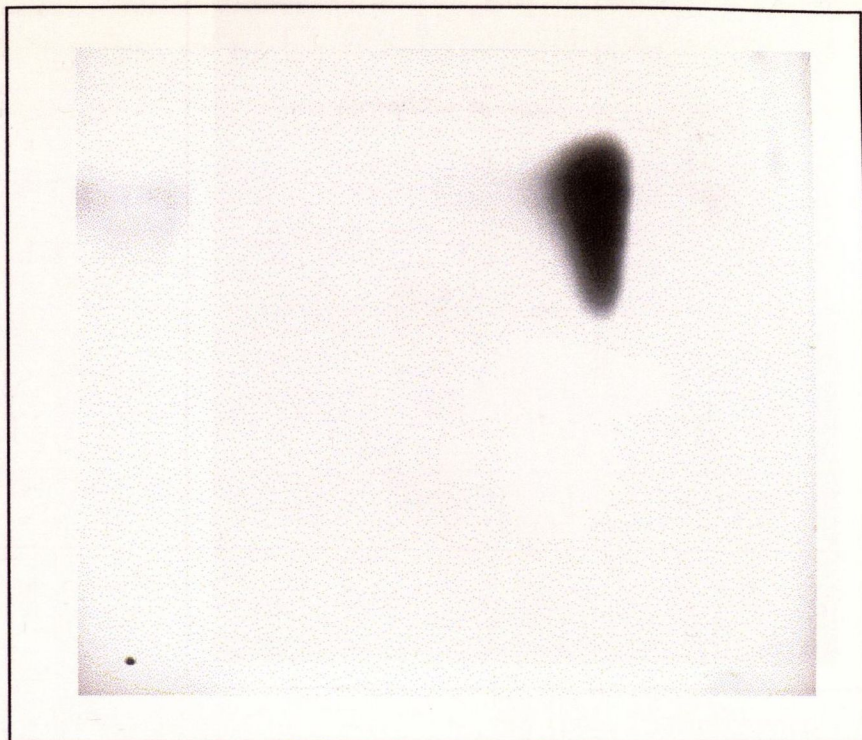


Fig. 3.2 Autoradiograph showing the position of [^{35}S]-methionine, following separation of radiolabelled methionine and its non-radiolabelled derivatives by 2D-TLC as described in section 3.3.3. Labelled methionine can be seen in the upper top right hand corner of the TLC plate. This confirms the position of methionine following 2-D TLC. A small amount of the methionine remained at the origin of the second solvent.

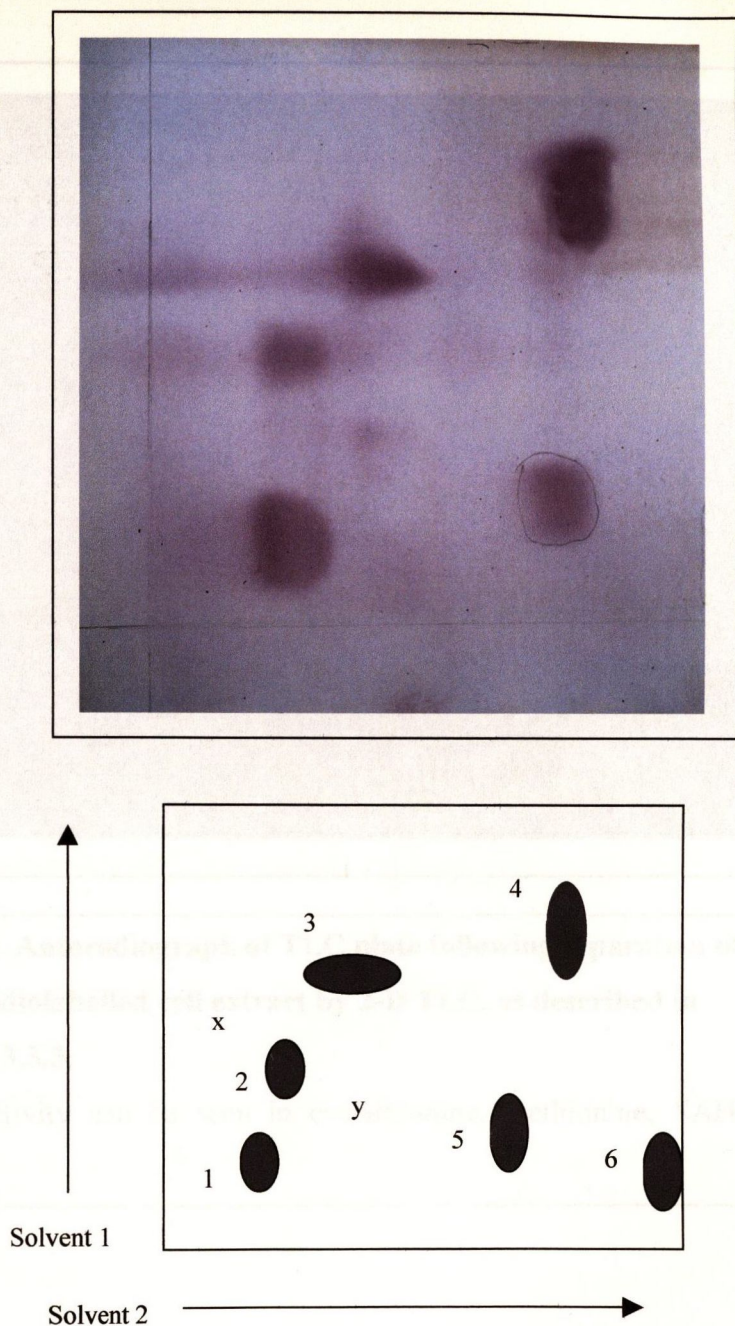


Fig. 3.3 Separation of methionine and its metabolic derivatives by 2-D TLC as described in section 3.3.3. The amino acids were visualised by ninhydrin staining. The adenosylated products SAH and SAM fluoresce under UV light and were thus visualised.

- 1: Cystathionine (cysta)
- 2: Cysteine (cys)
- 3: Homocysteine (hcy)
- 4: Methionine (met)
- 5: S-adenosyl homocysteine (SAH)
- 6: S-adenosyl methionine (SAM)
- x: homocysteine sulphinic acid
- y: homocystine

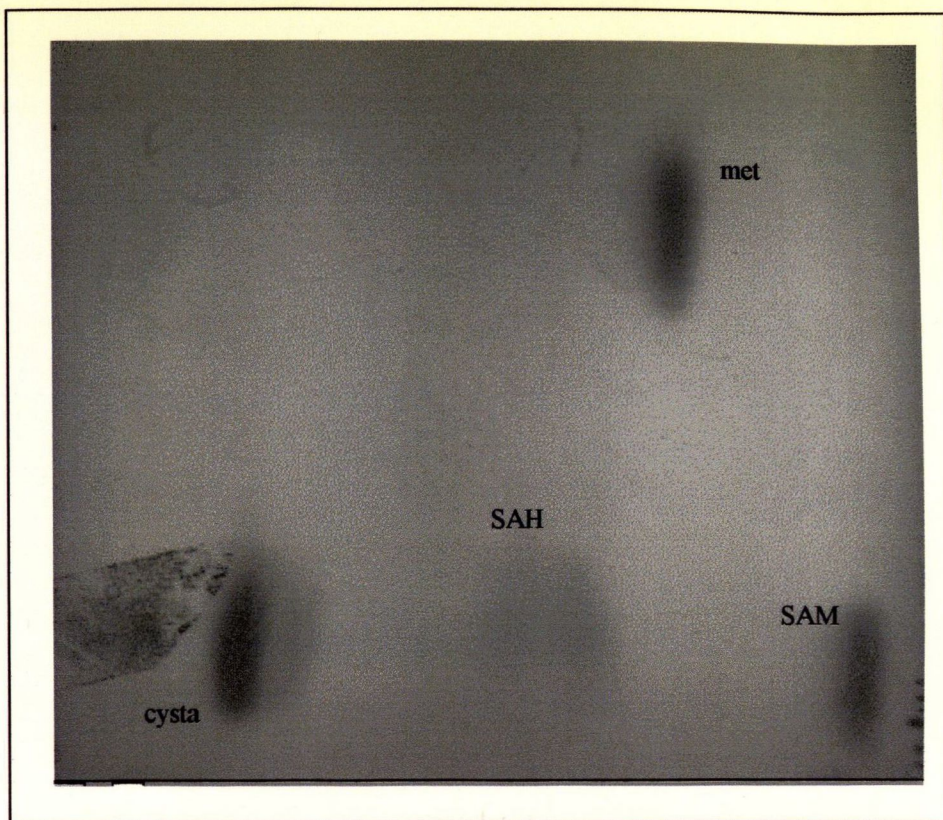


Fig. 3.4 Autoradiograph of TLC plate following separation of [^{35}S]-radiolabelled cell extract by 2-D TLC, as described in section 3.3.3.

Radioactivity can be seen in cystathionine, methionine, SAH and SAM.

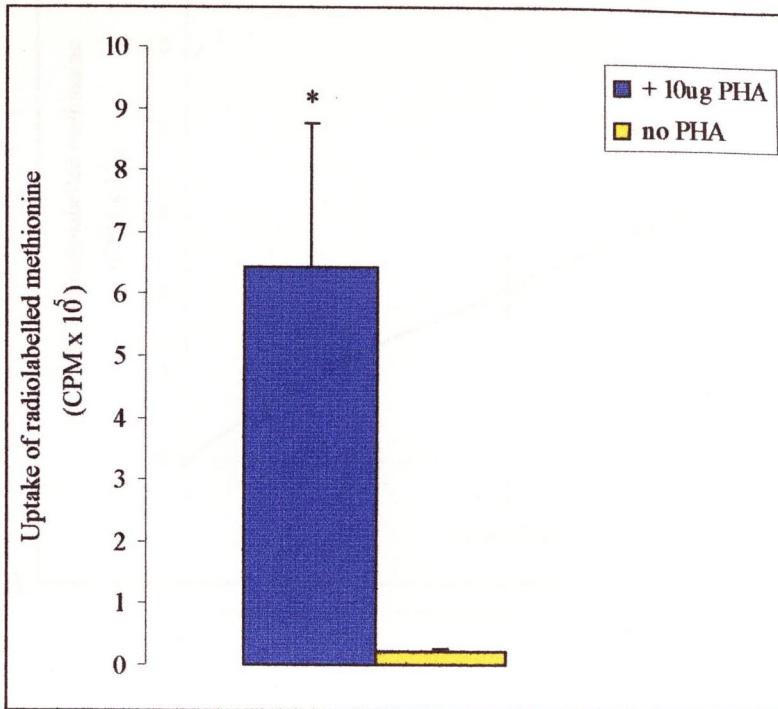


Fig. 3.5 Uptake of [³⁵S]-methionine by PHA (10µg/ml) and non-PHA treated PBMC.

Cells were cultured at a density of 5×10^5 cells/ml, for three days in the presence/absence of PHA and then exposed to radiolabelled methionine for four hours. Total uptake was calculated by counting radioactivity (counts per minute/CPM) in the cell extracts and in the acid precipitate (protein). Results shown represent mean $\pm$ SEM (n=6). Uptake of labelled methionine was significantly increased (* $p < 0.05$) by exposure to PHA.

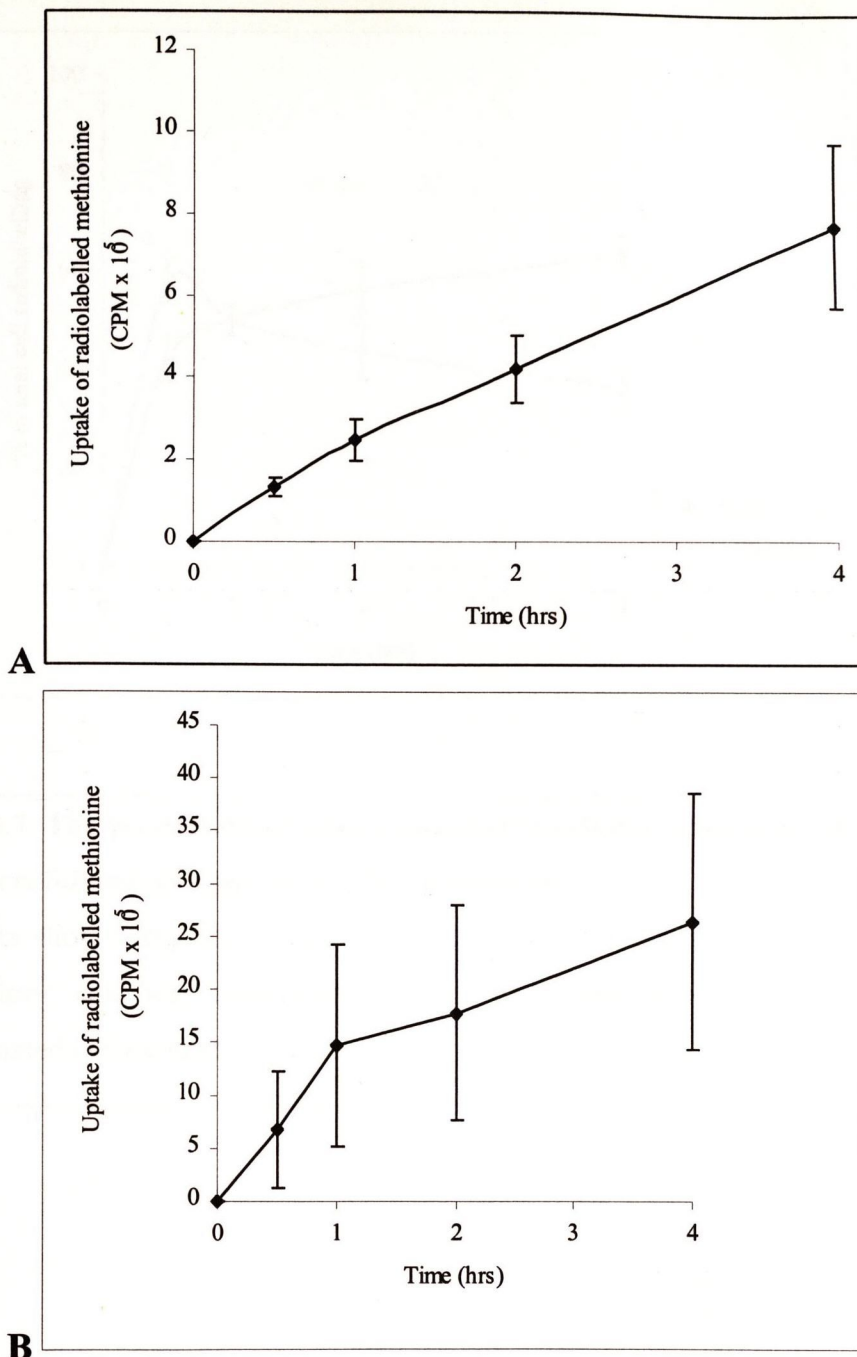


Fig. 3.6 Uptake of [³⁵S]-methionine (CPM) by cultured PBMC over time, following a three day exposure to PHA.

A: Continuous uptake of the label up to four hours by a typical PBMC culture (Data shown represents the mean $\pm$ range of values found for duplicate cultures of a single PBMC donor).

B: Continuous uptake of the label up to four hours was observed in all cell cultures examined. Results shown represent mean $\pm$ SEM ($n=6$, ie six individual donors).

The *extent* of labelling varied substantially but the *patterns* were repeated in each case, ie continuous uptake of the label. Based on these results, a four hour exposure to radiolabelled methionine was deemed sufficient for cell labelling studies.

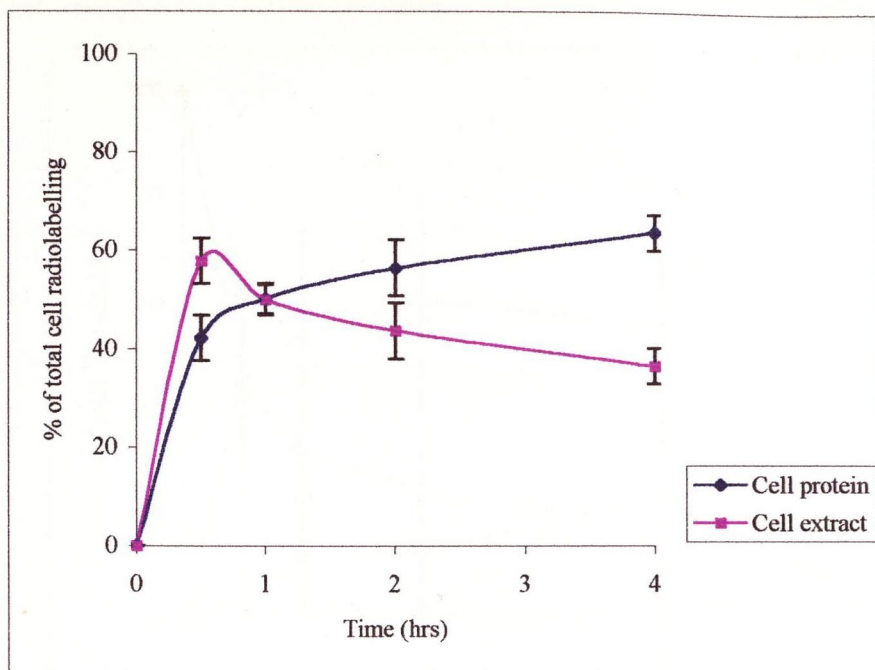


Fig. 3.7 The proportion of label appearing in cellular protein vs cellular extracts following exposure to $[^{35}\text{S}]$ -methionine.

Results shown represent mean $\pm$ SEM ($n=6$). Any material precipitated by perchloric acid was considered protein, while acid soluble material was designated cell extract.

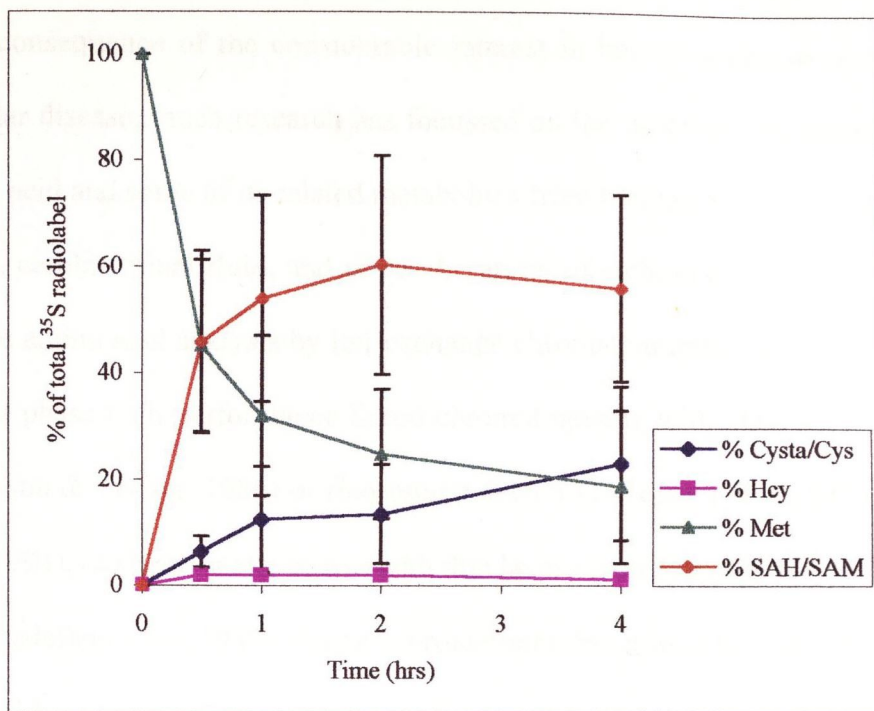


Fig. 3.8 Typical "methionine metabolic profile" of a non-hyperhomocysteinemic individual, generated by the methods described in section 3.3.

Error bars represent the range of values found in duplicate experiments. There was rapid transfer of the [^{35}S]-label out of methionine through the transsulphuration pathway (appearance of label in cysta/cys) and the remethylation pathway (appearance of label in SAH/SAM). There was no accumulation of the label in homocysteine.

3.6. DISCUSSION.

As a consequence of the considerable interest in homocysteine as a risk factor for vascular disease, much research has focussed on the isolation and quantitation of this amino acid and some of its related metabolites from biological samples such as plasma, serum, cerebrospinal fluid, and urine. A variety of techniques have been used. These include amino acid analysis by ion-exchange chromatography (Andersson *et al*, 1989), reverse phase high-performance liquid chromatography with electrochemical detection (Thomson & Tucker, 1986) or fluorescent detection (Refsum *et al*, 1989 and Jacobsen *et al*, 1994), radioenzymatic assays with thin layer chromatography (Kredich *et al*, 1981 and Chadeaux *et al*, 1989) and gas chromatography-mass spectrometry (Stabler *et al*, 1993). More recently, non-chromatographic immunoassays have been developed *eg* IMx analyser (Pfeiffer *et al*, 1999).

The system proposed in section 3.2 to study methionine metabolism, relied on the quantitation of radiolabel in methionine, cystathionine, cysteine, homocysteine, SAH and SAM. It was therefore necessary to devise a method to extract the labelled amino acids from a cell culture and to adequately separate and identify these metabolites, allowing their recovery for subsequent quantitation by scintillation counting. Although some of the analytical methods described above are capable of resolving 2-4 of the aforementioned methionine derivatives, to our knowledge no method has been described for the simultaneous separation of all six.

Sep-pak C₁₈ columns were initially examined as a possible means of separating the sulphur amino acids. While the columns proved useful in the isolation of the adenosylated products (SAH and SAM), they were found not to be suitable for this specific study. SAH and SAM could be eluted in methanol, while the sulphur amino acids were eluted in water and then subject to further separation by 2-D TLC. Significant dilution of methionine, homocysteine, cystathionine and cysteine by their

elution from the columns meant that only low levels of label were available for quantitation by liquid scintillation counting, thus having a deleterious effect on the sensitivity of the assay. In addition, colour quenching by ninhydrin-staining of the amino acids was approximately 20%, while quenching of the adenosylated products by methanol was insignificant. This made it difficult to combine the two sets of results to produce an accurate estimation of methionine cycle function. These drawbacks drove the search for a new method of separating all six amino acids in one step.

Thin layer chromatography proved adequate for the separation of methionine and its derivatives. Physical separation of molecules by TLC is a consequence of their different partitioning between the solid and mobile phases. In the case of TLC the solid phase is the gel matrix of the TLC plate, in this instance cellulose. The mobile phase is the solvent in which the plate is partially immersed so that the solvent may move up the plate by capillary action. A substance which reacts with the solid phase will be retarded in its progress up the plate with the solvent front and *vice versa*. A range of solvent systems were investigated before the pair of solvents (described in section 3.3.3) was found to provide the greatest resolution of the amino acids in question. In these solvents, the sulphur amino acids ran to discrete positions on the TLC plate as can be seen in Fig. 3.3. This separation method was consistent, economical and required no specialised equipment. Because there was no overlap in the positioning of the amino acids following this separation, the spots representing each amino acid could be scraped from the plate and the amount of radiolabel in each quantified by scintillation counting, allowing the composition of "methionine profiles".

A peripheral blood mononuclear cell preparation is composed predominantly of mononuclear lymphocytes (95%), while granulocytes and erythrocytes make up the remainder (Boyum, 1968). MS, CBS and MTHFR enzyme activities have been demonstrated in human lymphocytes (Peytremann *et al*, 1975; Tan *et al*, 1993 and

Frosst *et al*, 1995). It was therefore hypothesised that these cells could be utilised to study the fate of homocysteine in hyperhomocysteinemic individuals.

When cultured *in vitro* these cells are relatively metabolically inactive, thus their uptake of [^{35}S]-methionine is low (Fig. 3.5) and CBS activity is not readily detectable (Goldstein *et al*, 1973 and Tan *et al*, 1993). To effectively monitor the activity of the pathways, it was essential that the cells take up a substantial quantity of the tracer. This could be achieved in one of two ways, by using a methionine-deficient culture medium or by activating cells so that their needs for methionine were increased. Preliminary work showed that cells cultured in methionine-deficient medium accumulated only low levels of the label and that the label tended to remain predominantly in methionine and showed a relatively low flux through the transsulphuration pathway. This may have occurred because concentrations of SAM, a proposed activator of CBS, would be low due to the limited supply of its precursor, methionine (Jencks *et al*, 1987). Similar profiles were generated by quiescent cells cultured in a full medium. Their metabolic inactivity resulted in the persistence of the label in methionine, with very little of the label being transferred to the other metabolites. In effect, the activity of the transsulphuration and remethylation pathways could not be monitored using non-mitogen stimulated cells.

Enhanced uptake of [^{35}S]-methionine by rapidly dividing cells has been reported (Katz *et al*, 1992). Phytohaemagglutinin, derived from extracts of *Phaseolus vulgaris*, is known to stimulate lymphocyte mitosis in cell culture (Cooper *et al*, 1963 and Tan *et al*, 1993). Indeed, PHA has been used to induce CBS activity in lymphocytes, (where it is not usually detectable) to compare activity of this enzyme in healthy subjects and in those with homocystinuria (Goldstein *et al*, 1973). For this reason, the effect of PHA on the uptake of [^{35}S]-methionine by cultured PBMC was investigated. SAM plays a pivotal role in an innumerable number of biochemical reactions, including methylation

of DNA and RNA, proteins and phospholipids (Chiang *et al*, 1996). PHA, by inducing mitosis, stimulates numerous metabolic pathways, increases the cells needs for SAM and therefore increases the cells needs for methionine, its sole precursor. This is evident in the increased mean uptake (26.9 ± 14.6 fold) of [^{35}S]-methionine by the cell cultures in response to PHA (Fig. 3.5). This increase in cellular labelling ranged from a factor of 7.9 to 44.8. Such a range in magnitude may reflect variable responsivity of the lymphocytes from different individuals to the mitogen or the nutritional status of each individual donor. All blood samples collected for PBMC preparation, were non-fasting samples. Depending on the quality of the diet in previous weeks and on the protein content of the meal consumed prior to blood donation, cellular levels of methionine may have differed between donors. This would directly affect the cells needs for methionine when stimulated and therefore the extent to which they utilised [^{35}S]-methionine from the culture medium. Regardless of this, methionine profiles are constructed using *percentages* of total cellular (acid-soluble) label in each metabolite so that the actual amount of radioactivity in each metabolite is irrelevant when comparing these profiles. By stimulating the cells with PHA, the need for both methionine and cysteine for protein synthesis is thought to have been increased. As anticipated, this led to an increased flux through *both* the transsulphuration and remethylation pathways, thereby providing more information with regards to the activity of these pathways.

Progressive accumulation of the [^{35}S]-label, in an almost linear fashion, was observed in the PHA-stimulated cells over a four hour period, providing further evidence as to the metabolically active state of the cells (Fig. 3.6). This linear increase in labelling has been previously reported in tumour cells (Katz *et al*, 1992).

[^{35}S]-label was found in both an acid soluble and non-acid soluble (protein) fraction (Fig. 3.7). Up to 30mins after addition of the labelled methionine, the majority of the label was found in the cell extracts (*ie* acid-soluble material). Thereafter, the label

predominated in the protein fraction. Rapid labelling of proteins, (presumably via [^{35}S]-methionine and cysteine), a refection of protein synthesis, points again to the highly activated state of the cells in the presence of PHA.

By analysis of sulphur-containing amino acid labelling, it was hoped that potential abnormalities in the methionine cycle could be highlighted. A typical "metabolic profile" is shown in Fig. 3.8. The label was rapidly transferred from methionine to the other amino acids in its metabolic pathway. In this case there appeared to be considerable activity of the remethylation pathway, (as indicated by the high proportion of CPM in SAH/SAM and by the persistence of the label in methionine). The increasing appearance of radioactivity in cystathionine/cysteine was suggestive of transsulphuration. No significant accumulation of the label in homocysteine was apparent. This was expected, as the fasting homocysteine level for this individual was $6.8\mu\text{M}$, *ie* non-hyperhomocysteinemic.

In summary, the TLC separation method described in section 3.3.3 proved satisfactory for the purposes of this study, as it allowed the simultaneous separation of, identification of and quantitation of the amount of radiolabel in, methionine and its five major metabolites. Used in conjunction with the cellular labelling study described in section 3.3.2, it could be successfully used to monitor cellular activity of both the homocysteine remethylation and transsulphuration pathways.

Chapter 4

Cellular methionine metabolism in hyperhomocysteinemic subjects and its relationship to the MTHFR TT and other genotypes

Cellular methionine metabolism in hyperhomocysteinemic subjects and its relationship to the MTHFR TT and other genotypes.

4.1 INTRODUCTION.

The homozygous C to T transition at position 677 of the MTHFR coding sequence (TT genotype), which converts an alanine to a valine residue is associated with thermolability of the enzyme, and reduced specific activity (Frosst *et al*, 1995 and van der Put *et al*, 1995). Consequently, and it is often the case, elevated plasma homocysteine levels are observed in the presence of this polymorphism. However, the effects of the TT genotype are not always this clear cut. In the presence of adequate folate, homocysteine levels remain in the normal range (Christensen *et al*, 1997). It is also believed that impaired remethylation due to the TT genotype may induce higher activity of the transsulphuration pathway, thereby preventing the accumulation of homocysteine (Woodside *et al*, 1997). Alternatively, additional factors such as polymorphisms in the other genes regulating methionine metabolism (*eg* I278T (CBS) or D919G (MS)) and other nutritional deficiencies (*eg* B₆, B₁₂) may independently affect homocysteine metabolism and/or exacerbate underlying genetic defects (Selhub *et al*, 1994; Tsai *et al*, 1996b and Harmon *et al*, 1999).

While elevated homocysteine levels have been frequently demonstrated in a significant proportion of cardiovascular disease patients, not all studies have found a significant association between the TT MTHFR genotype and cardiovascular disease (Brattström *et al*, 1998). This issue raises questions as to the relevance of the TT genotype not only to homocysteine metabolism but also to vascular disease risk.

With this in mind, a study was undertaken to investigate methionine metabolism at the cellular level in both healthy non-hyperhomocysteinemic subjects (controls) and in

vascular disease subjects (both with and without mild hyperhomocysteinemia) and to find what, if any, is the influence of the MTHFR TT genotype and of recognised mutations in the CBS and MS genes, on methionine metabolism. In order to do so, the system described in Chapter 3 was used.

Subjects were recruited from the community through the Adelaide Hospital, 2007, who had previously participated in a study (Graham *et al.*, 1997). Subjects were of both sexes and ranged in age from 20 to 60 years.

4.3 METHODS

4.3.1 Blood Sampling

Blood from subjects was collected for fasting homocysteine measurement by venipuncture. All blood samples were centrifuged at 1000g. Plasma was separated and stored at -20°C until analysis. Cells for peripheral blood mononuclear cell (PBMC) isolation were stored at -20°C.

2.2.1. Blood samples were collected at 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 hours post-infusion.

4.3.2 Cellular metabolism and DNA

Cellular metabolism was measured by measuring the release of ¹⁴C-methionine from cells. Cells were cultured in the presence of ¹⁴C-methionine and the release of ¹⁴C-methionine was measured.

In addition, in the absence of ¹⁴C-methionine, the release of homocysteine from cells was measured. Cells were cultured in the presence of homocysteine and the release of homocysteine was measured.

of homocysteine (100 µM) was added to the cells. Cells were then subjected to 4-6 hours of treatment (2 hours) (2 hours).

corresponding to 4-6 hours of treatment were added to the cells.

amount of radioactivity in each amino acid was determined by scintillation counting as described in section 3.3.4.

4.3.3 Measurement of biochemical parameters.

Fasting plasma homocysteine, serum folate and B₁₂ were measured as described in section 2.2.4 – 2.2.6.

4.3.4 Identification of MTHFR TT genotype.

The presence of the cytosine to thymidine polymorphism at position 677 of the MTHFR coding sequence (TT MTHFR) was determined by a modification of a previously described method, whereby a region of the MTHFR gene is amplified by PCR (Frosst *et al*, 1995). The C to T transition mutation creates a *Hinf*I restriction site, which the wild-type genotype lacks. Digestion of the PCR products with *Hinf*I therefore indicates which genotype is present, through the appearance of an additional band when the C-T polymorphism occurs.

Genomic DNA was prepared from frozen whole blood samples (in lithium-heparin), using the Isolate II DNA isolation kit according to the manufacturer's instructions.

The primers and PCR conditions used to amplify a specific 198bp region in exon 4 of the MTHFR gene are described in Table 4.1.

PCR products (5µl) were digested by an overnight incubation with the restriction enzyme *Hinf*I, at 37°C, according to manufacturers' instructions. The products of the PCR digest were separated by 12%(w/v) acrylamide gel electrophoresis using a vertical gel electrophoresis unit (Model SG 125-02, CBS Scientific Co, Del Mar, California). Visualisation of the fragments was accomplished by ethidium bromide staining (Sambrook *et al*, 1989). The presence of the base thymine at position 677 of the cDNA

sequence allows digestion of the 198bp DNA fragment, generating two fragments of 23 and 175bp in size.

4.3.5. Identification of CBS I278T genotype.

The I278T polymorphism arises due to T-C base pair change at position 833, in exon 8 of the CBS gene (Sperandeo *et al*, 1996). The presence of this polymorphism creates a *Bsr* I restriction site.

The primers and conditions described in Table 4.1 were used to amplify a 139bp region of the CBS gene surrounding this polymorphism.

Following PCR amplification, digestion reactions using *Bsr* I were set up as outlined by manufacturer's instructions and were allowed to proceed for 3hrs at 65°C. The products of the digestion reaction were then separated by acrylamide gel electrophoresis and visualised by ethidium bromide staining (Sambrook *et al*, 1989). The presence of the transition mutation allows digestion of the 139bp PCR product, generating fragments of 111 and 28bp.

As discussed in section 1.4.2.1, this polymorphism often arises *in cis* with a 68bp insert (Tsai *et al*, 1996b). The presence of the 844ins68 was detected by sizing of the PCR products by agarose gel electrophoresis (the presence of the insert results in a PCR product of 207bp). If this insert was present in conjunction with the T-C polymorphism, digestion by *Bsr* I, would generate products of 111 and 96bp.

4.3.6. Identification of MS D919G genotype.

The aspartic (D) acid to glycine (G) amino acid substitution is caused by an A to G transition at position 2756 of the coding sequence of the MS gene. The presence of this mutation was determined by digestion of a PCR amplified 265bp region of the MS gene

as previously described by van der Put *et al* (1997). The presence of the mutation creates a restriction site for the restriction enzyme *Hae* III.

This amplification was carried out using the primers and conditions described in Table 4.1.

The digestion reaction was set up as outlined by manufacturer's instructions and was allowed to proceed overnight at 37°C. The products of the digestion reaction were then separated by acrylamide gel electrophoresis and visualised by ethidium bromide staining (Sambrook *et al*, 1989). The presence of the polymorphism allows digestion of the 265bp PCR product by *Hae* III, generating fragments of 180 and 85bp.

4.3.7. Verification of genotyping

This was accomplished by cloning and sequencing of the amplified MTHFR/CBS/MS gene fragments.

4.3.7.1. Purification of gene fragments amplified by PCR.

The MTHFR, CBS and MS gene fragments were amplified as described above, with one modification – the use of *Pfu* polymerase in place of *Taq* polymerase. This was used because it has a proof-reading ability that *Taq* polymerase lacks and also because the cloning vector used is designed for blunt-ended ligation of *Pfu*-generated PCR products.

PCR products were separated by agarose TAE gel electrophoresis. The band of interest was then excised from the gel and the DNA fragment was isolated by the Wizard® PCR Preps DNA Purification System. The kit was used according to the manufacturer's instructions (Promega, WI, USA). The PCR products were then concentrated by ethanol precipitation.

4.3.7.2. Ethanol precipitation of purified PCR products.

Absolute ethanol (2vol) and 3M sodium acetate (1/10th vol) was added to the purified PCR products. The samples were then stored at -70°C for 15-30mins to allow precipitation of DNA. This was followed by a 2 minute spin in the microfuge. The supernatant was removed and discarded and the pellet was washed by the addition of 2 volumes 70% (v/v) ethanol and spinning for 2mins in the microfuge. The supernatant was removed, the DNA pellet was air dried and resuspended in 5-10µl of nuclease-free H₂O.

4.3.7.3. Cloning of purified gene fragments.

The purified fragments were ligated into the pBluescript derivative, pPCR-Script AMP SK (+) and cloned into Epicurian Coli XL-1 Blue MRF' Kan supercompetent cells, according to the manufacturers instructions (Stratagene®, UK). These cells were then grown overnight at 37°C, on L-agar plates containing 50µg/ml ampicillin, IPTG (20µl of a 23.8mg/ml (H₂O) stock) and X-gal (35µL of a 50mg/ml (dimethylformamide) stock). Clones which contain an insert appear as white colonies on these plates, while those without insert appear blue. Positive clones were subcultured onto L agar* master plates containing 50µg/ml ampicillin, grown overnight at 37°C and screened by PCR using the MTHFR/CBS/MS primers, for insert size.

*L-agar: 10g/L tryptone, 5g/L yeast extract, 5g/L NaCl, 15g/L Agar in deionised H₂O.

4.3.7.4. Purification of plasmid DNA.

Using a sterile inoculating loop, a sample of each positive colony containing the correct insert, was taken and added to 10mls of Lennox broth (L-broth)* containing 50µg/ml ampicillin and grown overnight at 37°C in a shaking incubator (200rpm). Plasmid

DNA was recovered using the Promega Wizard® *Plus* SV miniprep kit as instructed by the manufacturers.

*L-broth: 10g/L tryptone, 5g/L yeast extract, 5g/L NaCl in deionised H₂O.

4.3.7.5. Sequencing of cloned gene fragments.

Plasmid DNA was sequenced using the Amersham Thermo Sequenase fluorescent labelled primer cycle sequencing kit. A master mix was prepared for each clone and contained 1µg of plasmid DNA, 10pmol of primer and H₂O to a final volume of 25µl. To 6µl of the master mix, 2µl of Reagent A/C/G/T was added. (Each reagent contains buffer, dNTPs, DNA polymerase and a dideoxy analogue of one particular base). The reaction mixture was overlaid with 20µl of mineral oil to prevent evaporation during thermocycling. The samples were then cycle sequenced under the following conditions: 94°C x 45s (denaturing), 55°C x 45s (annealing), 72°C x 45s (extension), for 30 cycles followed by a final extension of 72°C x 2mins.

Formamide stop solution (4µl) was then added to each sample. The purpose of this was two-fold - i) it prevented further DNA synthesis and ii) it contained loading dye for electrophoresis. 8µl of the A, C, G, T reactions were loaded onto 12% (v/v) acrylamide gels on the Alf Express™ automated DNA sequencer and the sequence of the insert was thus determined.

4.3.7.6. BLAST homology search.

All sequences generated as outlined above were aligned with the known DNA/cDNA sequences of the MTHFR, MS and CBS genes in the Genome sequence database using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al*, 1990). This verified that the cloned insert was that of the PCR amplified region of the MTHFR, MS or CBS

4.4. RESULTS.

4.4.1. Study subject details.

Tables 4.2 and 4.3 illustrate some of the parameters of the subjects investigated in this study. Control subjects were healthy laboratory staff volunteers, who showed no elevation in fasting plasma homocysteine (*ie* fasting plasma homocysteine $<12\mu\text{M}$) and were replete in both folate and vitamin B₁₂ (Graham *et al*, 1997).

Vascular disease patients were recruited by Dr. Raymond Meleady, at the Adelaide Hospital, Dublin. The vascular disease group comprised of seven patients, who suffered from coronary heart disease, cerebrovascular disease or peripheral vascular disease. Four of these were considered to be hyperhomocysteinemic (fasting plasma homocysteine $\geq 12\mu\text{M}$) and three were considered to have homocysteine levels within the normal range. The vascular disease patients who participated in the study presented here, were not chosen at random. Six of the seven vascular disease subjects had participated in the COMAC study, thus their homocysteine status was known and they were deemed suitable for this particular study which required hyperhomocysteinemic vascular disease subjects. The remaining subject (TC3) was chosen from those attending the Adelaide Hospital, as he presented with vascular disease and did not have hyperlipidemia. The biochemical data presented in Tables 4.2 and 4.3, represent the status of the individuals at the time of this study and in the case of the vascular disease subjects, were in close agreement with the values determined two years previously, for the COMAC study.

Statistical analyses, using unpaired Students t-tests, of the differences in the parameters determined in the control and vascular disease groups, are presented in Table 4.4. In the control group (Table 4.2) the subjects ranged from 23 to 50 years of age, with a mean of 28.2yrs. The mean age of the vascular disease group (48.7yrs) was significantly higher compared to these controls ($p < 0.05$).

Plasma homocysteine was significantly elevated not only when the hyperhomocysteinemic vascular disease subjects ($n=4$) were compared to the controls but also when the vascular disease group as a whole was compared to the controls ($p < 0.05$).

Differences in plasma homocysteine appeared to be related to serum folate levels. Folate levels and not vitamin B₁₂ levels, were notably reduced in the vascular disease group compared to the healthy controls ($p < 0.04$). These differences in folate were even more significant when the controls were compared to the hyperhomocysteinemic contingent of the vascular disease group ($p < 0.03$).

In Fig. 4.1 some associations between homocysteine and age, folate and B₁₂ are presented, using all the subjects involved in the study and one elderly subject whose metabolic profile was not determined. The general trends illustrated, *ie* a trend towards a positive correlation between homocysteine and age, and a negative correlation between homocysteine and folate and B₁₂, follow those described in the literature (Selhub *et al*, 1999). However, the only variable in this study to have any statistically significant effect on plasma homocysteine was folate ($t = 2.49$, $p < 0.05$). This link had also been suggested by the data presented in Table 4.3, where significant differences in plasma homocysteine were mirrored by differences in serum folate but not B₁₂ levels.

4.4.2. Methionine cycle enzyme genotypes.

The presence of common genotypes associated with an elevation in plasma homocysteine were assessed as described in section 4.3.4 – 4.3.6. The single base pair changes associated with the MTHFR TT, CBS I278T and the MS D919G genotypes were detected by PCR-RFLP (Fig. 4.2 - 4.4). In panel A of Figs. 4.2 - 4.4, the separation of the digested PCR products is shown. These point to the presence or absence of the particular polymorphism. In each case, an attempt was made to verify

the presence of the RFLP by sequencing, followed by BLAST analysis (Figs. 4.2 - 4.4, panel B and C). This sequencing verified the presence of the single base change in both the MTHFR and MS gene fragments. The CBS I278T gene fragment proved more difficult to sequence and 100% identity could not be accomplished. In this case, sequencing verified the presence of the 68bp insert but the presence of the T-C base change, that results in the I278T genotype could not be confirmed (Fig 4.3, panel B and C).

None of the vascular disease subjects had either the MS D919G variant or the CBS I278T variants (Table 4.3). Three of the controls were heterozygous for the I278T genotype (Table 4.2). However, in each case, the genotype was present in conjunction with the 68bp insert (844ins68bp) that as a consequence of alternative splicing, results in the production of a functional protein (Tsai *et al*, 1996b). Two controls (IS6 and JF5) demonstrated the D919G polymorphism, in the homozygous and heterozygous state respectively. Three controls and three vascular disease subjects had the MTHFR C677T substitution. The possible impact of these genotypes will be discussed later.

4.4.3. Methionine metabolic profiles of the control subjects.

Table 4.5 provides a summary of the labelling patterns seen in each individual, 2hrs after the addition of [35 S]-methionine to the cell cultures. Each of the control metabolic profiles (like that in Fig. 3.8) demonstrated labelling patterns suggestive of activity of both the remethylation and transsulphuration pathways. Fig. 4.5 is a representation of the composite methionine metabolic profile of the nine control (non-hyperhomocysteinemic) vascular disease-free subjects. Panel A shows the distribution of the label in the metabolites of the methionine metabolic pathway.

The label, supplied in the form of [35 S] -methionine, was found to accumulate in the cells over the course of a four hours exposure (Fig. 3.6, Chapter 3). As can be seen in

Fig. 4.5, panel A, over the course of this exposure, the proportion of the label in methionine fell, indicating that it was metabolised by the cells. Its appearance in cystathionine, cysteine, homocysteine, SAH and SAM indicate that it was being effectively utilised by the methionine catabolic pathway. Its appearance in protein suggests that it was being used in protein synthesis, either as methionine or perhaps as cysteine (Panel B). Alternatively, its appearance in protein could result, to perhaps a slight extent, from the binding of labelled species *eg* homocysteine.

While the label was lost from methionine, it appeared predominantly in the adenosylated products SAH and SAM, suggesting extensive activity of the methionine remethylation pathway. In all cases, the majority of this label (>65%) was found in SAM.

Activity of the transsulphuration pathway was also evident as indicated by the appearance and accumulation of the label in cystathionine and cysteine. With the exception of subject TF6, in all cases examined, the majority of the labelling accredited to cystathionine/cysteine was found in cystathionine (>65%). The remethylation pathway appeared to be relatively more active, with $55.9 \pm 5.9\%$ (mean $\pm$ SEM) of the label found in products of the remethylation pathway and $22.9 \pm 4.8\%$ (mean $\pm$ SEM) found in products of the transsulphuration pathway at four hours. At no time was there any evidence of accumulation of the label in homocysteine. Maximum labelling of homocysteine occurred at 2hrs, where $1.9 \pm 0.04\%$ (mean $\pm$ SEM) of the label was found in this amino acid.

As can be seen from the data presented in Table 4.5, the percentage of the radioactivity appearing in the transsulphuration products varied considerably, so that the controls could be broadly classified into two discrete groups - those who demonstrated a relatively high activity of the transsulphuration pathway (>10% labelled cystathionine/cysteine) and those who demonstrated a lower rate (<10% labelled

cystathionine/cysteine). Based on this data, composite profiles representing these two subsets of the controls were constructed (Fig. 4.6). The labelling of homocysteine and methionine did not differ significantly between the two groups. Where labelling of the transsulphuration products was high (Fig. 4.6, panel A), the labelling of the remethylation products was concurrently low and *vice versa* (Fig. 4.6, panel B). Therefore it appears that although the activities of the remethylation and transsulphuration pathways vary to a certain degree in different control subjects, they operate at a level sufficient to prevent cellular accumulation of the label in homocysteine.

The control subjects, who did not have elevated plasma homocysteine, demonstrated that steady state conditions, whereby homocysteine was metabolised *via* the transsulphuration and remethylation pathways, were established rapidly, preventing significant accumulation of homocysteine.

Overall, the vascular disease group demonstrated a much greater heterogeneity with respect to these labelling patterns. As a consequence of this, their profiles are presented individually in Figs. 4.7 - 4.13.

An increased labelling of cellular protein over the four hour study period, was observed in the controls. This indicates that the cells were highly metabolically active, synthesising new proteins (Fig. 4.5, panel B).

SAM plays an important role in cell metabolism, as discussed in Chapter 1 and might be expected to be elevated in highly active cells. Based on this hypothesis that elevated SAM may be a reflection of protein synthesis, a plot of the extent of radiolabelling of proteins *vs* the extent of radiolabelling of SAM was constructed. A positive correlation between SAM and protein manufacture was found ($t=5.9$, $p<0.01$)(Fig 4.7).

4.4.4. Methionine metabolic profiles of the vascular disease subjects.

The methionine metabolic profile of MF1, a 61yr old female with peripheral vascular disease is presented in Fig 4.8. Her metabolic profile was in striking contrast to that of the controls. The loss of label from methionine was more dramatic, 30mins after the addition of the label to the cells. While in the controls it fell to approximately 45%, it fell to 18% in this subject and remained low over the following four hours. One hour after labelling, the extent of remethylation was comparable to the controls, but by 2hrs was just half that of the controls and less again at 4hrs. Very high rates of transsulphuration were observed, higher than was seen in any of the controls. The proportion of the label increased in the transsulphuration products over time, with almost 80% of the label appearing in cystathionine and cysteine at 4hrs. Consistent with the biochemical findings there was no accumulation of the label in homocysteine. There appeared to be no significant differences in the partitioning of the label between the protein and non-protein fraction (Fig 4.8, panel B). This subject did not have an elevated fasting plasma homocysteine, was replete in folate (8.7µg/L) and B₁₂, although at 167.3ng/L, her B₁₂ was close to deficiency. In addition, it was determined that this patient was homozygous for the C₆₇₇T MTHFR genotype, and was wild-type for both the CBS I278T and MS D919G genotypes.

The methionine metabolic profile of JC2, a 55yr old male with coronary heart disease is presented in Fig 4.9. Here, the most striking difference compared to the control profile is the almost complete lack of activity in the transsulphuration pathway, with the label never exceeding 5% in both cystathionine and cysteine. The label did not accumulate in homocysteine over the study period of 4hrs. This is most likely due to the high activity of the remethylation pathway. An excess of label was found in the protein fraction in comparison to the controls, at each time point (Fig 4.9, panel B). This is in keeping with the hypothesis that remethylation may be a reflection of protein synthesis. This subject

had a normal fasting plasma homocysteine, was replete in both folate (11.8 μ g/L) and B₁₂ (353.0ng/L). In addition, it was found that this patient was homozygous for the C677T MTHFR genotype. The presence of the CBS I278T genotype and the MS D919G could not be assessed, as the patient was unavailable when that part of the study was undertaken.

Fig. 4.10 shows the methionine metabolic profile of TC3, a 37yr old male who had coronary artery disease. The profile was quite similar to that of the controls, particularly, the controls who demonstrated low labelling of cystathionine and cysteine. Again in line with the biochemical findings, there was no accumulation of homocysteine at the cellular level. There was no apparent difference in the partitioning of the label between the protein and cell extracts, compared to the controls. This patient was wild-type with respect to the three genotypes investigated. This was the third of the vascular disease patients who had a normal fasting plasma homocysteine (10.3 μ M) and was replete in both folate (5.5 μ g/L) and B₁₂ (727.5ng/L).

CM4 was a 54yr old male with peripheral vascular disease. The methionine metabolic profile for this patient is shown in Fig. 4.11. There appeared to be no significant difference in the labelling of the transsulphuration products (cystathionine and cysteine) compared to the controls, but a significant increase in the labelling of the remethylation intermediates, SAH and SAM was seen. There was a trend towards increased accumulation of the label in homocysteine and a lower labelling of methionine, in comparison to the controls. There appeared to be a trend towards increased cellular labelling of proteins compared to controls. This trend was mirrored by extensive labelling of SAH/SAM (Fig 4.11, panel B). This patient was deemed to be hyperhomocysteinemic as he had a fasting plasma homocysteine of 21.9 μ M. Fasting serum folate and B₁₂ were 1.1 μ g/L and 250.1ng/L respectively, thus making him

deficient in folate. This subject was found to be wild-type for the genotypes investigated.

The methionine metabolic profile for vascular disease subject HK5 is presented in Fig. 4.12. The profile was comparable to the controls, but differed in a marked accumulation of the label in homocysteine. Labelling of the transsulphuration products was comparable to, though slightly lower than that of, the low transsulphurating controls. No reduction in the activity of the remethylation pathway was observed in this patient who had the MTHFR TT genotype. The evidence points to defective transsulphuration being the possible cause of the accumulation of homocysteine in this individual. There was no significant difference in the labelling of cellular protein compared to the controls. This 56yr old male was hyperhomocysteinemic (fasting plasma homocysteine of $13.8\mu\text{M}$), and was replete in both folate and B_{12} ($5.0\mu\text{g/L}$ and 251.8ng/L respectively).

The 26yr old cerebrovascular patient TF6 presented the most striking methionine metabolic profile (Fig. 4.13a). There was rapid and significant transfer of the label to homocysteine, with modest accumulation in cystathionine/cysteine, and significantly reduced labelling of SAH and SAM. Activity through the transsulphuration pathway was comparable to that of the low transsulphurating controls. Therefore it appears that the accumulation of the label in homocysteine may be as a result of defective remethylation. The subject did not appear to have any gross vitamin deficiency, and although his folate was one of the lowest of the study group, it did fall within the normal range. This individual was wild-type with respect to the MTHFR genotype. MS and CBS genotypes were not assessed, as the subject was unavailable at the time of this analysis.

It is interesting to note that while labelling of SAH/SAM remained low, so too did labelling of protein (Fig 4.13, panel B). A shift in the proportions of radiolabelling of

the protein and non-protein fractions, in comparison to the controls, took place. This was the only subject studied who demonstrated a majority of labelling in the non-protein cellular extract. Unfortunately, this patient was unavailable for further study and so the experiment was never duplicated. However, in an effort to verify this unusual profile an autoradiographic image of one of the extracts separated by TLC was prepared. It verified the extensive labelling of the homocysteine in the cell extracts of this subject (Fig. 4.13b).

EB7 was a 52yr old hyperhomocysteinemic peripheral vascular disease patient (fasting plasma homocysteine of $15.6\mu\text{M}$), replete in folate and B_{12} ($6.6\mu\text{g/L}$ and 221.4ng/L respectively). There were no significant differences in his metabolic profile compared to the controls (Fig. 4.14). While there was a trend towards increased labelling of homocysteine up to one hour but this was not sustained. Thus although an elevated plasma homocysteine was observed, it was not followed by a significantly enhanced accumulation of homocysteine at the cellular level. There was a slight bias towards increased labelling of the protein *vs* the non-protein components of the cell preparations, compared to the controls (Fig.4.14, panel B). Because this subject had presented with an elevated plasma homocysteine, and did not show an accumulation of homocysteine at the cellular level, its presence in the culture medium was assessed, as described in section 4.3.2. There appeared to be no significant difference in the proportion of label in homocysteine in the medium of this subject compared to a control. However, no conclusions could be drawn from these results as (and this will be discussed later), the methodology did not prove sensitive enough. When radiolabelled methionine was added to culture medium (that had not been exposed to cells) and subjected to separation by thin layer chromatography, the results were almost identical, *ie* the excess of radiolabelled methionine in the culture medium, makes the accurate quantitation of radiolabelled homocysteine, exported from the cells, impossible.

Primer	Sequence	Reference
MTHFR 1	5' TGA AGG AGA AGG TGT CTG CGG GA 3'	Frosst <i>et al</i> , 1995
MTHFR 2	5' AGG ACG GTG CGG TGA GAG TG 3'	Frosst <i>et al</i> , 1995
S:I3	5' GCA GTT GTT AAC GGC GGT AT 3'	Gallagher, PhD thesis, 1996
A: I7	5' TGC GAG GAT GGA CCC TTC GG 3'	Gallagher, PhD thesis, 1996
MS28F	5' GTT GTG TTC CCA GCT GTT AGA TG 3'	Van der Put <i>et al</i> , 1997
MS30R	5' GAC ACT GAA GAC CTC TGA TTT GAA C 3' (intronic)	Van der Put <i>et al</i> , 1997

	Cycling conditions	Product Size	[MgCl ₂]	[dNTP]	[Primer]
MTHFR	94°C 1 min, 62°C 1 min, 72°C 1 min, 40 cycles.	198bp	1.5mM	300µM	0.2µM
CBS	94°C 1 min, 62°C 2 min, 72°C 1 min, 35 cycles.	139/207bp	1.0mM	200µM	0.5µM
MS	92°C 1 min, 56°C 1 min, 72°C 1.5min, 35 cycles.	265bp	1.0mM	200µM	0.4µM

Table 4.1 Primer sequences and conditions used to amplify a region of the MTHFR, CBS and MS genes.

A: This shows the primer sequences used for PCR-RFLP analysis.

B: This shows the concentrations of the components used in each PCR using the primers described above. Each of the reactions had 100ng DNA as template, 0.5IU of *Taq* polymerase and the appropriate buffer and water were added to a final volume of 25µL. Each reaction was overlaid with 5µL of mineral oil to prevent evaporation during thermocycling. In addition, each PCR was subject to a "hot start" (95°C x 5mins) to ensure complete denaturation of the DNA and a final extension of 72 °C for 10mins.

Subject	Age	Sex	Plasma hcy	Serum folate	Serum B ₁₂	MTHFR	CBS	MS
	(yrs)		(μ M)	(μ g/L)	(ng/L)	(C677T)	(I278T)	(D919G)
VB1	24	F	6.5	7.6	491.5	--	--	--
CH2	23	F	8.8	23.2	479.6	--	Hetero	--
EF3	23	F	9.1	7.3	234.1	Homo	--	--
DO4	50	F	10.4	14.6	383.0	--	--	--
JF5	41	F	9.0	11.7	254.0	Homo	--	Hetero
IS6	23	M	11.4	7.1	244.4	--	--	Homo
BD7	21	M	7.3	8.4	658.0	--	--	--
DH8	23	M	9.1	12.5	244.2	--	Hetero	--
DC9	26	M	8.6	11.4	407.0	Hetero	Hetero	--

Table 4.2 Biochemical parameters, genotypes and age of the control (vascular disease-free) subjects.

The age of the subjects ranged from 21-50yrs. The above subjects were healthy laboratory staff volunteers.

Hyperhomocysteinemia was defined as a fasting plasma homocysteine of $>12\mu$ M (Graham *et al*, 1997). None of these subjects was considered to be hyperhomocysteinemic. Normal ranges of B₁₂ and folate were accepted as fasting serum levels of 160-970ng/L and $>1.5\mu$ g/L respectively. Homocysteine, B₁₂ and folate analysis were done on fasting samples.

None of the subjects were considered to have a deficiency in either vitamin.

M: male, F: female, --: wild type genotype, Hetero: heterozygous for that polymorphism, Homo: homozygous for that polymorphism.

Subject	Age (yrs)	Sex	Plasma hcy (μ M)	Serum folate (μ g/L)	Serum B ₁₂ (ng/L)	MTHFR (C677T)	CBS (I278T)	MS (D919G)
MF1	61	F	7.2	8.7	167.3	Homo	--	--
JC2	55	M	9.9	11.8	353.0	Homo	ND	ND
TC3	37	M	10.1	5.5	727.5	--	--	--
CM4	54	M	21.9*	1.1†	250.1	--	--	--
HK5	56	M	13.8*	5.0	251.8	Homo	ND	ND
TF6	26	M	15.7*	4.4	674	--	ND	ND
EB7	52	M	15.6*	6.6	221.4	--	--	--

Table 4.3 Biochemical parameters, genotypes and age of the vascular disease subjects.

The age of the subjects ranged from 26-61yrs. The above subjects were being treated at the Adelaide Hospital, Dublin for vascular disease (cerebral, peripheral and coronary). Hyperhomocysteinemia was defined as a fasting plasma homocysteine of $>12\mu$ M (Graham *et al*, 1997).

Normal ranges of B₁₂ and folate were accepted as 160-970ng/L and $>1.5\mu$ g/L respectively. Homocysteine, B₁₂ and folate analysis were done on fasting samples.

* indicates subjects that were considered to be hyperhomocysteinemic.

† indicates nutritional deficiency in that vitamin.

M: male, F: female, --: wild type genotype, ND: analysis not done, Hetero: heterozygous for that polymorphism, Homo: homozygous for that polymorphism.

	Age (yrs)	Hcy (μ M)	Folate (μ g/L)	B ₁₂ (ng/L)
Controls (n=9)	28.2 $\pm$ 3.4	8.9 $\pm$ 0.5	11.5 $\pm$ 1.7	377.3 $\pm$ 49.2
Vascular disease subjects (n=7)	48.7 $\pm$ 4.4 *	13.5 $\pm$ 1.7*	6.2 $\pm$ 1.2*	377.9 $\pm$ 80.8
Hyperhomocysteinemic vascular disease subjects (n=4)	47.0 $\pm$ 6.3*	16.8 $\pm$ 1.6*	4.3 $\pm$ 1.0*	349.0 $\pm$ 97.3

Table 4.4 Parameters of the study groups.

Results are presented as mean $\pm$ SEM. Values for homocysteine, folate and B₁₂ represent fasting levels. Controls were healthy laboratory staff volunteers. It should be noted that the hyperhomocysteinemic vascular disease subjects are a subset (4/7) of the vascular disease group.

* denotes statistically significant differences when compared to the control group (Students t-test).

	Cysta	Cys	Hcy	Met	SAH	SAM
Controls						
VB1	23.0	7.0	1.1	35.6	11.1	22.2
CH2	17.5	5.2	2.1	45.1	2.7	27.4
EF3	14.5	6.4	2.0	33.1	4.6	39.4
ID6	9.3	1.4	1.2	23.5	5.9	58.7
DH8	5.0	1.2	1.6	13.8	6.9	71.5
DC9	5.4	0.6	1.8	4.3	5.1	82.8
D04	5.1	1.1	2.5	23.4	8.5	59.4
BD7	3.4	1.0	2.2	18.2	7.5	67.7
JF5	3.7	0.9	1.9	64.6	7.9	21.0
Vascular disease subjects						
MF 1	60.8	2.6	1.1	9.3	1.3	24.9
JC 2	2.8	0.4	1.4	27.6	8.4	59.4
TC 3	6.3	2.4	1.2	34.7	4.8	50.6
CM 4	16.3	3.4	2.6	14.7	6.6	56.4
HK 5	4.0	0.4	4.9	29.2	7.8	53.7
TF 6	3.5	3.9	85.1	3.9	0.5	3.1
EB 7	10.4	1.5	1.5	20.6	4.8	61.2

Table 4.5 Percentage CPM in methionine metabolites of control and vascular disease subjects following 2hrs exposure to radio-labelled methionine.

Numbers represent % of radio-label in that metabolite, and are the mean of two experiments. Both experiments were highly comparable, so that each set of duplicate values were within 10% of one another.

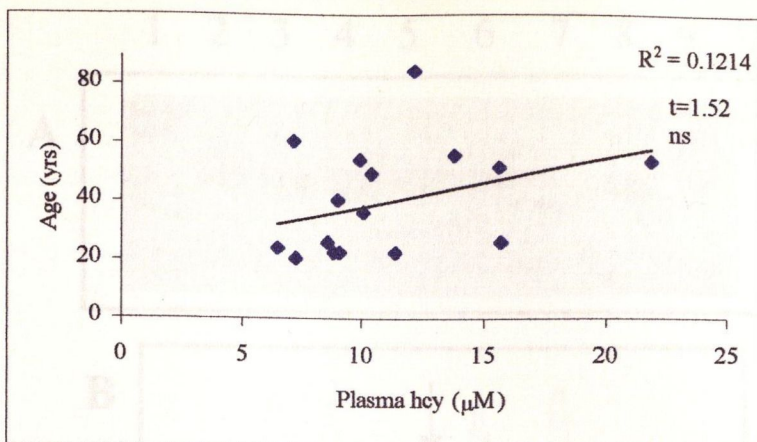
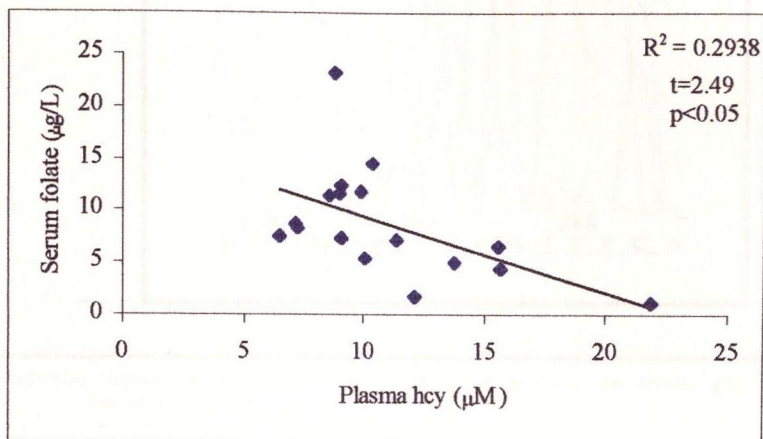
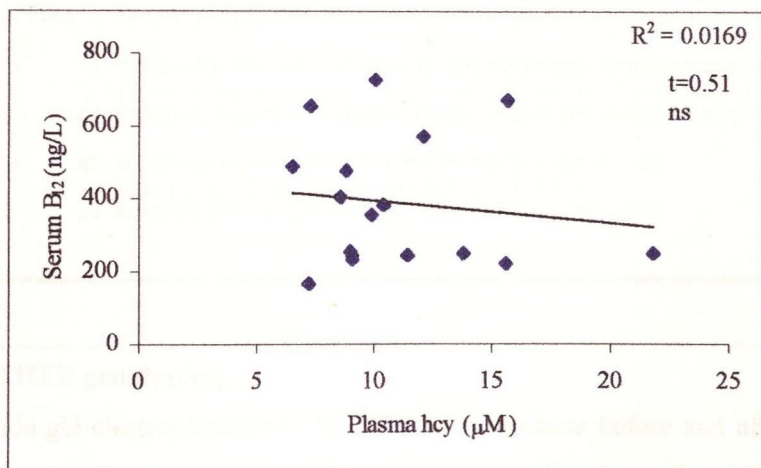
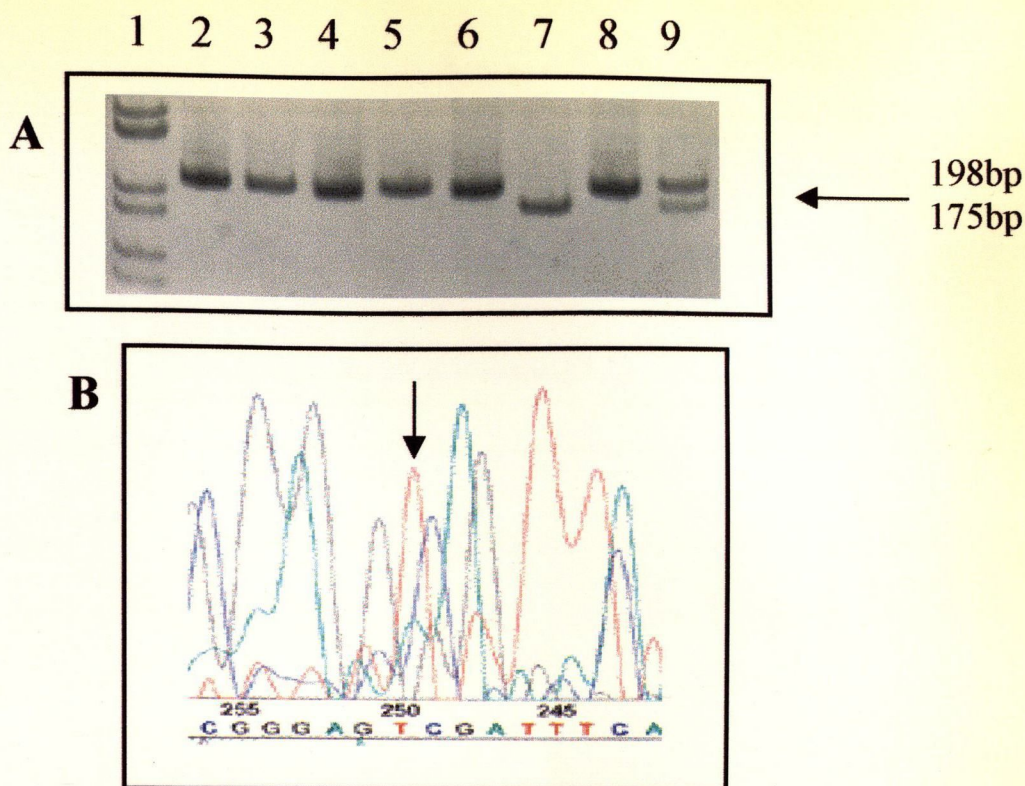
A**B****C**

Fig. 4.1 Correlations between age (A), folate status (B), vitamin B_{12} status (C) and homocysteine, for all subjects in the study, *ie* both healthy and vascular disease subjects ($n=17$).

Best fit lines and R^2 and p values were calculated by Microsoft® Excel 97 graphics and statistics package. None of the correlations were particularly strong (implied by the R^2 and t values) but some trends were suggested. There was a weak positive correlation between homocysteine and age, a negative correlation between homocysteine and folate and a much weaker negative correlation between homocysteine and B_{12} levels. Folate was the only variable that had a statistically significant effect on homocysteine concentration ($p < 0.05$). (ns: non-significant).



C

>gb:HSU09806 Human methylenetetrahydrofolate reductase mRNA, partial cds.
Length = 2187

Score = 486 (134.3 bits), Expect = 5.5e-46, Sum P(2) = 5.5e-46
Identities = 98/99 (98%), Positives = 98/99 (98%), Strand = Plus / Plus

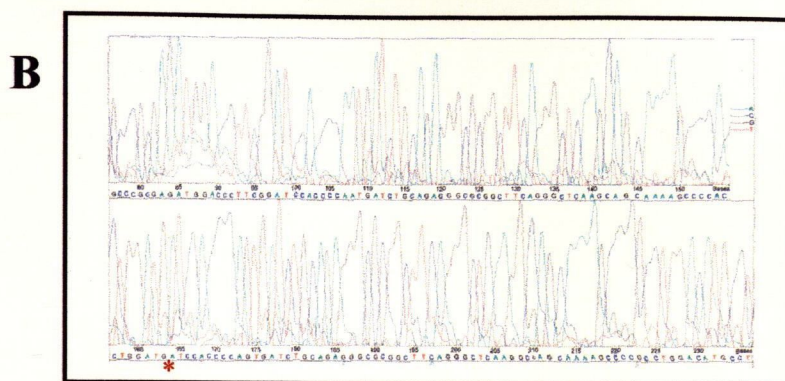
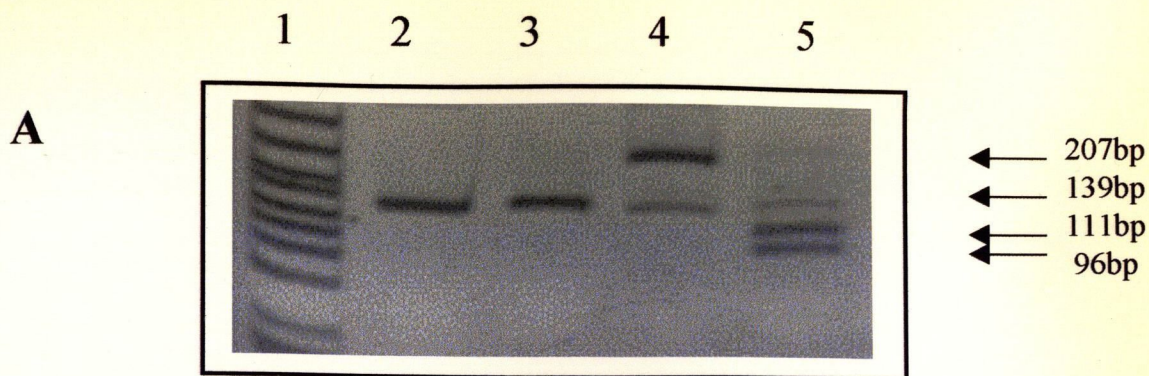
JF5 : 2 TGAAGGAGAAGGTGTCTGCGGGAGTCGATTTTCATCATCACGCAGCTTTTCTTGAGGCTG 61
|||||
MTHFR: 644 TGAAGGAGAAGGTGTCTGCGGGAGCCGATTTTCATCATCACGCAGCTTTTCTTGAGGCTG 703
*
JF5 : 62 ACACATTCTTCCGCTTTGTGAAGGCATGCACCGACATGG 100
|||||
MTHFR: 704 ACACATTCTTCCGCTTTGTGAAGGCATGCACCGACATGG 742

Fig. 4.2 MTHFR genotyping.

A: Acrylamide gel electrophoresis of MTHFR PCR products before and after digestion with *HinfI*. Lane 1: pBS DNA *Hae* III/*Bam* HI molecular weight marker, lanes 2 and 3: pre- and post-digestion (VB1), lanes 4 and 5: pre- and post-digestion (EB7), lanes 6 and 7: pre- and post-digestion (JF5), lanes 8 and 9: pre- and post-digestion (DC9). The presence of the C-T substitution at base pair 677, allows digestion of the PCR products to 175 and 23bp. VB1 and EB7 are wild-type for this polymorphism. JF5 is a C-T homozygote and DC9 is heterozygous for this polymorphism.

B: DNA sequence of the TT homozygote identified in panel A. This confirms the presence of the C-T transition (indicated by the arrow) that allows digestion by *HinfI*.

C: Alignment of the sequence (illustrated in panel B) with the human methylenetetrahydrofolate reductase gene, using BLAST. The C-T substitution is marked by an asterisk *.



C

gb|S78267|S78267 cystathionine beta-synthase {insertion mutant} [human, homocystinuria patient 12, skin fibroblasts, Genomic; Mutant, 120 nt]
Length = 120

Score = 157 bits (79), Expect = 7e-37
Identities = 95/98 (96%), Gaps = 3/98 (3%)
Strand = Plus / Minus

Query: 6 gatggacccttcgg-atccacccaatgatctgcagagggcgcggttcagggtcaag- 63
|||||
Sbjct: 98 gatggacccttcgggatccacccaatgatctgcagagggcgcggttcagggtcaagc 39

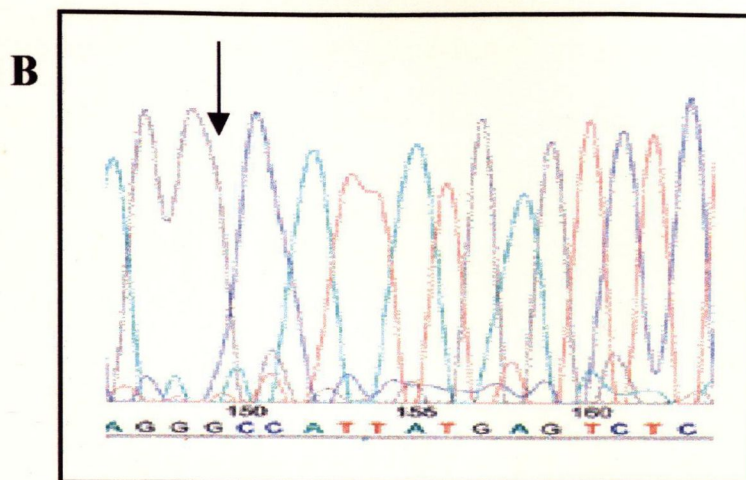
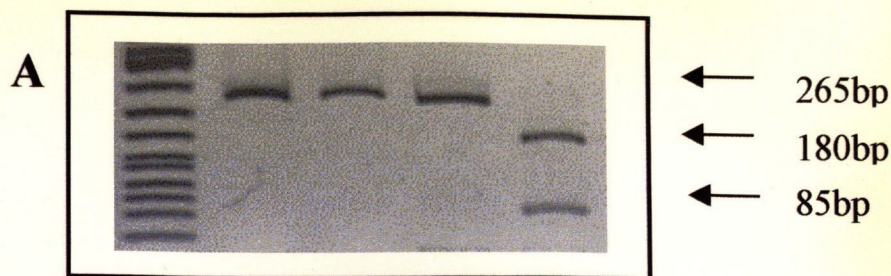
Query: 64 -cagcaaaaagccccacctggatgatccacccagtgat 100
|||||
Sbjct: 38 ccagcaaaaagccccacctggatgatccacccagtgat

Fig. 4.3 CBS genotyping.

A: Acrylamide gel electrophoresis of I278T PCR products before and after digestion with *Bsr* I. Lane 1: ρ X174 DNA/*Hinf*I molecular weight marker, lanes 2 and 3: pre- and post- digestion (VB1), lanes 4 and 5: pre- and post- digestion (CH2). The presence of the polymorphism allows digestion by *Bsr* I to yield products of 111 and 28bp. However, in this case, the polymorphism occurs in *cis* with a 68bp insert, so that the resulting PCR product is 207bp and the products of digestion by *Bsr* I, in the presence of the I278T polymorphism are 111 and 96bp, as can be seen on the gel above. VB1 is wild-type and CH2 is a heterozygote for this polymorphism and the insert.

B: DNA sequence of the heterozygote identified in panel A. The insert is indicated by *

C: Alignment of the sequence illustrated in panel B, with the human cystathionine beta synthase gene, using BLAST. This confirms the presence of the insert, (indicated by underlining).



C

gb|U75743|HSU75743 Human methionine synthase mRNA, complete cds
Length = 7122

Score = 198 bits (100), Expect = 4e-49
Identities = 103/104 (99%)
Strand = Plus / Plus

Query: 2 ggtgtgttccagctgtagatgaaaatctaaaggatgaatactttgaggaaatcatgga 61
|
Sbjct: 2959 ggtgtgttccagctgtagatgaaaatctaaaggatgaatactttgaggaaatcatgga 3018

Query: 62 agaatatgaagatattagacagggccattatgagtctctcaagg 105
|
Sbjct: 3019 agaatatgaagatattagacaggaccattatgagtctctcaagg 3062

*

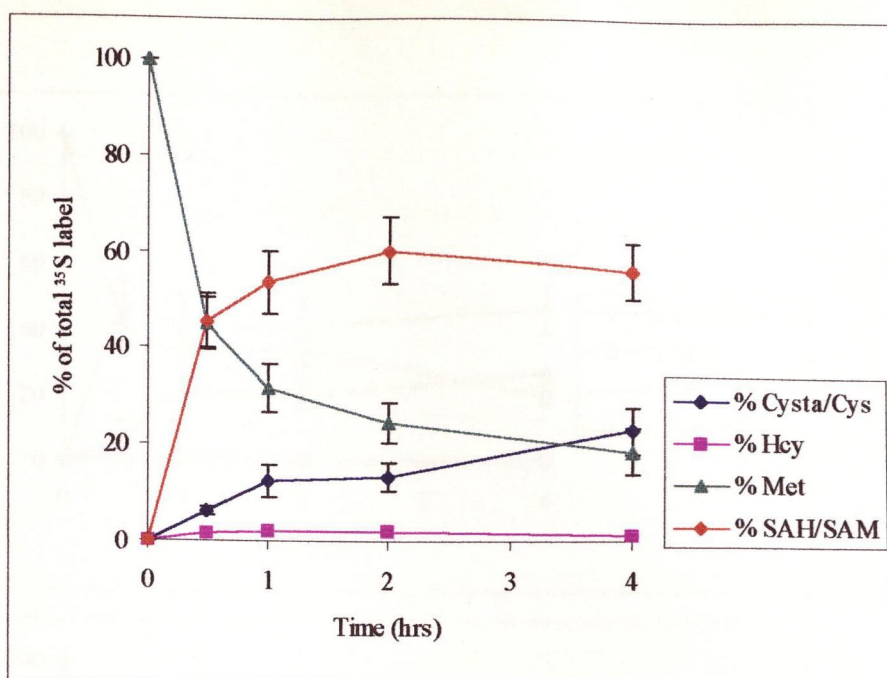
Fig. 4.4 MS genotyping.

A: Acrylamide gel electrophoresis of MS PCR products before and after digestion with *Hae* III. Lane 1: ρ X174 DNA/*Hinf*I marker, lanes 2 and 3: pre- and post- digestion (VB1), lanes 4 and 5: pre- and post- digestion (IS6). The presence of the G-A transition allows digestion by *Hae* III to yield products of 180 and 65bp. VB1 is wild-type and IS6 is homozygous for this polymorphism.

B: DNA sequence of the homozygote identified in panel A. This confirms the presence of the G-A transition (indicated by the arrow) that allows digestion by *Hae* III.

C: Alignment of the sequence illustrated in panel B, with the human methionine synthase gene, using BLAST. The G-A transition is marked by an asterisk *.

A



B

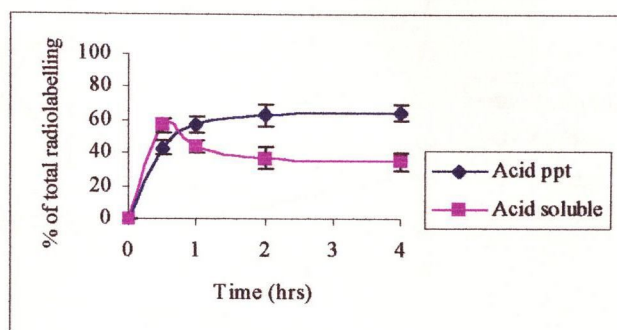


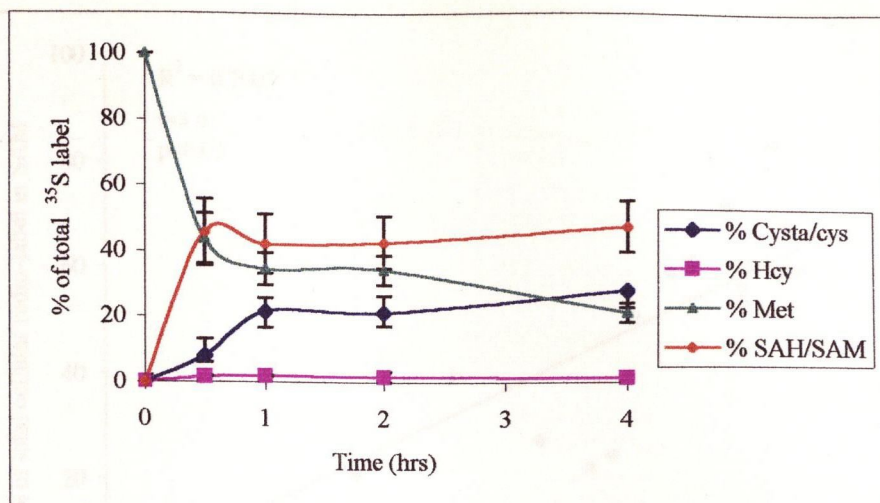
Fig. 4.5 Composite methionine metabolite profile of non-hyperhomocysteinemic vascular disease-free (control) subjects.

A: This represents the profiles of nine individuals, who were each analysed in duplicate as described in Chapter 3. Data shown represents mean $\pm$ SEM (n=9).

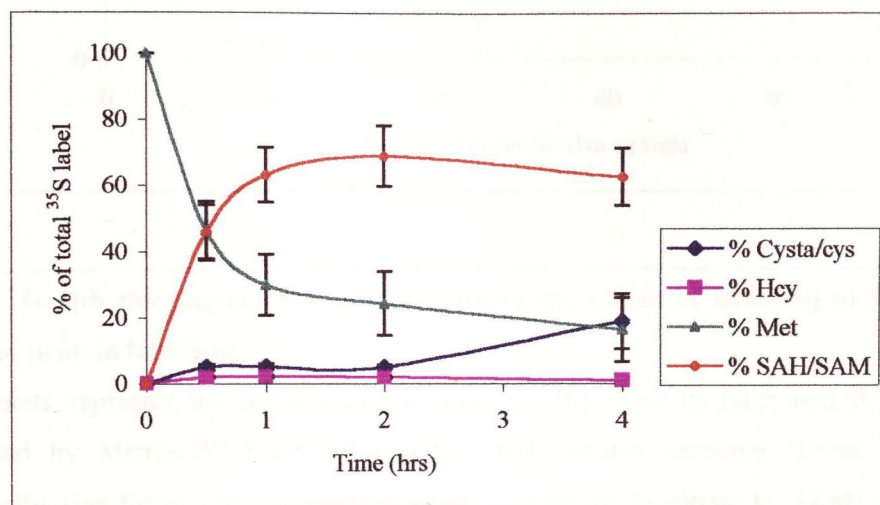
The proportion of radioactivity in cystathionine and cysteine (Cysta/cys) indicates activity of the transsulphuration pathway, while activity in the remethylation pathway is reflected by the proportion of radioactivity in SAH, SAM (SAH/SAM) and methionine.

The profile demonstrates modest activity of the transsulphuration pathway and extensive activity of the remethylation pathway, with no significant accumulation of the label in homocysteine.

B: This demonstrates the proportions of radioactivity that appeared in the acid precipitate (protein) and acid soluble (cell extract) fractions. At the earlier time points (0.5 and 1hr), radioactivity was almost equally represented in both fractions, but over time, more of the label appeared in the protein fraction, suggesting extensive protein synthesis.



A



B

Fig. 4.6 Methionine metabolic profile of non-hyperhomocysteinemic vascular disease-free (control) subjects.

A: A composite profile of the control subjects who demonstrated a relatively high rate of transsulphuration (n=4).

B: A composite profile of the control subjects who demonstrated a lower rate of transsulphuration (n=5).

Values shown represent mean $\pm$ SEM.

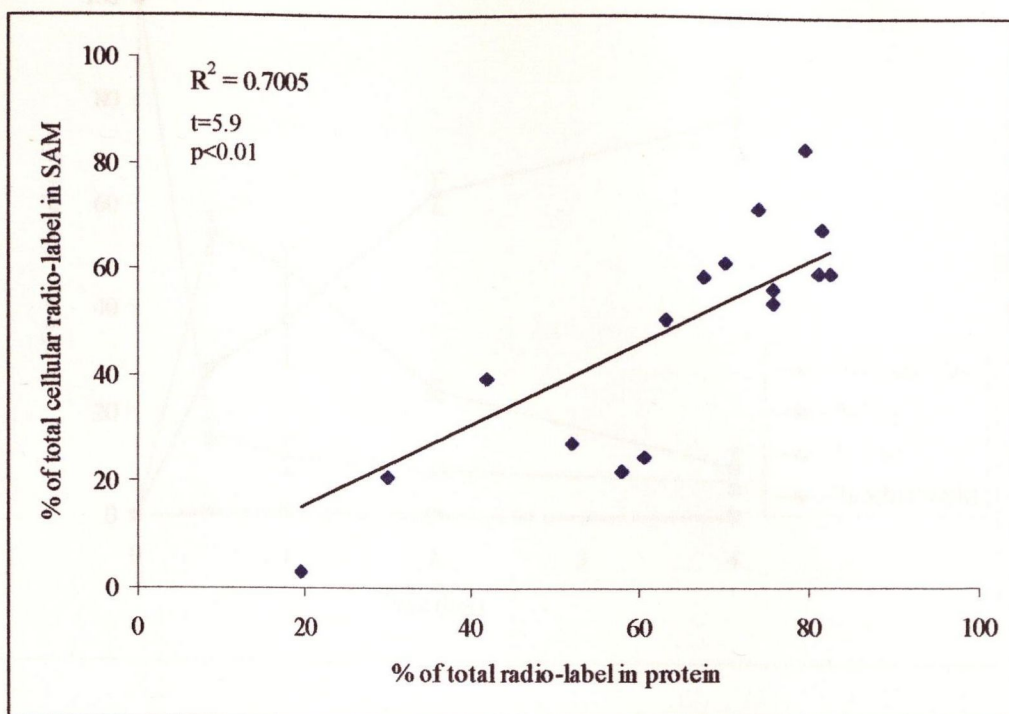
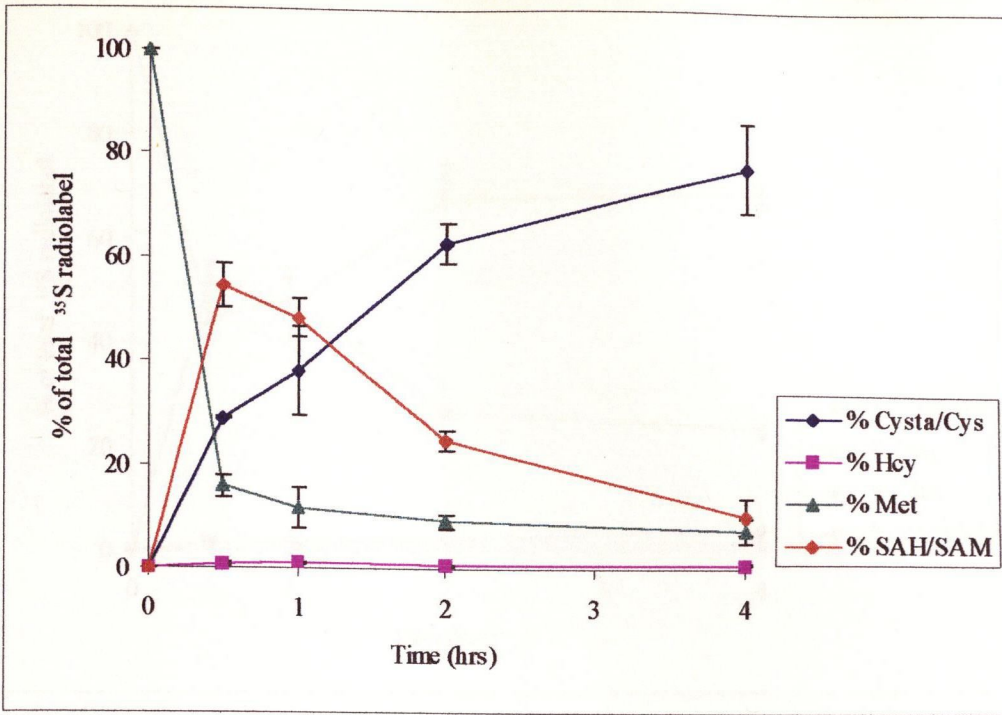


Fig 4.7 Graph showing the correlation between the extent of labelling of SAM and the extent of protein labelling.

Data points represent all subjects in the study ($n=16$). Best fit lines and R^2 values were calculated by Microsoft® Excel 97 graphics and statistics package. There was a direct statistically significant correlation between the extent of labelling in SAM and extent of labelling in protein ($p<0.01$).

A



B

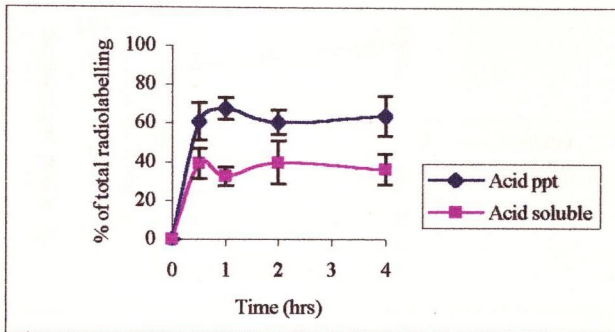


Fig. 4.8 Methionine metabolic profile of vascular disease subject MF1.

The illustration represents the distribution of ^{35}S radiolabel at the times specified above, after its addition to the cell culture media in the form of [^{35}S]-methionine.

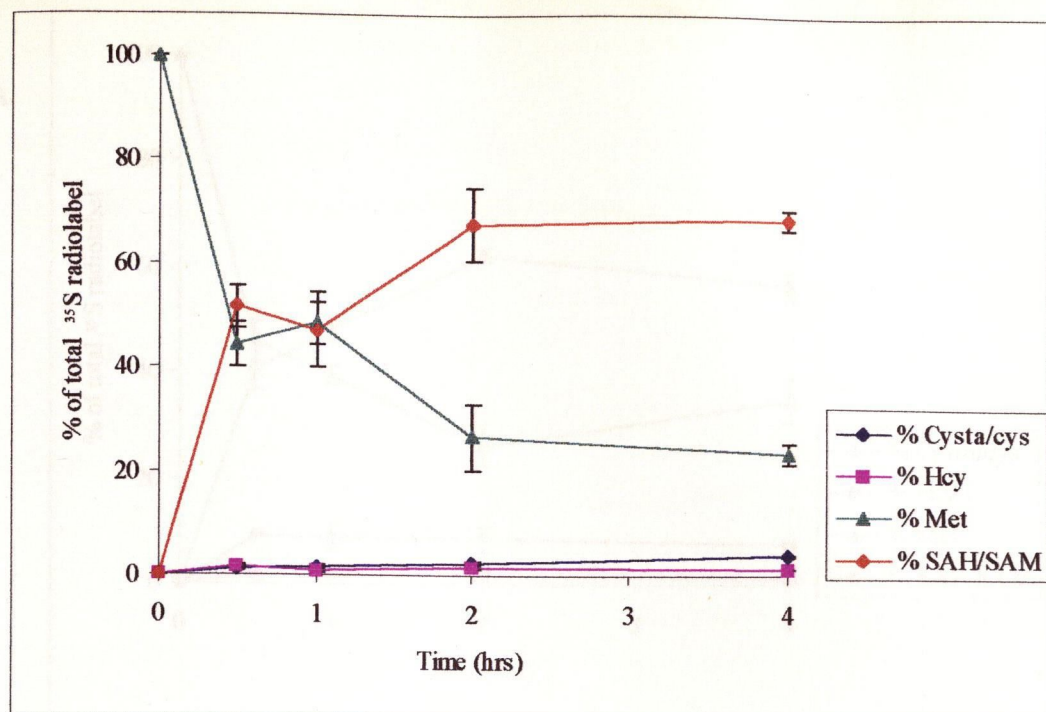
Data shown represents mean $\pm$ range of values found in duplicate experiments.

A: The proportion of radioactivity in Cysta/cys indicates activity of the transsulphuration pathway, while activity in the remethylation pathway is reflected by the proportion of radioactivity in SAH/SAM. The profile indicates early activity of the remethylation pathway, followed by extensive activity of the transsulphuration pathway and shows no accumulation of the label in homocysteine.

B: This demonstrates the proportions of radioactivity that appeared in the acid precipitate (ppt) (protein) and acid soluble (cell extract) fractions. At 0.5hrs, radioactivity was almost equally represented in both fractions, but over time, more of the label appeared in the protein fraction, suggesting extensive protein synthesis.

This subject (MF1) had a fasting homocysteine level within the normal range ($7.2\mu\text{M}$), demonstrated no nutritional deficiency and possessed the MTHFR TT genotype.

A



B

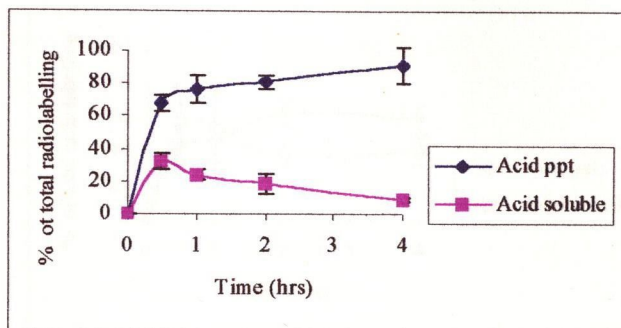


Fig. 4.9 Methionine metabolic profile of vascular disease subject JC2.

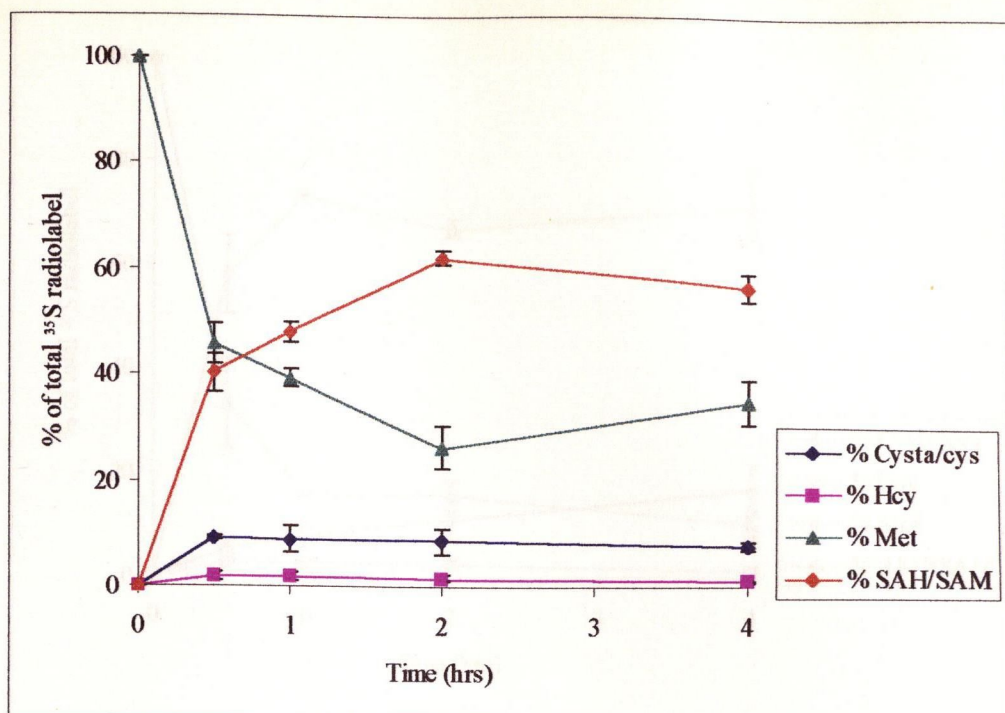
A: The illustration represents the distribution of ^{35}S radiolabel at the times specified above, after its addition to the cell culture media in the form of [^{35}S]-methionine.

Data shown represents mean $\pm$ range of values found in duplicate experiments. The proportion of radioactivity in Cysta/cys indicates activity of the transsulphuration pathway, while activity in the remethylation pathway is reflected by the proportion of radioactivity in SAH/SAM and methionine. The profile indicates very little activity of the transsulphuration pathway, while there appears to be extensive activity of the remethylation pathway with no accumulation of the label in homocysteine.

B: This demonstrates the proportions of radioactivity that appeared in the acid precipitate (protein) and acid soluble (cell extract) fractions. At each time point, the majority of the label was found in the protein fraction, suggesting extensive protein synthesis, in excess of that demonstrated by the controls (Fig. 5.2).

This subject (JC2) had fasting homocysteine levels within the normal range ($9.9\mu\text{M}$), demonstrated no nutritional deficiency and possessed the MTHFR TT genotype.

A



B

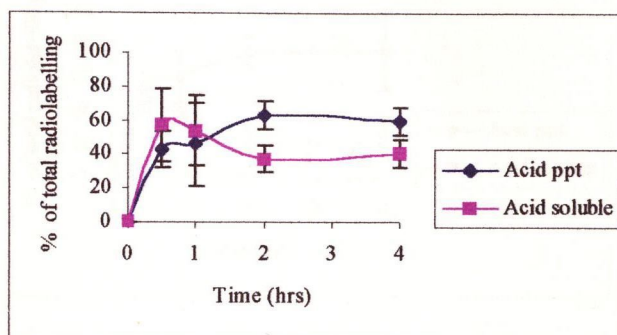


Fig. 4.10 Methionine metabolic profile of vascular disease subject TC3.

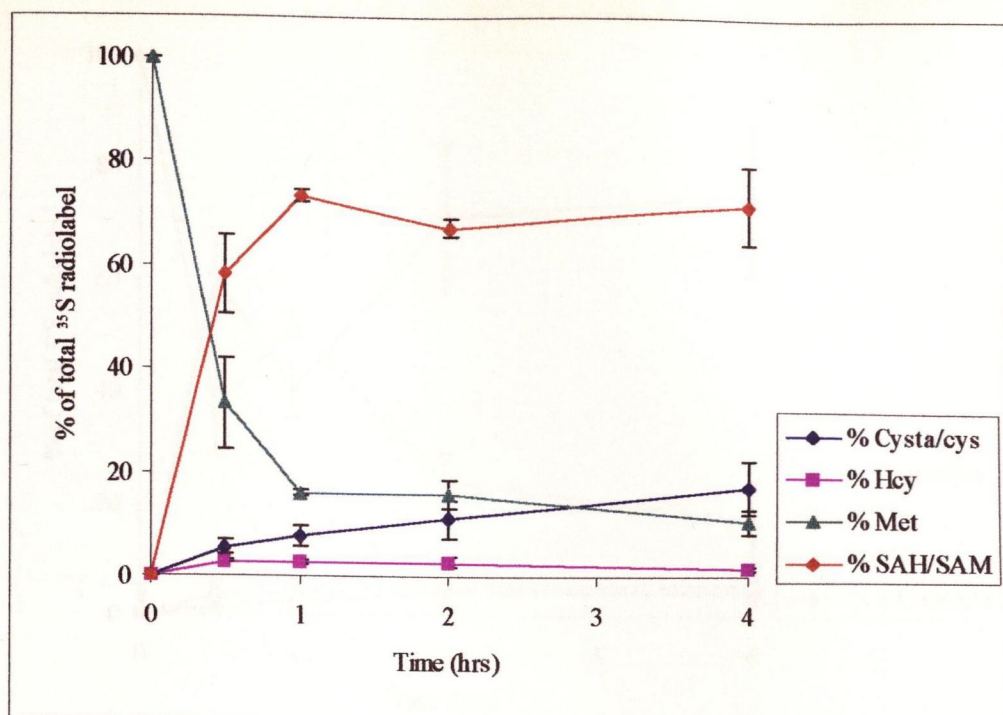
The illustration represents the distribution of ^{35}S radiolabel at the times specified above, after its addition to the cell culture media in the form of [^{35}S]-methionine. Data shown represents mean $\pm$ range of values found in duplicate experiments.

A: The proportion of radioactivity in Cysta/cys indicates activity of the transsulphuration pathway, while activity in the remethylation pathway is reflected by the proportion of radioactivity in SAH/SAM and methionine. The profile indicates substantial activity of the remethylation pathway, with some activity of the transsulphuration pathway and shows no significant accumulation of the label in homocysteine.

B: This demonstrates the proportions of radioactivity that appeared in the protein and cell extract fractions. The majority of the label was found in the cell extracts up to one hour. At 2 and 4hrs, the majority of the label was found in the protein fraction.

This subject (TC3) had fasting homocysteine levels within the normal range ($10.3\mu\text{M}$) and demonstrated no nutritional deficiency. This patient was wild-type with respect to the three genotypes investigated.

A



B

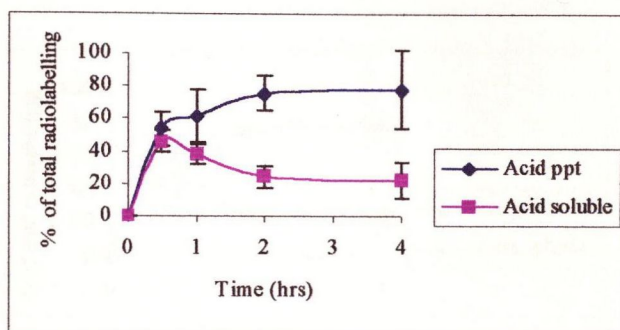


Fig. 4.11 Methionine metabolic profile of vascular disease subject CM4.

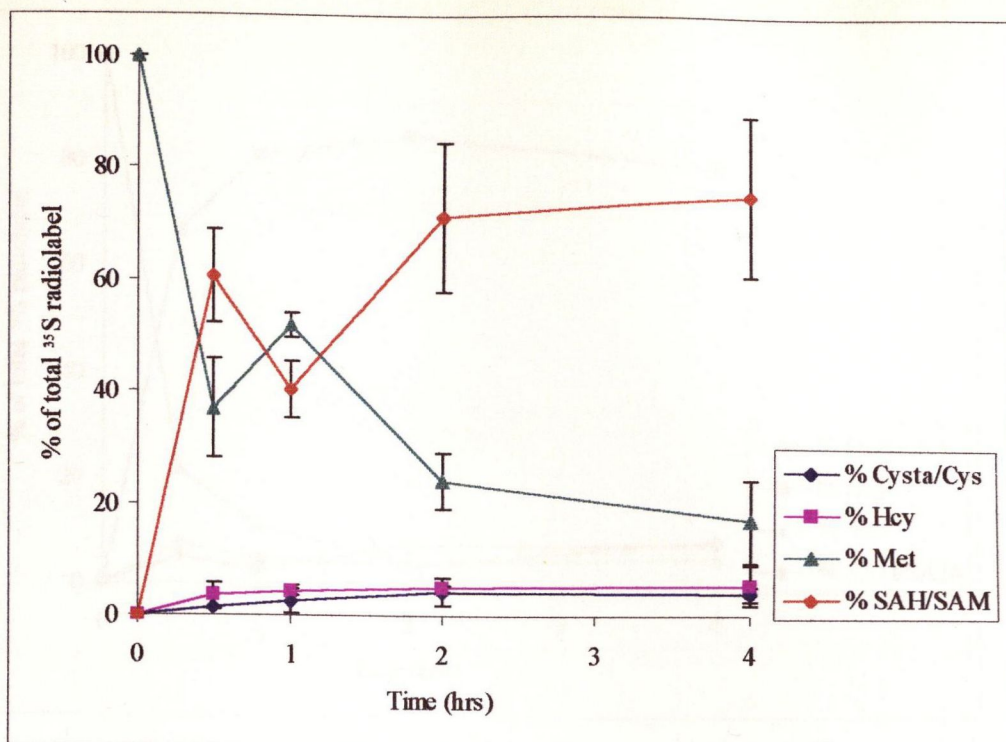
A: The illustration represents the distribution of ^{35}S radio-label at the times specified above, after its addition to the cell culture media in the form of [^{35}S]-methionine.

Data shown represents mean $\pm$ range of values found in duplicate experiments. The proportion of radioactivity in Cysta/cys indicates activity of the transsulphuration pathway, while activity in the remethylation pathway is reflected by the proportion of radioactivity in SAH/SAM and methionine. The profile indicates modest activity of the transsulphuration pathway, and extensive activity of the remethylation pathway. While the proportion of label in homocysteine remains low, it is nonetheless statistically higher than that of the controls.

B: This demonstrates the proportions of radioactivity that appeared in the acid precipitate (protein) and acid soluble (cell extract) fractions. At 0.5hrs, there was approximately equal labelling of both fraction but at later time points, the majority of the label was found in the protein fraction.

This subject (CM4) had fasting homocysteine levels outside the normal range (21.9 μM) (hyperhomocysteinemic), was deficient in folate (1.1ng/L) and was wild type with respect to the MTHFR TT, CBS I278T and MS D919G genotypes.

A



B

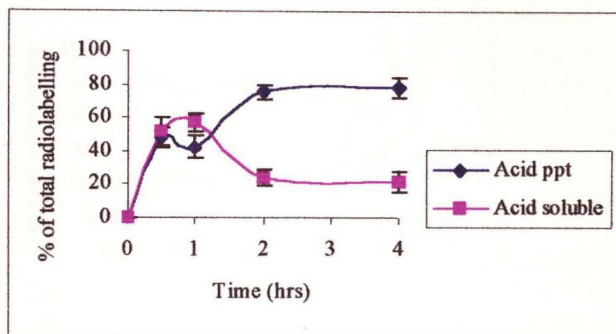


Fig. 4.12 Methionine metabolic profile of vascular disease subject HK5.

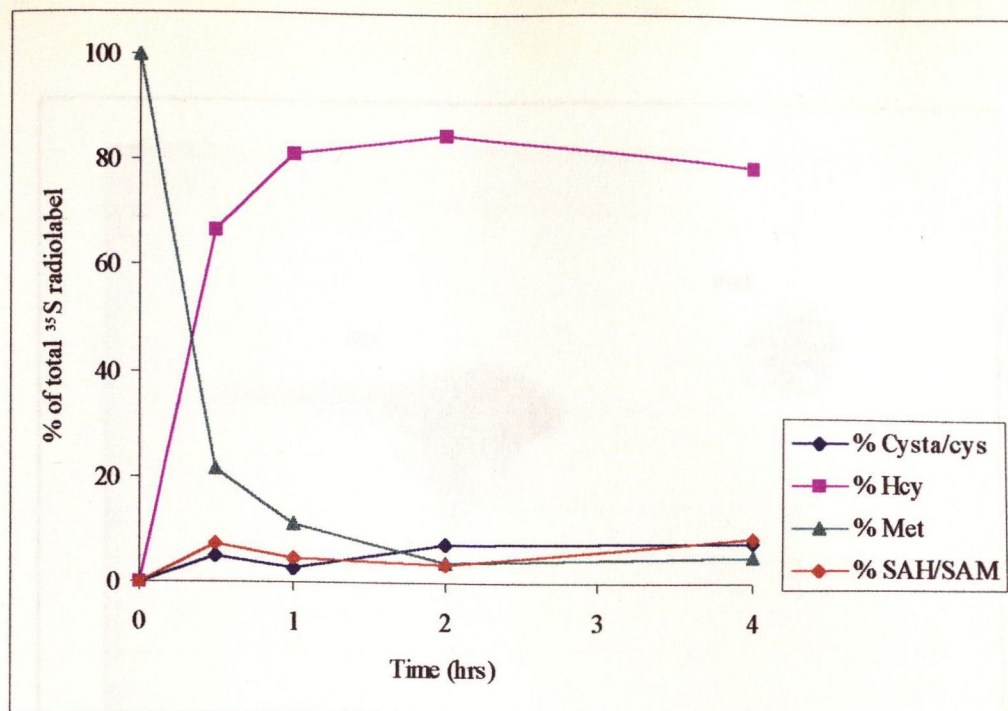
A: The illustration represents the distribution of ³⁵S radiolabel at the times specified above, after its addition to the cell culture media in the form of [³⁵S]-methionine.

Data shown represents mean $\pm$ range of values found in duplicate experiments. The proportion of radioactivity in Cysta/cys indicates activity of the transsulphuration pathway, while activity in the remethylation pathway is reflected by the proportion of radioactivity in SAH/SAM and methionine. The profile indicates significant activity in the remethylation pathway and minimal activity of the transsulphuration pathway with more label accumulating in homocysteine than in the intermediates of the transsulphuration pathway.

B: This demonstrates the proportions of radioactivity that appeared in the acid precipitate (protein) and acid soluble (cell extract) fractions. Up to one hour, an excess of label was found in the cell extract, but at 2 and 4hrs, the majority of the label was found in the protein fraction.

This subject (HK5) had an elevated fasting plasma homocysteine (13.8 μ M), demonstrated no nutritional deficiency and possessed the MTHFR TT genotype.

A



B

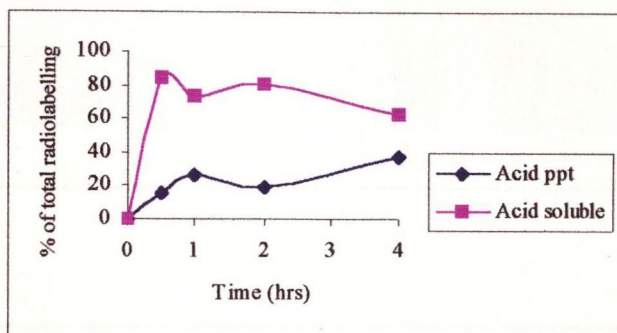


Fig. 4.13a Methionine metabolic profile of vascular disease subject TF6.

The illustration represents the distribution of ^{35}S radiolabel at the times specified above, after its addition to the cell culture media in the form of [^{35}S]-methionine. Data shown represents the results of just one experiment. However, a methionine loading experiment carried out in parallel with this experiment appeared to substantiate these results. Also, the presence of the majority of the label in homocysteine was verified by autoradiography of the TLC plate (Fig. 4.13b).

A: The proportion of radioactivity in Cysta/cys indicates activity of the transsulphuration pathway, while activity in the remethylation pathway is reflected by the proportion of radioactivity in SAH/SAM and methionine. The profile demonstrates very little activity in either the remethylation or transsulphuration pathways, with the rapid accumulation of the label in homocysteine.

B: This demonstrates the proportions of radioactivity that appeared in the acid precipitate (protein) and acid soluble (cell extract) fractions. In contrast to the control profile (Fig. 4.5), the majority of the label was found in the cell extract.

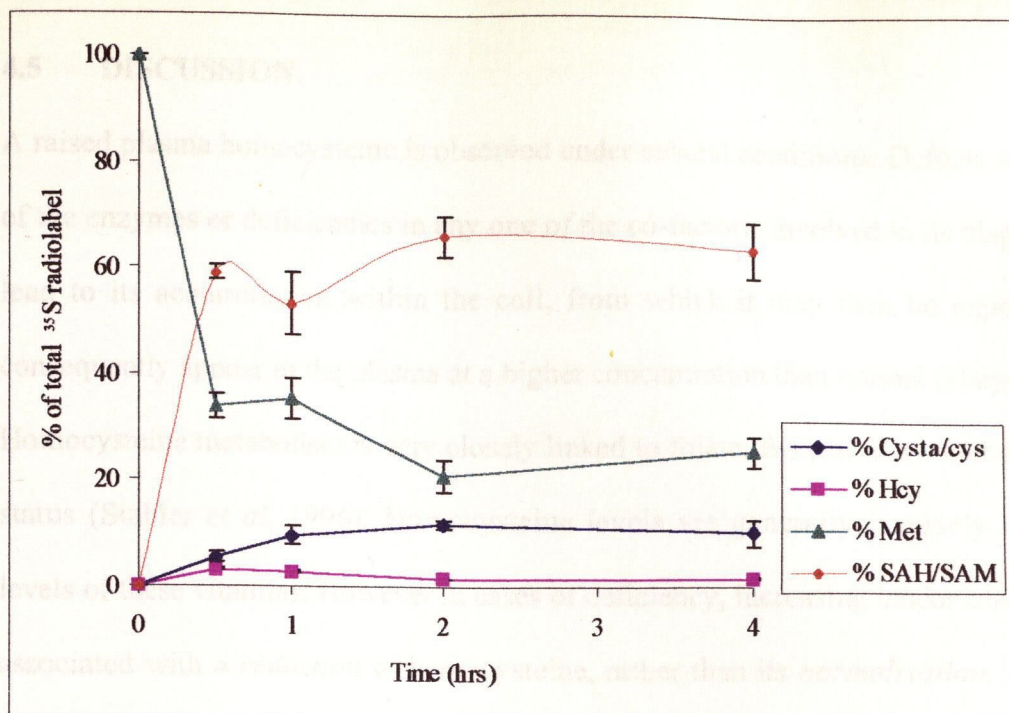
This subject (TF6) had an elevated fasting plasma homocysteine ($15.7\mu\text{M}$), demonstrated no nutritional deficiency and was wild type with respect to the MTHFR TT genotype.



Fig. 4.13b Autoradiograph of cell extract of subject TF6, following 2-D TLC.

The cell extract was prepared 1hr after the addition of [^{35}S]-methionine to the cells and subjected to 2-D TLC as described in section 3.3.3. The TLC plate was then exposed to photographic film for 3 days at -70°C and developed. The autoradiograph verifies the profile shown in Fig 4.13a. The majority of the radioactivity was found in homocysteine, with some in methionine and negligible amounts in cysta/cys and SAH/SAM.

A



B

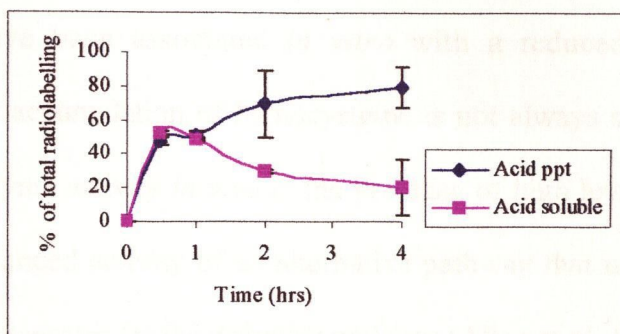


Fig. 4.14 Methionine metabolic profile of vascular disease subject EB7.

The illustration represents the distribution of ^{35}S radiolabel at the times specified above, after its addition to the cell culture media in the form of [^{35}S]-methionine. Data shown represents mean $\pm$ range of values found in duplicate experiments.

A: The proportion of radioactivity in Cysta/cys indicates activity of the transsulphuration pathway, while activity in the remethylation pathway is reflected by the proportion of radioactivity in SAH/SAM and methionine. The profile demonstrates modest activity of the transsulphuration pathway and extensive activity of the remethylation pathway, with little accumulation of the label in homocysteine.

B: This demonstrates the proportions of radioactivity that appeared in the acid precipitate (protein) and acid soluble (cell extract) fractions. Up to one hour, approximately equal labelling of the protein and cell extract took place. Following this, the majority of the label was found in the protein fraction.

This subject (EB7) had an elevated fasting plasma homocysteine ($15.6\mu\text{M}$), demonstrated no nutritional deficiency ($6.6\mu\text{g/L}$ folate and 221.4ng/L B_{12}) and was wild type with respect to the MTHFR TT, CBS I278T and MS D919G genotypes.

4.5 DISCUSSION.

A raised plasma homocysteine is observed under several conditions. Defects in any one of the enzymes or deficiencies in any one of the co-factors, involved in its disposal, can lead to its accumulation within the cell, from which it may then be exported and consequently appear in the plasma at a higher concentration than normal (Harjai, 1999). Homocysteine metabolism is very closely linked to folate, B₁₂ and to a lesser degree B₆ status (Stabler *et al*, 1996). Homocysteine levels are generally inversely related to levels of these vitamins. However in cases of deficiency, increasing intake is sometimes associated with a *reduction* of homocysteine, rather than its *normalisation*. A similar situation exists with regards to enzyme activity. While genetic lesions in MTHFR, MS and CBS have been associated *in vitro* with a reduced activity, the expected physiological accumulation of homocysteine is not always seen. This may be due to enhanced enzyme activity *in vivo* in the presence of high levels of a co-factor or may represent enhanced activity of an alternative pathway that utilises homocysteine and therefore compensates for the defective pathway (Allen *et al*, 1993). In addition, marginal vitamin deficiency is not uncommon, particularly among the elderly. Several common detrimental polymorphisms have been identified in the genes that regulate homocysteine metabolism, *eg* MTHFR TT polymorphism. Considering the frequency of these factors, there is strong possibility that hyperhomocysteinemia arises not just from a single element but from the interaction of a number of these factors (Wang *et al*, 1999).

The relevance of the C→T transition in the MTHFR gene to the genesis of hyperhomocysteinemia has been recently attracting significant attention. The effects of this polymorphism are thought to be largely abrogated by the presence of sufficient folate (Schwartz *et al*, 1997). In addition, Woodside *et al*, have recently provided evidence to suggest that in the presence of the TT genotype, the transsulphuration

pathway may work at a higher rate in an effort to catabolise homocysteine (Woodside *et al*, 1997). Therefore, the presence of the TT genotype alone may not be sufficient to induce hyperhomocysteinemia. As a risk factor for hyperhomocysteinemia, it would seem logical that the TT genotype would therefore be a risk factor for vascular disease but epidemiological evidence on this issue is conflicting and raises further doubts as to the relationship between this genotype and homocysteine metabolism (Kluijtmans *et al*, 1996 and Brugada & Marian, 1997). Folate status and enhanced transsulphuration may account for the apparent ambiguous relationship between the TT genotype and cardiovascular risk.

There have been few reports on the activity of the methionine cycle as a whole in hyperhomocysteinemic subjects. This study sought to investigate the fate of homocysteine at the cellular level in healthy and vascular disease subjects, both in those with and without hyperhomocysteinemia. It was also hoped that these studies would throw further light on the role of the MTHFR TT genotype in homocysteine disposal. When this part of the work was completed, it was decided to investigate the presence of two other mutations thought to effect homocysteine metabolism, namely, the MS D919G genotype and the CBS I278T genotype (discussed in Chapter 1) to determine if they were related to a specific effect on homocysteine metabolism at the cellular level.

In this study, a fasting plasma homocysteine of $\geq 12\mu\text{M}$ was defined as hyperhomocysteinemic. This was the cut-off used in the COMAC study in which many of the vascular disease patients participated and is in line with the findings of other studies (Chu & Hall 1988 and Graham *et al*, 1997).

This is by no means an epidemiological study. Yet, despite the small number of subjects studied, general trends in agreement with those previously reported (Selhub *et al*, 1993), between homocysteine and age, folate and B₁₂ could be seen (Fig. 4.1). Folate was the only variable to prove statistically significant. The lack of statistical

significance of B₁₂ and age on homocysteine is more than likely a result of the small numbers in the study (n=17). The statistical significance of folate, especially in this small a study demonstrates its importance as a determinant of plasma homocysteine.

Peripheral blood mononuclear cells (which are predominantly a lymphocyte preparation) were chosen to study methionine/homocysteine metabolism. These cells are relatively easy to isolate and culture and most importantly have demonstrated CBS activity (when stimulated by PHA), MAT and MS activity (Goldstein *et al*, 1976; Kotb *et al*, 1985; Tan *et al*, 1993 and van der Put *et al*, 1997). It was therefore hoped that a study of methionine metabolism in these cells, would provide an accurate reflection of the activity of the methionine cycle in the individuals studied.

This study demonstrated that radio-labelled methionine can be used to follow the methionine cycle in human cells. [³⁵S]-methionine was efficiently taken up by the cells and was rapidly metabolised, appearing not only in the intermediates of the methionine cycle but also in proteins. By looking at the distribution of the label in the different metabolites, inferences could be drawn as to the extent of activity of the enzymes of the transsulphuration and remethylation pathways.

The tracer used in these studies was [³⁵S]-methionine. Once this methionine has entered the cells, it may enter the methionine cycle to participate in transmethylation reactions or it may be directly channelled towards protein synthesis (Storch *et al*, 1988). The path it takes depends on the cells' needs. PHA-stimulated cells are metabolically very active and protein synthesis is a pre-requisite for which methionine is essential. A high rate of protein synthesis would thus be expected. As can be seen from the graph in Fig. 4.5, panel B, there was extensive labelling of proteins following the addition of [³⁵S]-methionine. This suggests that the majority of the label was incorporated into newly synthesised proteins, needed by these stimulated cells. It should be noted that five of the seven vascular disease subjects showed a greater appearance of the label in protein than

the controls. The exceptions were TC3 and TF6 who resembled the average control labelling pattern (Fig. 4.5, panel B). These differences in labelling patterns will be discussed later.

As can be seen in Fig. 4.5, panel A, activity of both pathways was evident in the controls. There was no accumulation of labelled homocysteine. The extent to which both pathways contributed to this, appeared to vary between individuals with some demonstrating a higher transsulphuration rate than others (Fig. 4.6). The activity of the remethylation pathway, as judged by the appearance and persistence of the label in SAH and SAM was always higher than that of the transsulphuration pathway. The results in Fig. 4.5 suggest that the controls dispose of homocysteine predominantly through the remethylation pathway.

It is important to consider the conditions under which homocysteine metabolism was studied. In the presence of PHA, the cells have been driven into a mitogenic state, a state where SAM plays a crucial role, as described in detail in Chapter 1 and where protein synthesis is upregulated. In this context the extent of protein labelling is of interest. An elegant study of methionine metabolism in healthy men using an infusion of stable isotope tracers, by Storch *et al*, estimated that more than one third of the homocysteine formed daily is remethylated (Storch *et al*, 1988). Based on these findings approximately 33% of the label would have been expected to persist in methionine, SAH and SAM, and not 60-80% as was observed. The findings here of a higher remethylation rate than expected may be directly related to the amount of protein synthesis occurring in these activated cells. The metabolic consequences of the addition of PHA to lymphocytes include the production of RNA, proteins and phospholipids, all of which require the presence of SAM, to act as a methyl donor (Chiang *et al*, 1996). It is therefore possible that the upregulation of the remethylation pathway seen here, is a reflection of increased metabolism, including protein production. To this end, a plot of

the percentage of radiolabel in proteins versus the percentage of radiolabel in SAM, demonstrated a significant and positive correlation between the incorporation of the label into proteins and its persistence in SAM (Fig 4.7).

The appearance of radio-label was monitored in cystathionine, cysteine, homocysteine, methionine, SAH and SAM. Cellular concentrations of these intermediates are controlled both by the actual production/utilisation of that particular amino acid and also its ability to leave the cell. Intracellular labelling of cysteine, homocysteine and SAH was low. SAH has a particularly short half-life and is rapidly de-adenosylated to yield homocysteine. This might explain why the proportion of label in SAH was never particularly high.

Of the reported radiolabelling of cystathionine/cysteine, the majority of the labelling was found in cystathionine. The low level of intracellular labelling of cysteine is most likely not an indication of a low production rate of cysteine but of its incorporation into protein and glutathione and possibly, its export from the cell. Despite this, any measurement of intracellular labelled cysteine would be directly proportional to its production and in conjunction with a measurement of the persistence of the label in cystathionine, which is not exported from the cell, could provide an accurate index of the activity of the transsulphuration pathway.

No intracellular accumulation of the label in homocysteine was observed in the controls. This indicates that homocysteine was effectively salvaged by the remethylation pathway and utilised by the transsulphuration pathway. Alternatively, this low-level labelling of homocysteine could be a reflection of its export to the culture medium, when its rate of formation exceeds its metabolic capacity. Christensen *et al*, have studied homocysteine export by a number of cell types, including cultured lymphocytes. They have demonstrated that under conditions similar to those used in the experiments described here, homocysteine export does indeed occur, with

homocysteine concentrations in the medium rising from 0.5 μ M to approximately 3 μ M, 69hrs after the addition of PHA (Christensen *et al*, 1991). However, they also demonstrated that the *rate* of homocysteine export had begun to decrease 60hrs after the addition of PHA. The values reported in this study on the amount of radiolabelled homocysteine within the cell is probably a good indication of its utilisation by the transsulphuration and remethylation pathways, and probably does not reflect its export from the cell to any great extent, for two reasons. Firstly, in the experiments reported here, where the methionine tracer was added 72hrs after the addition of the PHA to the cells, it is expected that the rate of homocysteine export would not be at its peak. Secondly, Christensen *et al*, reported an export rate of approximately 0.12nmoles/hr/10⁶cells, 72hrs after the addition of PHA. This would mean that in the healthy controls studied here, a very small amount of labelled homocysteine would be exported from the cells in the four hours for which they were studied.

In an effort to estimate the amount of homocysteine export from cells, an attempt was made to quantify the amount of radioactive homocysteine in the cell culture medium. This was done by adding homocysteine and methionine carriers to the medium and separating these two amino acids by 1-D TLC. Separation was successfully achieved but as it is estimated that homocysteine would account for only 0.01% of the label in the culture medium, (the majority being [³⁵S]-methionine added to the culture medium) no meaningful estimation of the proportion of label in homocysteine could be made.

In summary, the controls demonstrated activity of both the transsulphuration and remethylation pathways, with no significant accumulation of homocysteine. Although activity of the transsulphuration pathway was evident in all controls, there appeared to be two distinct groups, *ie* those who presented with a relatively high degree of labelling in cystathionine and cysteine and those who presented with a lower level of cystathionine/cysteine labelling. The high activity in the remethylation pathway

possibly occurred as a consequence of the heightened metabolic activity of the cells. Nonetheless, it is expected that a major metabolic defect in either would be detected because both pathways are up-regulated by PHA.

The vascular disease subjects, both those with and without hyperhomocysteinemia, presented very different methionine metabolic profiles.

Only two of the seven vascular disease patients presented with a nutritional deficiency of folate or B₁₂. These were MF1 (fasting serum B₁₂ of 167.3ng/L) and CM4 (fasting serum folate of 1.1µg/L). In the profile of MF1 (Fig. 4.8), it appears that there was defective activity of the remethylation pathway, which might be attributed to defective activity of MTHFR due to the presence of the TT genotype. In comparison to the controls, where the label accumulated in SAH and SAM over time, here it appeared early but was not sustained. Despite this, no accumulation of homocysteine occurred. This may have been due to an excessively active transsulphuration pathway, as suggested by the high proportion of radioactivity in cystathionine and cysteine. While this subject had adequate folate, her B₁₂ was bordering on inadequate. This taken together with the presence of the TT genotype may represent a compound defect in the remethylation pathway and may explain the high rate of transsulphuration observed in this subject. Cobalamin present in the medium might have been expected to compensate for a low dietary intake of vitamin B₁₂, but did not appear to do so in this case. Inadequate utilisation of dietary cobalamin may be at fault.

In the profile of CM4, there was a notable increase in the labelling of SAH/SAM, and a trend towards increased labelling of homocysteine, and apparently decreased labelling of methionine. Taken together with the deficiency in folate, this points to a reduction in the cycling of homocysteine through the remethylation pathway. The label appears to be transferred to both SAM and SAH and from there to homocysteine. Here it is utilised by the transsulphuration pathway but is not efficiently remethylated possibly due to the

folate deficiency, thereby leading to a reduction in the transfer of the label back to methionine and consequently, an accumulation of the label SAH/SAM and to a lesser extent in homocysteine. Excess folate is supplied by the medium, but if a defect exists in one of the enzymes involved in its metabolism, it may be unavailable in a utilisable form. In comparison to the controls, a slightly higher proportion of the label appeared in the protein fraction compared to the controls (Fig. 4.11, panel B). Possibly the reduction in labelled methionine in the cell extract fraction may be a consequence of its use in the manufacture of new proteins by the cell.

The presence of the MTHFR C-T, CBS I278T and MS D919G genotypes were determined by PCR-RFLP. The polymorphisms in both the MTHFR and MS genes were verified by sequencing of PCR-amplified gene fragments (Fig. 4.2 and 4.4). However, while sequencing verified the identity of the CBS PCR-product, and also identified the presence of the 68bp insert, sequence quality was insufficient to confirm the presence of the T-C base change (Fig. 4.3). The 68bp insert sequence perfectly matches that of the intron-exon junction at the 5' end of exon 8 (53 and 15 bases upstream and down stream of the splicing site, respectively) (Sperandeo *et al*, 1996). This can be seen in Fig 4.4. Based on the specificity of the primers, the activity of the restriction enzyme (*Bsr* I) and the size of the products generated by both the PCR and the digestion, it can be assumed that the CBS I278T genotyping was correct.

In all, six of the subjects in the study presented with the C677T MTHFR polymorphism (five in the homozygous and one in the heterozygous state). Three of these were vascular disease patients (MF1, JC2 and HK5). Of these, only one (HK5) presented with an elevated plasma homocysteine. The profile for MF 1 has been discussed above. JC2, despite the presence of the TT genotype maintains effective remethylation rates, probably as a consequence of his high folate levels (Fig. 4.9). The rate of transsulphuration was comparable to the low transsulphurating controls.

From the profile of HK5, there appears to be no defect in the remethylation pathway, despite the presence of the TT genotype. Residual activity in the thermolabile variant may have been enhanced either due to his serum folate or folate supplied in the medium. Increased labelling of homocysteine compared to the controls was observed. Labelling of cystathionine and cysteine was comparable to, though slightly lower than, the low transsulphurating controls. The lack of defective remethylation, points to defective transsulphuration being the cause of the elevated plasma homocysteine seen in this individual. This could arise as a result of a genetic defect or may reflect nutritional deficiency. Vitamin B₆ plays a crucial role in the transsulphuration pathway, therefore any deficiency in this vitamin could be detrimental. Unfortunately, serum B₆ levels were not measured in this study and therefore, the reason for the reduced transsulphuration in this individual can only be speculated upon. While serum B₁₂ was deemed adequate, it was at the lower end of the normal range. This may correspond to a low dietary intake and may exacerbate another nutritional deficiency, particularly in this case, a deficiency in B₆. Although a defect in the transsulphuration pathway is implied by these results, the additional role of the TT genotype in raising plasma homocysteine in this individual cannot be ruled out, due to the potential masking of its effects by folate supplied in the medium.

Three control subjects were found to carry the C677T transition mutation; one in the heterozygous state (DC9) and two in the homozygous state (EF3 and JF5). JF5 was also a heterozygous carrier for the D919G polymorphism. None of these subjects had elevated plasma homocysteine. They did not accumulate homocysteine at the cellular level and did not demonstrate a reduced activity in the remethylation pathway.

Based on the lack of correlation between the MTHFR TT genotype and the accumulation of homocysteine in plasma and at the cellular level, this albeit small study seems to suggest that this genotype does not have a major role to play in homocysteine

accumulation. These results are by no means conclusive and it should be noted that in each of these individuals with the TT genotype, none was lacking in folate and even if this was the case, folate supplied by the culture medium might mask a defective remethylation rate.

None of the subjects (controls and vascular disease subjects) possessed the I278T polymorphism in isolation, *ie* it was present with the 844ins68bp, thereby rendering this potentially detrimental mutation benign (Tsai *et al*, 1996b).

Two subjects (both controls) presented with the D919G polymorphism (JF5 and IS6, Table 4.2). One subject (JF5) was a heterozygous carrier for the D919G polymorphism and was also a TT MTHFR homozygote, while another (IS6) was found to be homozygous. The D919G polymorphism, particularly in the homozygous state (DD) is thought to lead to impaired activity of MS and may therefore contribute to hyperhomocysteinemia (Leclerc *et al*, 1996). Both of these subjects had normal fasting homocysteine levels, did not accumulate homocysteine at the cellular level, and showed no impairment in methionine recycling. The expression of this genotype is not as sensitive to folate and B₁₂ status and so if a defect did exist, it would probably have been detected by this system.

The most striking profile was generated by TF6 (Fig. 4.13a). Here, the majority of the label persisted in homocysteine over the four-hour study period. The reason for this accumulation can only be speculated upon. The high rate of intracellular labelling of homocysteine points to an eventual accumulation of homocysteine within the cell in time. At such high levels of labelling, one might expect an accompanying high export rate but this is not evident from this subjects plasma levels. CM4 had a fasting plasma homocysteine in excess of this individual and did not show such an extent of intracellular homocysteine labelling. From studies in lymphoma cells, Christensen *et al*, have suggested that if methionine needs to be conserved, homocysteine egress from the

cell may be reduced (Christensen *et al*, 1991). This may explain the accumulation of homocysteine *in* the cell. The subject did not appear to have any gross vitamin deficiency, and although his folate was one of the lowest of the study group, it did fall within the normal range. Possibly, a defect in methionine synthase is at fault. This individual was wild-type with respect to the MTHFR genotype but this of course does not rule out the possibility of other mutations present in the gene. This was the only subject studied who demonstrated a majority of labelling in the non-protein cellular extract. Unfortunately, this patient was unavailable for further study and so the experiment was never duplicated. As can be seen in Fig 4.13b, autoradiographic imaging of a cell extract, after 2-D TLC substantiated these results.

There was a significant accumulation of the label in homocysteine in one of the hyperhomocysteinemic subjects (TF6); a notable, though small, accumulation of the label in homocysteine in one other (HK5) and a trend towards increased labelling in the remaining two hyperhomocysteinemic subjects (CM4 and EB7) (Table 4.5). These accumulations were not large and this is possibly to be expected as the subjects under investigation could be classified as having *mild* hyperhomocysteinemia. In addition, the short exposure of the cells to the label might not have been sufficient to detect an accumulation that may occur *in vivo* over a period of months.

The influence of vitamins (B₆, B₁₂ and folate) supplied by the culture medium cannot be ruled out. In particular, the presence of folate could abrogate the effects of a TT genotype that *in vivo* leads to homocysteine accumulation. RPMI 1640 has sufficient folate to sustain PHA-stimulated lymphocytes for three days (1mg/L). In addition, folate and other vitamins may be supplied by foetal calf serum. These would undoubtedly contribute to the methionine cycle and may have prevented a more severe accumulation of homocysteine than that seen in three of the four hyperhomocysteinemic individuals (CM4, HK5 and EB7). Although folate-deficient

RPMI is readily available, folate and other vitamins may be supplied by foetal calf serum, the composition of which cannot be controlled. Moreover, cultured cells (particularly mitogen-stimulated cells) do not survive for prolonged periods of time in the absence of folate.

As alluded to earlier in this discussion, there was evidence of considerably more cell protein biosynthesis by the lymphocytes of the vascular disease subjects compared to the controls. While there is no obvious reason as to why this should occur, it is perhaps possible that the vascular disease-state of the subjects may have rendered their circulating lymphocytes more responsive to the mitogen. Whatever the reason, the diversion of methionine, cysteine and SAM into protein biosynthesis may in part explain the lack of evidence of cellular homocysteine accumulation. Another possibility is that increased labelling of homocysteine went undetected as it was incorporated into proteins. Homocysteine incorporation into proteins is post-translational, reflecting homocysteinylation of protein lysine residues by homocysteine thiolactone. Jakubowski reported that in endothelial cells in which MS was inhibited by folate limitation, homocysteine incorporation into proteins represented 36% of the incorporation of methionine (Jakubowski, 2000). This is a substantial amount and may explain both the low level of labelling of homocysteine in three of the hyperhomocysteinemic subjects (CM4, HK5 and EB7) and might also explain the general trend towards increased labelling of protein by these three hyperhomocysteinemic subjects.

While the study outlined here and in the previous chapter may prove useful in studying methionine metabolism and its control in healthy cells, it may not be the most suitable for studying metabolism in those who may have a predisposing nutritional deficiency or a genotype that is very sensitive to its nutritional environment, *eg* the MTHFR TT genotype, due to the relatively high vitamin content of the culture medium. This may

mask a nutritional deficiency and could give a misleading picture of the cells' metabolic capacity.

These studies were quite labour intensive and time-consuming and are therefore of little practical relevance as is. However, with some alterations, the system could be adapted to suit a bigger throughput. In preliminary experiments carried out on the completion of the work described above, it was found that the activity of the methionine cycle could be monitored in whole blood using the same principle as the one utilised by this study, *ie* uptake and utilisation of ^{35}S -tagged methionine. The label could be detected in methionine and its metabolic derivatives within two hours, thus reducing the time of experiment considerably. In addition, the use of whole blood preparations would overcome the problem of exogenous vitamins masking a defect. Analysis of the distribution of the radio-label at just one time after the addition of [^{35}S]-methionine to the blood may suffice, *ie* after 2hrs the labelling patterns may provide sufficient information as to the activity of the pathways. Furthermore, by developing a HPLC system that would allow the separation of the methionine metabolites, the speed at which the samples could be processed would be greatly enhanced.

Nonetheless, the experiments described here have shown activity of the pathways involved in homocysteine generation and utilisation. Although in the case of the hyperhomocysteinemic subjects the study has not provided conclusive results, it has indicated which pathways may be at fault.

*TNF alpha expression by monocyte/macrophages in response to homocysteine***5.1. INTRODUCTION**

More than 75 clinical and epidemiological studies have shown a relationship between total homocysteine levels and coronary artery disease, peripheral artery disease, stroke and venous thrombosis (Berger et al, 1997). However the exact mechanisms by which homocysteine causes vascular disease remain unresolved.

Chapter 5

TNF alpha expression by monocytes/macrophages in response to homocysteine.

endothelial cells (Berger et al, 1993 and Goto & Lavi, 1993). Within the atherosclerotic plaque, TNF is produced primarily by macrophages (including foamy cells) and to a lesser extent by T lymphocytes (Berger et al, 1993). TNF is also produced in human atherosclerosis by smooth muscle cells and endothelial cells (Lavi et al, 1993).

Homocysteine has been shown to induce endothelial dysfunction, stimulate muscle cells proliferation, and induce foam cell formation through the production of reactive oxygen species (Berger et al, 1993, Lavi et al, 1993 and Lavi & Berger, 1999). These may all have an effect on the expression of TNF by monocytes/macrophages within the atherosclerotic plaque. The effect of homocysteine on these cells has been all but ignored. TNF is a cytokine produced by macrophages in response to stress (Jorring et al, 1993). As a pro-inflammatory cytokine, it was decided to study the effect of homocysteine on TNF production by monocyte/macrophages.

TNF alpha expression by monocyte/macrophages in response to homocysteine.

5.1. INTRODUCTION.

More than 75 clinical and epidemiological studies have shown a relationship between total homocysteine levels and coronary artery disease, peripheral artery disease, stroke and venous thrombosis (Nygård *et al*, 1997). However the exact mechanisms by which homocysteine causes vascular disease remain unresolved.

Cytokines regulate a number of processes of atherogenesis. TNF α appears to be of particular importance in atherosclerosis, due to its pro-atherogenic/thrombotic properties, as discussed in Chapter 1. For instance, TNF α is known to stimulate smooth muscle cell proliferation and migration and to induce a pro-coagulant state in endothelial cells (Morisaki *et al*, 1993 and Grau & Lau, 1993). Within the atherosclerotic plaque, TNF α is produced primarily by macrophages (including those transformed to foam cells), and to a lesser extent by smooth muscle cells and endothelial cells (Barath *et al*, 1990a).

Homocysteine has been shown to induce endothelial dysfunction, stimulate smooth muscle cells proliferation, and enhance lipid peroxidation through the production of reactive oxygen species (Heinecke *et al*, 1993; Tsai *et al*, 1994 and Lee & Wang, 1999). These may all have a role in promoting atherosclerosis. Despite an abundance of macrophages within the atherosclerotic plaque, the effect of homocysteine on these cells has been all but ignored. TNF α expression is thought to be sensitive to oxidative stress (Jovinge *et al*, 1996). As a potential source of oxidative stress, it was decided to study the effect of homocysteine on TNF α production by monocytes/macrophages.

5.2. STRATEGY.

To investigate the influence of homocysteine on TNF α expression of macrophages, the monocytic cell lines U-937 and THP-1 were used. Differentiation to macrophages was induced by exposure to phorbol myristic acid (PMA). The monocytes/macrophages were cultured in medium containing homocysteine at various concentrations. At specified time points, cell medium was removed and assayed for release of TNF α by an enzyme-linked immunosorbent assay (ELISA). In addition, RNA from control and homocysteine-treated cells was prepared, to investigate the effect of homocysteine on TNF α gene expression.

5.3. MATERIALS AND METHODS.

5.3.1. Investigation of the induction of TNF α by PMA in the U-937 and THP-1 cell lines.

The human monocytic cell-lines, U-937 and THP-1, were cultured at a density of 5×10^5 cells/ml, as described in section 2.2.2 and 2.2.3. PMA at 0, 5 or 10ng/ml was included in the culture medium at various times up to 24hrs, cell medium was removed. Cell adherence was estimated by counting the number of cells in the medium as described in section 2.2.2 and subtracting this value from the number plated originally.

$$\text{Percentage of adherent cells} = \frac{(\text{total number plated} - \text{number in suspension})}{\text{total number plated}} \times 100$$

TNF α levels in the culture medium were determined by ELISA as described in the next section.

5.3.1.1. Estimation of TNF α concentrations in cell culture medium by ELISA.

A 96-well microtitre plate was coated with 100 μ l of capture antibody (mouse anti-human TNF α , 2 μ g/ml) and incubated overnight at 4°C. Wells were washed 5 times with wash buffer (Phosphate buffered saline (PBS) containing 0.05% (v/v) Tween 20) and blocked with 250 μ l of blocking buffer (PBS containing 1% (w/v) bovine serum albumin (BSA)) for one hour at 37°C. The blocking buffer was then decanted from the wells and 100 μ l of standards and samples were added in duplicate and incubated at 37°C for one hour. Wells were washed as described above, 100 μ l of second antibody (biotinylated rabbit anti-human TNF α) was added (0.5 μ g/ml) and allowed to incubate at 37°C for one hour. Wells were washed again, 100 μ l of Streptavidin-HRP was added to each well and incubated at 37°C for 15 minutes. This was removed by washing, 100 μ l of tetramethylbenzidine (TMB) substrate reagent was added and the colour allowed to develop for 10 minutes. The reaction was stopped by the addition of 100 μ l of 0.5M H₂SO₄. This resulted in the formation of a yellow colour, the absorbance of which was read in a multi-plate reader at 450nm. The concentration of TNF α in culture medium was determined by comparing absorbances to those of a standard curve (Fig. 5.1).

5.3.1.2. Investigation of the effect of homocysteine on the ELISA.

To ensure that homocysteine did not interfere with the performance of the ELISA, TNF α standards were assayed in triplicate in the presence and absence of homocysteine (15, 100, and 200 μ M) and standard curves were constructed. A "best-fit" straight line was fitted to each curve and the slope of the line (the value of which is used to convert absorbance readings to TNF α concentrations) was calculated using the Microsoft®

Excel 5.0 graphics and statistics package. The statistical significance of the differences found in the calculated slope values was assessed using paired Students t-tests.

5.3.2. Investigation of TNF α production by U-937 and THP-1 monocytes, in response to homocysteine.

The human monocytic U-937 and THP-1 cells were cultured at a density of 5×10^5 cells/ml, as described in section 2.2.2-2.2.3. Homocysteine at 0, 15 or $100\mu\text{M}$ concentrations was included in the culture medium. A positive control (PMA supplemented (10ng/ml) medium) was included in each set of experiments. At specific time points, culture medium was removed. Adherence of the cells was estimated as described in section 5.3.1.

The cells in suspension were then precipitated by centrifugation ($5000g$ for 5mins). The supernatant was removed and stored at -70°C for TNF α estimation by ELISA as described in section 5.3.1.1.

5.3.3. Investigation of the effects of *pre-exposure to homocysteine* on PMA-induced TNF α production by the U-937 cell-line.

U-937 cells were cultured at a density of 5×10^5 cells/ml, as described in section 2.2.3 in medium containing homocysteine at 0 or $100\mu\text{M}$ concentrations. This medium was then removed and the cells were re-plated at a density of 5×10^5 cells/ml with fresh medium containing PMA (10ng/ml). At 4, 10, 18 and 24hrs , cell medium was removed and adherence and TNF α production were assessed as described in section 5.3.1 and 5.3.1.1.

5.3.4. Investigation of TNF α production by the U-937 cell-line, in response to simultaneous exposure to PMA and homocysteine.

The human monocytic U-937 cells were cultured at a density of 5×10^5 cells/ml, as described in section 2.2.3. PMA at a concentration of 10ng/ml was included in the medium, as was homocysteine at 0, 15 or 100 μ M concentrations. After 4, 8, 18 and 24hrs, the culture medium was removed, cell adherence was estimated as described above and the cells in suspension were then precipitated by centrifugation (5000g for 5mins). The supernatant was removed and stored at -70°C for TNF α estimation by ELISA as described in section 5.3.1.1.

5.3.5. Investigation of TNF α production by pre-PMA-stimulated U-937 and THP-1 macrophages, in response to homocysteine.

The human monocytic U-937 and THP-1 cells were cultured at a density of 5×10^5 cells/ml, as described in section 2.2.3. The cells were exposed to PMA (10ng/ml) for 18hrs to induce differentiation to macrophages. Non-adherent cells were removed, adherent cells were washed in PBS and fresh medium containing 0, 15, 50, 100 or 200 μ M homocysteine (U-937) and 0, 15 or 100 μ M homocysteine (THP-1) was added. After 4hrs, cell culture medium was removed and stored at -70°C for TNF α estimation by ELISA as described in section 5.3.1.1.

5.3.6. Investigation of TNF α gene expression by PMA-stimulated U-937 macrophages, in response to homocysteine.

In order to obtain sufficient RNA for this analysis, 10mls of U-937 cells were seeded at a density of 5×10^5 cells/ml, in 75ml culture flasks and PMA was added at a concentration of 10ng/ml. After 18hrs, non-adherent cells were removed, adherent cells were washed with PBS and medium containing 0 or 100 μ M homocysteine was added.

At specific time points, cell medium was removed and the adherent cells were lysed in 1ml of Ultraspec™ and stored at -70°C. RNA was then prepared and quantitated as described in section 2.2.7 – 2.2.8. A 1ml sample of the cell culture medium was also taken for TNF α estimation by ELISA as described in section 5.3.1.1.

Levels of TNF α mRNA expression were assessed by Northern blotting as described in section 2.2.10. GAPDH expression was assessed to ensure that equal quantities of mRNA were present in each RNA preparation.

5.3.7. Investigation of homocysteine cytotoxicity.

U-937 monocytes were cultured at a density of 5×10^5 cells/ml, as described in section 2.2.3, in the presence of 0, 15, 100, 200 μ M or 10mM homocysteine. After 24hrs, culture medium was removed and homocysteine cytotoxicity was investigated by assessment of LDH release (section 2.2.11). Viability of monocytes exposed to homocysteine of concentrations up to 200 μ M was also investigated by calculating the number of dead cells in the culture, by acridine orange staining and comparing this to the control cultures (non-homocysteine treated).

To investigate the cytotoxicity of homocysteine to macrophages, U-937 cells were cultured at a density of 5×10^5 cells/ml, as described in section 2.2.3. The cells were exposed to PMA (10ng/ml) for 18hrs to induce differentiation to macrophages. Non-adherent cells were removed, adherent cells were washed in PBS and fresh medium containing 0, 15, 50, 100 or 200 μ M homocysteine was added. At specific time points, cell culture medium was removed and homocysteine cytotoxicity was assessed by counting the number of viable and non-viable cells using acridine orange staining (section 2.2.2)

Homocysteine cytotoxicity was similarly investigated in the THP-1 cell-line.

5.3.8. Investigation of $\text{TNF } \alpha$ production by PMA-stimulated U-937 macrophages, in response to cysteine.

The human monocytic U-937 cells were cultured at a density of 5×10^5 cells/ml, as described in section 2.2.3. The cells were exposed to PMA (10ng/ml) for 18hrs to induce differentiation to macrophages. Non-adherent cells were removed, adherent cells were washed in PBS and fresh medium containing 0 or 100 μM cysteine was added. At specific time points, cell culture medium was removed and stored at -70°C for $\text{TNF } \alpha$ estimation by ELISA as described in section 5.3.1.1.

5.4. RESULTS.

5.4.1. Assessment of the ELISA.

The precision of the assay was determined and was found to be reproducible, demonstrating an intra-assay coefficient of variation (CV) of 9.26% (n=8), and an inter-assay CV of 5.28% (n=4). A typical standard curve is shown in Fig. 5.1. There was a strong linear correlation between absorbance at 450nm and concentration of tumour necrosis factor alpha ($r^2 = 0.99$). The best-fit, Pearson coefficient (" r^2 " value) and the equation for the best-fit line were calculated using the Microsoft® Excel 5.0 graphics and statistics package.

To ensure that homocysteine did not interfere with the performance of the ELISA, standards were analysed in the presence of 0, 15, 100 or 200 μ M homocysteine (n=3) (Fig. 5.2). Best-fit lines were fitted to the curves generated and "y values", equivalent to the slope of the lines fitted to the standard curves were calculated using the Microsoft® Excel 5.0 graphics and statistics package. The mean values for y were 0.0018 – 0.0020 in each case. The differences between the calculated "y values" were compared by paired Students t-tests. This showed that the presence of 15, 100 and 200 μ M homocysteine had no significant effect on the detection of TNF α compared to controls (no homocysteine) (p>0.5).

5.4.2. PMA-induced differentiation and TNF α production of monocytic cells.

Differentiation of several human and murine cells is known to occur in the presence of PMA (Tsuchiya *et al*, 1982). For this reason, in this work, unactivated U-937 and THP-1 cells are referred to as monocytes and PMA-stimulated cells are referred to as macrophages.

To determine the optimum concentrations of PMA required to induce differentiation and TNF α production by the U-937 and THP-1 cell lines, PMA at 0, 5 or 10ng/ml was included in the culture medium of cells plated at a density of 5×10^5 cells/ml. At various times up to 24hrs, adherence (a marker for differentiation to macrophages) was monitored, cell morphology was examined by light microscopy and cell medium was removed for determination of TNF α levels, as described in section 5.3.1.1.

Following the addition of PMA to the culture medium, U-937 cells rapidly showed signs of differentiation, *ie* they adhered to the culture plates and showed morphological changes, *eg* once they had adhered they lost their regular ovoid shape and their cell surface became more irregular in structure. After just 4hrs, about one fifth of the cells were adherent. This peaked at 84%, occurring after an 18hrs exposure to the phorbol ester. Adherence of the cells was almost equal in response to PMA at both 5 and 10ng/ml concentrations (Figure 5.3a, panel B).

Coinciding with this maturation, was the production of tumour necrosis factor alpha. TNF α production was never more than 10pg/ml in cells cultured in PMA-free medium. However, cells exposed to PMA at 5 or 10ng/ml demonstrated considerable induction of this cytokine, with higher levels of induction associated with the higher PMA dose. Significant induction was seen as soon as four hours after addition of PMA. PMA at 5ng/ml led to the production of 77.6 ± 14.9 pg/ml TNF α (n=3) and at 10ng/ml led to the production of 169.4 ± 17.9 pg/ml (n=3). TNF α continued to accumulate in the culture medium up to 18hrs, with peak production coinciding with maximum adherence of the cells (Figure 5.3a, panel A).

A similar response to PMA was found in the monocytic cell-line, THP-1. In the absence of PMA, these cells did not differentiate, grew as a single cell suspension and showed virtually no adherence or TNF α synthesis. In contrast, adherence of cells was obvious four hours after addition of PMA (at both 5 and 10ng/ml) (Fig. 5.3b, panel B). Cells

became progressively adherent over time, with a higher proportion of adherent cells seen in response to the higher PMA dose (10ng/ml). Adherence peaked at 24hrs; $76.7 \pm 4.2\%$ (n=3) and $86.7 \pm 8.3\%$ (n=3) in response to PMA at 5 and 10ng/ml, respectively. These levels of adherence are in agreement with those reported in the literature (Tsuchiya *et al*, 1982).

As with the U-937 response, the higher PMA dose was associated with a higher induction of TNF α in the THP-1 cells (Fig. 5.3b, panel A). Again, significant production of this cytokine had taken place within four hours after exposure to PMA. TNF α synthesis continued and peaked at 24hrs, where levels reached $404.8 \pm 39.8\text{pg/ml}$ (n=3) in the presence of 10ng/ml PMA, coinciding with maximum adherence ($86.7 \pm 8.3\%$).

5.4.3. Investigation of TNF α production by U-937 and THP-1 monocytes, in response to homocysteine.

Jovinge *et al*, have demonstrated that low levels of oxidised LDL can induce synthesis and release of TNF α by cultured human monocytes/macrophages (Jovinge *et al*, 1996). To test the hypothesis that oxidant stress in the form of homocysteine may activate monocytes and promote the synthesis of tumour necrosis factor alpha, U-937 and THP-1 monocytes were cultured in the presence of homocysteine. No differences were observed in relation to adherence or cell morphology in the presence of 15 and 100 μM homocysteine, compared to controls (*ie* no homocysteine). TNF α was not present in substantial quantities ($<10\text{pg/ml}$) in the cell culture medium of U-937 or THP-1 monocytes supplemented with 0, 15 or 100 μM homocysteine over 24hrs (n=3). A positive control, (18hrs exposure to PMA 10ng/ml), included in the same experiments showed TNF α in the culture medium at a concentration of $225 \pm 12\text{pg/ml}$ and $376.5 \pm$

28.3pg/ml for U-937 and THP-1 cells respectively (n=3). Thus, homocysteine, of itself, was not capable of inducing differentiation of monocytes or TNF α production.

5.4.4. Investigation of the effects of *pre-exposure to homocysteine* on PMA-induced TNF α production by U-937 cells.

In order to further investigate the effect of homocysteine on TNF α expression, U-937 monocytes were pre-exposed to 100 μ M homocysteine for 24hrs and their adherence and TNF α production in response to PMA was assessed as described in sections 5.3.1 and 5.3.1.1.

The results of this experiment are shown in Fig 5.4. Prior exposure of the monocytes to homocysteine led to an increased production of TNF α in response to PMA, that was statistically significant at 4, 10 and 24hrs (Students paired t-test, $p < 0.01$, $n = 3$) but not at 18hrs ($p > 0.05$). In contrast, the rate of PMA-induced cell adherence was significantly lower in the homocysteine-treated cells up to 18hrs but reached the same level of adherence as the control cells by 24hrs ($p > 0.1$) (Fig. 5.4, panel B).

5.4.5. Investigation of TNF α production by the U-937 cell-line in response *simultaneous exposure* to PMA and homocysteine.

To further investigate the role of homocysteine in the regulation of PMA-induced TNF α production the effect of simultaneous exposure to homocysteine and PMA was investigated in the U-937 cell-line as described in section 5.3.4.

The pattern of cell adherence was similar to that outlined in Fig. 5.3a. No difference in the extent of adherence was seen in the presence of homocysteine. In the control experiment (0 μ M hcy), TNF α secretion increased over time up to 24hrs (Fig.5.5). This pattern was repeated by the cells exposed to both 15 and 100 μ M homocysteine, up to 18hrs, but a fall in the levels of TNF α occurred at 24hrs. In addition, the level of TNF

α induction was attenuated, by the presence of homocysteine. In the presence of 15 μ M homocysteine, there was a trend towards reduced TNF α expression, but this was statistically significant only at 24hrs (as assessed by a Students paired t-test ($p < 0.02$, $n = 3$), where there was a $26.5 \pm 6.2\%$ reduction in TNF α production. At 4, 8, 18 and 24hrs, exposure to 100 μ M homocysteine, led to a decrease in PMA-mediated TNF α secretion, but these differences only became statistically significant (Students paired t-test) at 18hrs ($p < 0.02$, $n = 3$) and 24hrs ($p < 0.01$, $n = 3$). This attenuation by homocysteine appeared to be dose-responsive and was most pronounced in the cells exposed to 100 μ M homocysteine. Maximum attenuation occurred at 24hrs in the presence of 100 μ M homocysteine, where a $41.4 \pm 3.0\%$ ($n = 3$) reduction in TNF α secretion was observed.

5.4.6. Investigation of TNF α production by U-937 and THP-1 macrophages (*pre-PMA-stimulated*), in response to homocysteine.

Following on from the studies on monocytes and on differentiating monocytes (section 5.4.3 - 5.4.5), the effect of homocysteine on TNF α secretion by U-937 and THP-1 macrophages (*ie*, PMA-stimulated cells) was investigated as described in section 5.3.5. Earlier studies (section 5.4.2.) indicated that a maximum differentiation of these cells occurred after an 18hrs exposure to PMA at a concentration 10ng/ml. U-937 and THP-1 cells were so treated before the addition of fresh medium containing homocysteine. In agreement with the results of section 5.4.5, a decrease in the cell medium TNF α levels was observed in response to homocysteine, in both the U-937 and THP-1 macrophages. In the U-937 macrophages, after 4hrs, there was a significant dose-dependent decrease in TNF α expression ($p < 0.05$, $n = 3$) (Fig. 5.6). This did not reach statistical significance at 15 μ M homocysteine ($p > 0.05$). The largest reduction was seen in the presence of 200 μ M homocysteine ($52.3 \pm 5\%$ reduction in expression, compared to controls).

PMA-induced TNF α production, by cells cultured on different days, varied substantially. For instance, TNF α production by three different U-937 control cultures at 18hrs after addition of PMA, were 270.1, 695.5 and 375.3pg/ml (mean: 447.0 ± 221.6 pg/ml). However, regardless of the level of expression, the responses to homocysteine were consistent, *ie* homocysteine was associated with an inhibition of TNF α production and maximum inhibition occurred in the presence of 200 μ M homocysteine.

In order to determine if this effect of homocysteine was specific to this particular cell line, a similar study was undertaken in the THP-1 macrophages. A similar pattern was observed (Fig. 5.7). Again, there were no notable differences in cell adhesion in the presence of homocysteine (data not shown). As in the U-937 macrophages, homocysteine was associated with a decrease in TNF α production. THP-1 macrophages appeared to be more sensitive to this inhibitory effect of homocysteine than the U-937 macrophages. After 4hrs, 15 μ M homocysteine led to a 66.7 ± 10.4 % reduction in TNF α expression, while 100 μ M homocysteine was associated with a 76.3 ± 6.4 % reduction compared to controls (no homocysteine) ($p < 0.01$, $n=3$).

5.4.7. Effect of homocysteine on TNF α gene expression by U-937 macrophages (*pre-PMA-stimulated* U-937 cells).

To determine if the effects of homocysteine on macrophage TNF α expression are mediated at the transcriptional or translational level, the effects of homocysteine on TNF α gene expression were investigated by Northern blotting as described in section 5.3.6. RNA was prepared and quantitated as described in section 2.2.7. To ensure that the RNA was not degraded, each RNA sample was separated by denaturing gel electrophoresis (section 2.2.8). The RNA samples prepared using Ultraspec™ can be seen in Fig. 5.8. This gel demonstrates the integrity of the RNA. The intensity of the

28s band is almost twice that of the 18s band, verifying the quality of the RNA preparation. If degradation had occurred, there would be a smear of ethidium bromide-staining material between the 18 and 28s bands and below the 18s band and the relative intensities of the 28s and 18s bands would be altered.

The TNF α probe was synthesised by RT-PCR using primers specific to exon 1 and 2 of the TNF α gene (section 2.2.9 and 2.2.10). This resulted in the production of a 433bp probe, specific to TNF α . Amplification only occurred when human cDNA was used as template, *ie* these primers did not allow the amplification of this TNF α region from genomic DNA (Fig 5.9). The GAPDH probe (597bp) was produced in a similar manner and was a gift of Donncha Dunican, Dept of Biochemistry, RCSI.

To verify that equal amounts of mRNA were present in each RNA preparation, expression of GAPDH, a constitutively expressed house-keeping gene was assessed. As can be seen in Fig 5.10, panel A, there was no significant difference in expression of this gene between the eight different samples, thereby implying that similar amounts of mRNA were present in each sample. This was confirmed by densitometry, where the intensities of each band was measured (Table 5.1).

The level of TNF α in the culture medium of the cells used for the Northern blotting analysis are shown in Fig 5.10. Although TNF α secretion into the culture medium was reduced in those cells exposed to 100 μ M homocysteine, there appeared to be no marked differences in the expression of TNF α mRNA as assessed by densitometry, regardless of homocysteine treatment (Fig. 5.10, panel B). This suggests that homocysteine does not control tumour necrosis factor alpha expression at the transcriptional level. TNF α levels in the culture medium were 44, 43 and 36% reduced at 4, 12 and 24hrs after the addition of homocysteine. These responses to homocysteine are of a similar magnitude to those reported in section 5.4.6, where 100 μ M homocysteine caused almost a 40% reduction in TNF α levels after 4hrs, compared to controls.

Northern blotting analysis was carried out on two sets of RNA samples. The results shown in Fig 5.10 are from one of these experiments and are typical of the results found.

5.4.8. Homocysteine cytotoxicity.

Homocysteine cytotoxicity was investigated by counting the number of viable and non-viable cells by acridine orange staining (section 2.2.2) and by assaying for LDH release (section 2.2.11).

Lactate dehydrogenase (LDH) is a cytoplasmic enzyme. Its presence in the culture medium of cells is indicative of cell membrane damage (cytotoxicity) which allows its release from the cells. It is therefore commonly used as a measure of cytotoxicity. Exposure of monocytes to homocysteine up to 200 μ M did not lead to any significant increases in LDH release in comparison to the controls (non-homocysteine treated cells) (Fig 5.11). This indicates that homocysteine up to 200 μ M was not cytotoxic to these cells. Although there was a trend for increased release of the enzyme in the presence of increasing homocysteine, it became statistically significant only at 10mM ($p < 0.02$), *ie* a grossly unphysiological concentration.

Staining of cells by acridine orange revealed that a 24hr exposure of the monocytes to homocysteine up to 200 μ M in concentration did not effect viability or cell division (data not shown).

To investigate the potential cytotoxicity of homocysteine on macrophages, non-adherent cells (monocytes) were removed following PMA-induced differentiation of both U-937 and THP-1 cells, adherent cells were washed with PBS and fresh medium, containing homocysteine (0-200 μ M), was added. At several times after this medium had been added, the number of cells in the culture medium was assessed by acridine orange staining (section 2.2.2). Excess lifting of cells or an excess of dead cells in the

culture medium, in the presence of homocysteine, would indicate that homocysteine was cytotoxic.

Following differentiation, the cells remained predominantly adherent. After 24hrs there was an appearance of dead cells in the culture (approximately 5% of the total number of adherent cells). This occurred in both the control and homocysteine treated cells but did not occur to any greater extent in the homocysteine-treated cells and was probably due to the exhaustion of the culture medium by the cells. Homocysteine appeared to have no significant effect on cell viability of either U-937 or THP-1 macrophages.

Thus, two different methods have shown that homocysteine, at least at the concentrations used in these experiments, was not cytotoxic to U-937 and THP-1 monocytes/macrophages.

5.4.9. Effect of cysteine on TNF α production by U-937 macrophages (*ie* PMA-stimulated U-937 cells).

To ensure that the effect of homocysteine on TNF α production was specific to homocysteine and not a thiol-associated phenomenon, the effect of cysteine on macrophage TNF α expression was examined as described in section 5.3.8. Cysteine is a homologue of homocysteine and an amino acid present in the plasma at approximately 130 μ M (Wilcken & Gupta, 1979). Following PMA-induced differentiation of U-937 cells, the adherent cells were given fresh medium with supplemental cysteine (100 μ M). TNF α release by those cells supplemented with cysteine, did not differ to those with no added cysteine (n=3) (Fig. 5.12). Thereby showing that the effect of homocysteine on TNF α production is specific to that amino acid.

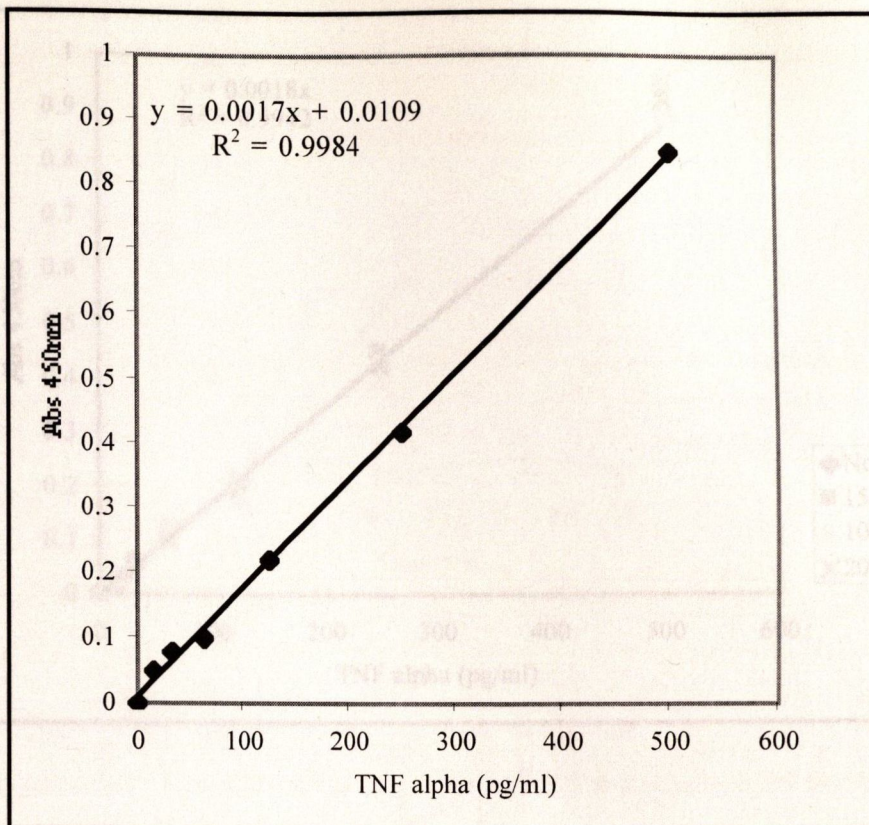


Fig 5.1 Typical standard curve for TNF α using an ELISA.

Fig 5.1 Typical standard curve for TNF α using an ELISA (Genzyme Diagnostics, UK).

Correlation between the absorbance at 450nm and the concentration of the cytokine is strong, as demonstrated by the high r^2 value (Pearson coefficient). The equation, of the type $y = mx + c$ (where m is equal to the slope of the line), was used to convert absorbances to TNF α concentrations. The best fit, " r^2 " value and the equation for the best-fit line were calculated using the Microsoft™ Excel '97 graphics and statistics package.

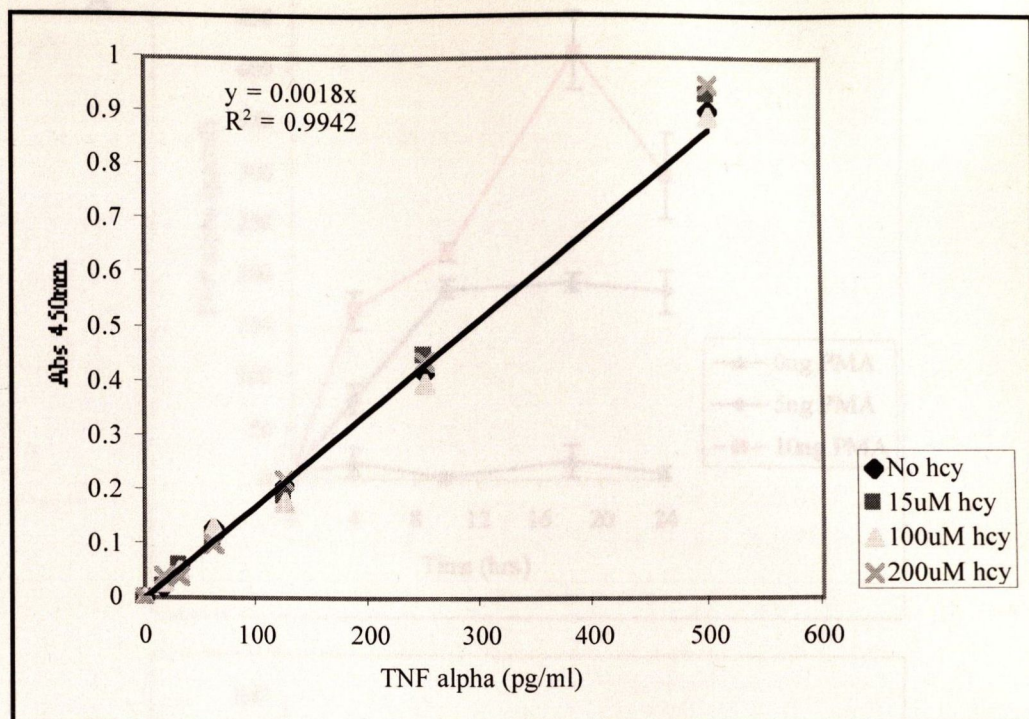


Fig 5.2 Effect of homocysteine on the TNF α ELISA.

A series of TNF α standards were assayed by the ELISA, in the presence and absence of homocysteine (0, 15, 100, 200 μ M (n=3)). The graph above shows the *mean* absorbances measured for each standard in the presence and absence of homocysteine. Statistical analysis using paired Students t-tests showed that there was no significant difference in the detection of TNF α in the presence of 15, 100 and 200 μ M homocysteine.

Fig. 5.3a. Dose effect of PMA on TNF α production and adherence of U-937 cells.

U-937 cells were cultured at a density of 5×10^5 cells/ml, and supplemented with 0, 5 or 10 ng/ml PMA. At the time points indicated in the graph, culture media was removed and adherence was assessed as described in section 5.3.1 and TNF α secretion was assayed by ELISA.

A: There was minimal production of TNF α in the absence of PMA. In the presence of PMA, there was a time and dose-dependent increase in TNF α production, up to 18 hrs.

B: Cell adherence increased up to 18 hrs and did not appear to be dose-dependent.

Results shown represent the mean $\pm$ SD of three experiments.

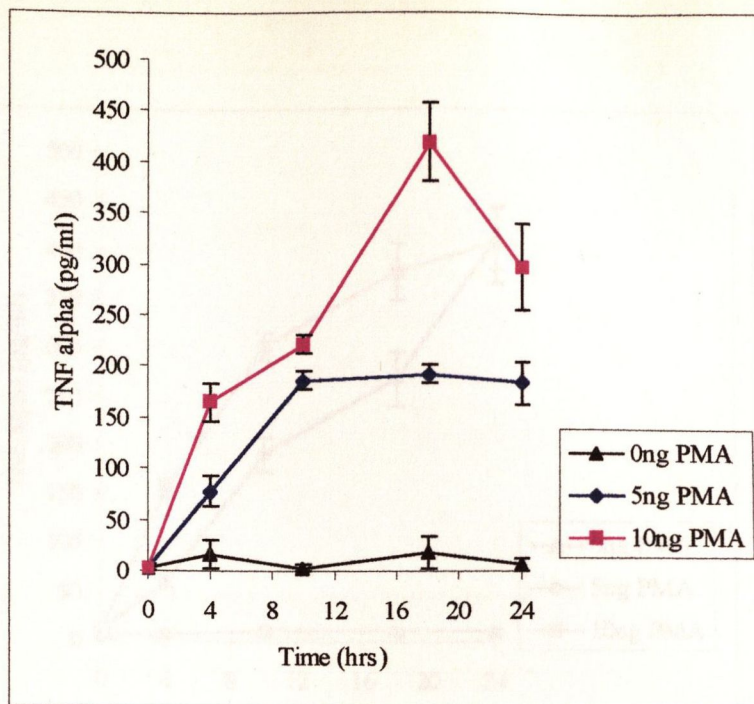
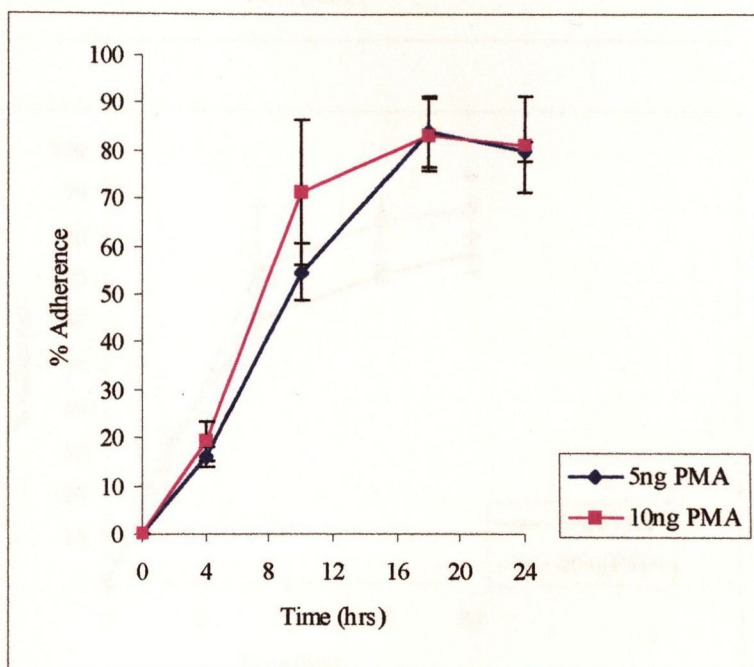
A**B**

Fig. 5.3a. Dose effect of PMA on TNF α production and adherence of U-937 cells.

U-937 cells were cultured at a density of 5×10^5 cells/ml, and supplemented with 0, 5 or 10ng/ml PMA. At the time points indicated in the graph, culture media was removed and adherence was assessed as described in section 5.3.1 and TNF α secretion was assayed by ELISA.

A: There was minimal production of TNF α in the absence of PMA. In the presence of PMA, there was a time and dose-dependent increase in TNF α production, up to 18hrs.

B: Cell adherence increased up to 18hrs and did not appear to be dose-dependent.

Results shown represent the mean $\pm$ SD of three experiments.

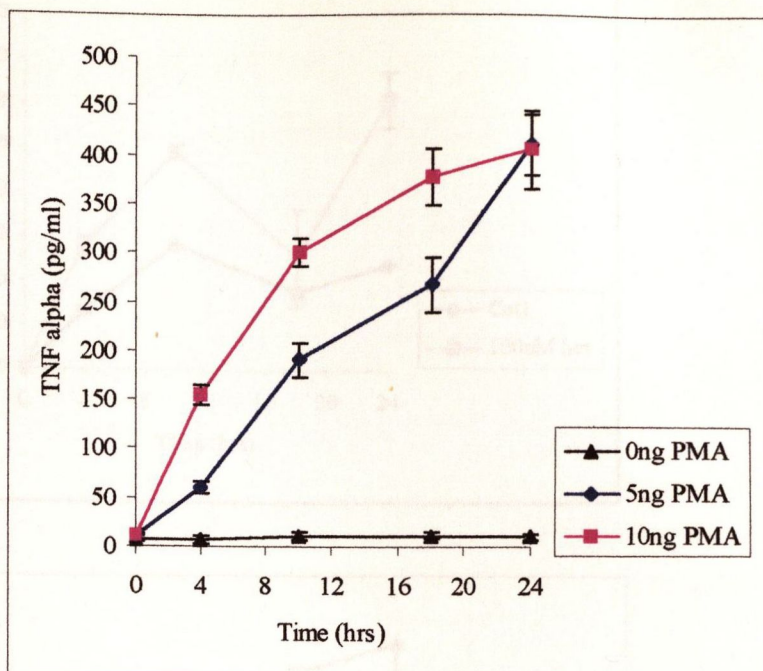
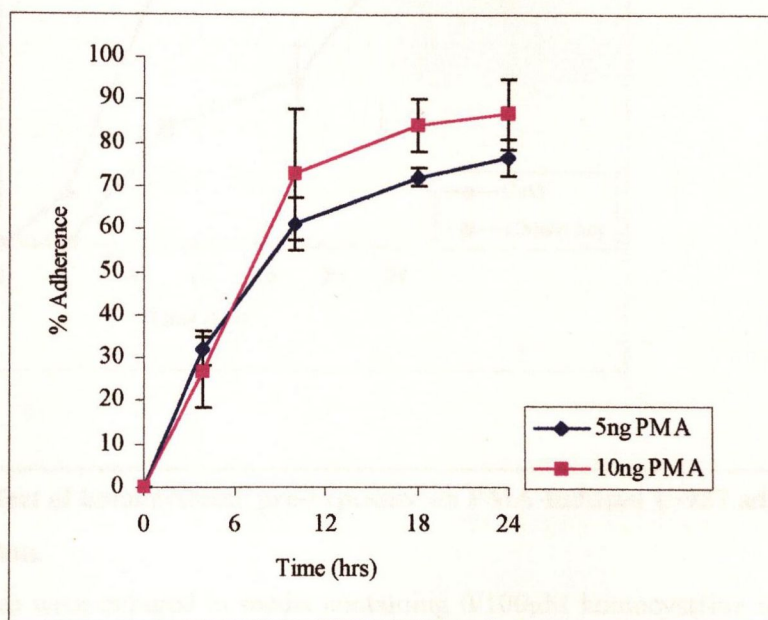
A**B**

Fig. 5.3b. Dose effect of PMA on TNF α production and adherence of THP-1 cells.

THP-1 cells were cultured at a density of 5×10^5 cells/ml, and supplemented with 0, 5 or 10ng/ml PMA. At the time points indicated in the graph, culture media was removed, cell adherence was monitored as described in section 5.3.1 and TNF α secretion was assayed by ELISA (section 5.3.1.1).

A: There was minimal production of TNF α in the absence of PMA. In the presence of PMA, there was a time and dose-dependent increase in TNF α production, up to 24hrs.

B: Adherence of cells increased up to 24hrs.

Results shown represent the mean $\pm$ SD of three experiments.

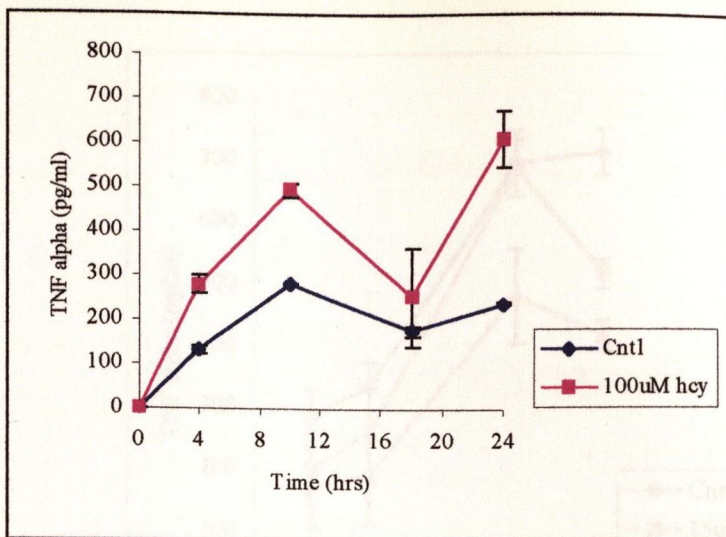
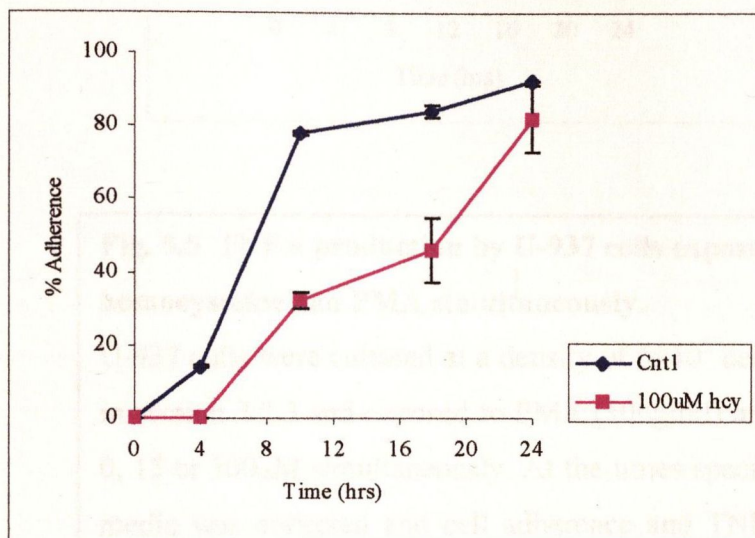
A**B**

Fig. 5.4 The effect of homocysteine pre-exposure on PMA-induced U-937 adherence and TNF α production.

U-937 monocytes were cultured in media containing 0/100 μ M homocysteine for 24hrs. They were then transferred to fresh media and PMA-induced adherence and TNF α production were monitored over the following 24hrs (section 5.4.4).

A: TNF α levels in the culture media. PMA-induced TNF α production appeared to be enhanced by prior exposure of the monocytes to 100 μ M homocysteine.

B: % Adherence (an indication of differentiation). Up to 18hrs, there appeared to be a decrease in PMA-induced adherence caused by a prior exposure of the monocytes to 100 μ M homocysteine. Homocysteine was however not cytotoxic to these cells as assessed by LDH release and acridine orange staining.

Results shown represent the mean $\pm$ SD of triplicate experiments.

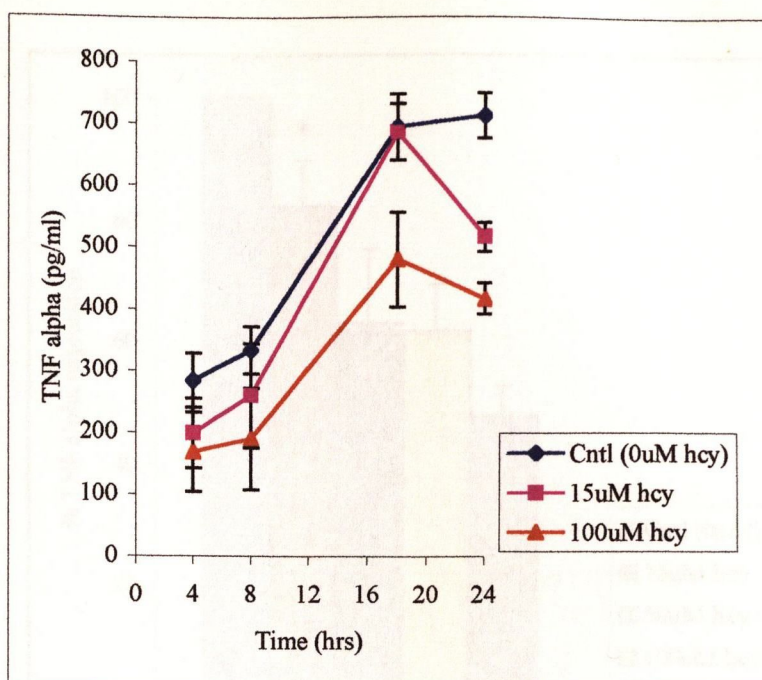


Fig. 5.5 TNF α production by U-937 cells exposed to homocysteine and PMA simultaneously.

U-937 cells were cultured at a density of 5×10^5 cells/ml, as described in section 2.2.3 and exposed to PMA (10ng/ml) and homocysteine at 0, 15 or 100 μ M simultaneously. At the times specified above, culture media was collected and cell adherence and TNF α secretion were assessed.

Homocysteine did not appear to effect cell adherence (data not shown). In the presence of 15 μ M hcy, there were no significant changes in PMA-induced TNF α expression, except at 24hrs ($p < 0.02$, $n = 3$). At 4, 8, 18 and 24hrs, exposure to PMA and 100 μ M hcy led to a decrease in the TNF α secretion compared to controls (no homocysteine), but was only statistically significant at 18 ($p < 0.01$) and 24hrs ($p < 0.01$) (Students paired t-test, $n = 3$).

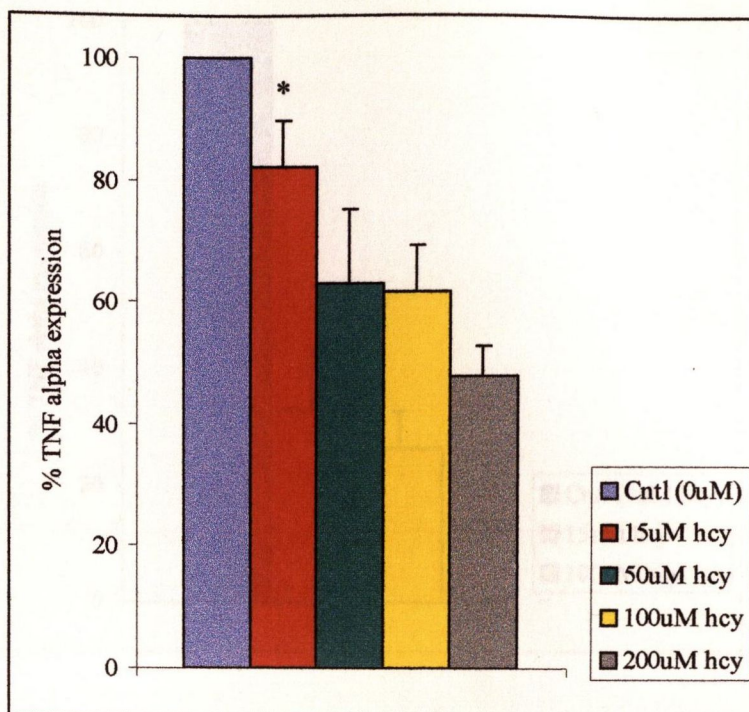


Fig. 5.6 Effect of homocysteine on TNF α production by U-937 macrophages (*ie* PMA-stimulated U-937 cells).

Differentiation of U-937s was induced by an 18hr exposure to PMA (10ng/ml). The macrophages were washed and supplemented with fresh media containing homocysteine at 0, 15, 50, 100 or 200 μ M. After 4hrs, media was collected to estimate TNF α production. With one exception (*), homocysteine caused a statistically significant reduction in the expression of TNF α , which was almost dose-dependent. Data presented are the results of three separate experiments (mean $\pm$ SD), each assayed in duplicate by ELISA. TNF α production by the cultures supplemented with homocysteine is expressed as a percentage of the production by the control cultures *ie* no homocysteine (designated 100%).

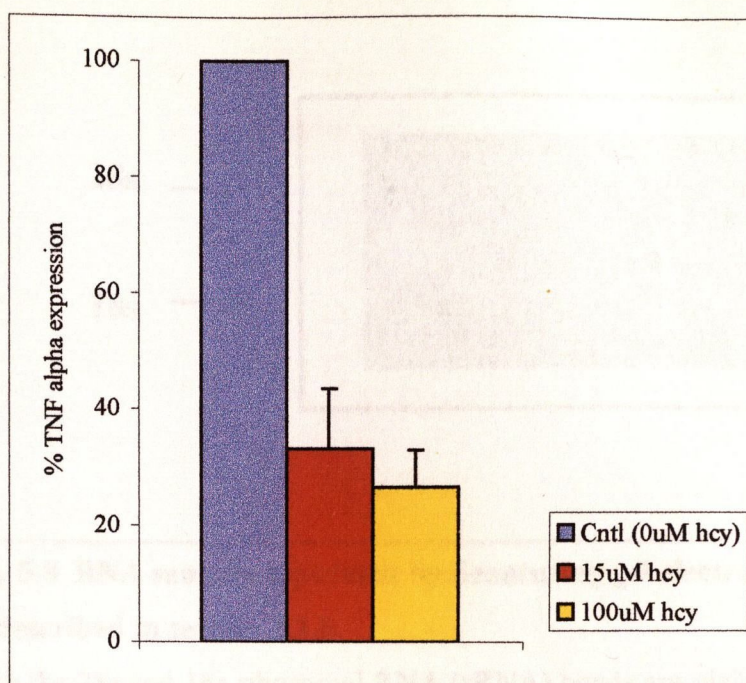


Fig. 5.7 Effect of homocysteine on TNF α production by THP 1 macrophages (*ie* PMA-stimulated THP-1 cells).

Differentiation of THP-1 monocytes was induced by an 18hr exposure to PMA (10ng/ml). The macrophages were washed and supplemented with fresh media containing homocysteine at 0, 15 or 100 μ M. After 4hrs, media was collected to estimate TNF α production. Homocysteine caused a statistically significant decrease in the expression of TNF α . Data presented are the results of three separate experiments (mean $\pm$ SD), each assayed in duplicate by ELISA. TNF α production by the cultures supplemented with homocysteine is expressed as a percentage of the production by the control cultures *ie* no homocysteine (designated 100%).

28s



18s

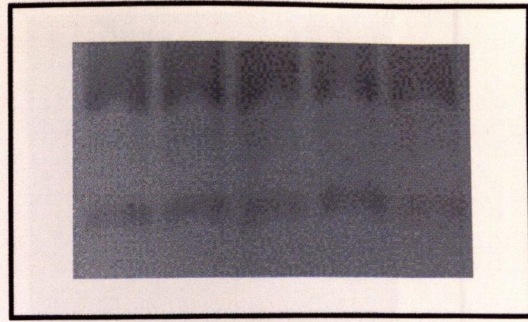


Fig. 5.8 RNA samples separated by denaturing gel electrophoresis, as described in section 2.2.8.

Both the 28s and 18s ribosomal RNA (rRNA) bands are visible, with the intensity of the 28s rRNA approximately twice that of the 18s rRNA. Degradation of RNA would result in loss of the two bands that would appear as a smear of ethidium bromide staining material. The above gel testifies to the quality of the RNA preparation and verifies that it is intact.

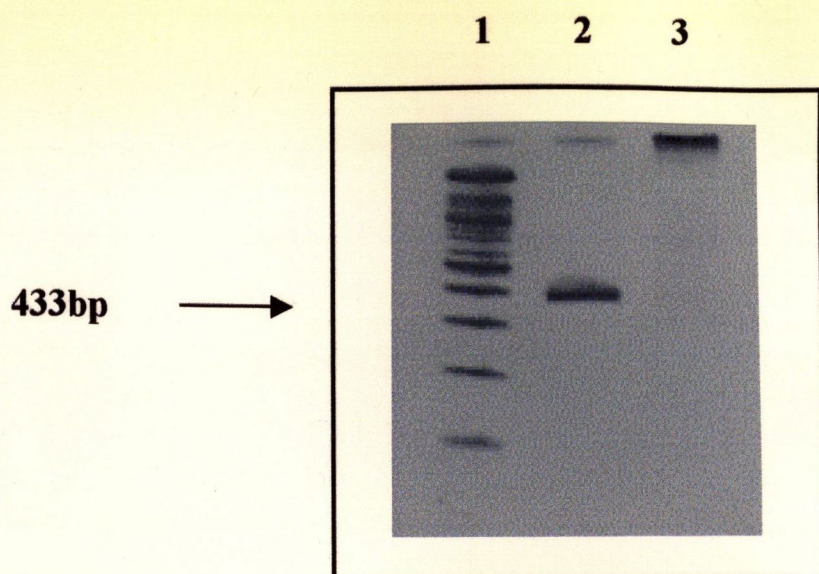


Fig. 5.9 Agarose gel (1.5%(w/v)) electrophoresis showing the TNF α probe used in Northern blotting.

Lane 1: 100bp ladder molecular weight marker

Lane 2: PCR product using cDNA as template

Lane 3: PCR product using genomic DNA as template.

Only when cDNA was used as template, was there amplification of the TNF α gene, giving a product of 433bp. This band was excised from the gel, subject to gel purification, labelled by α ^{32}P dCTP and used to detect TNF α gene expression by Northern blotting (section 2.2.10).

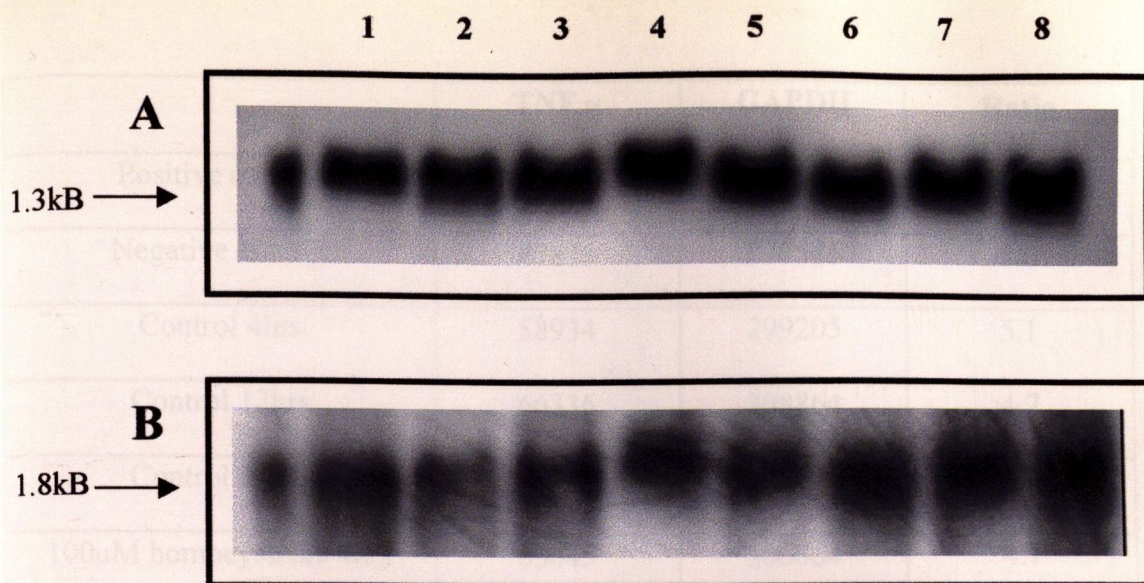


Fig. 5.10 Northern blot analysis of TNF α gene expression in response to 100 μ M homocysteine in U-937 macrophages.

A: GAPDH expression.

B: TNF α expression.

Lane	Sample	*TNF α
1	Positive control (PMA (10ng/ml))	228.9pg/ml
2	Negative control (No PMA)	8.7pg/ml
3	Cntl 4hrs	35.9pg/ml
4	Cntl 12hrs	51.7pg/ml
5	Cntl 24hrs	139.3pg/ml
6	100 μ M hcy 4hrs	20.3pg/ml
7	100 μ M hcy 12hrs	29.7pg/ml
8	100 μ M hcy 24hrs	93.1pg/ml

Differentiation of U-937 cells was induced by exposure to PMA (section 5.3.1). Macrophages were washed and fresh media containing 0 or 100 μ M homocysteine was added. At the times specified above, total RNA was prepared, blotted and probed as described in section 5.3.6 and TNF α levels in the culture media were assessed in duplicate by ELISA (section 5.3.1.1).

GAPDH expression was uniform in each sample, confirming that there was an equal amount of mRNA present in each sample. TNF α expression was also uniform in each sample except in the sample with no added PMA. Here (lane 2), TNF α expression was lower than in the positive control (10ng/ml PMA, lane 1).

*TNF α refers to the concentration of TNF α in the medium from which the RNA was harvested.

	TNF α	GAPDH	Ratio (GAPDH/TNF)
Positive control	61659	249360	4.1
Negative control	47876	277585	5.8
Control 4hrs	58934	299205	5.1
Control 12hrs	66336	308864	4.7
Control 24hrs	61706	302349	4.9
100 μ M homocysteine 4hrs	65645	306064	4.7
100 μ M homocysteine 12hrs	69514	310686	4.5
100 μ M homocysteine 24hrs	70003	321207	4.6

Table 5.1 Relative intensities (as measured by densitometry) of the labelled TNF α and GAPDH mRNAs of control and homocysteine-treated U-937 macrophages, as shown in Fig. 5.10.

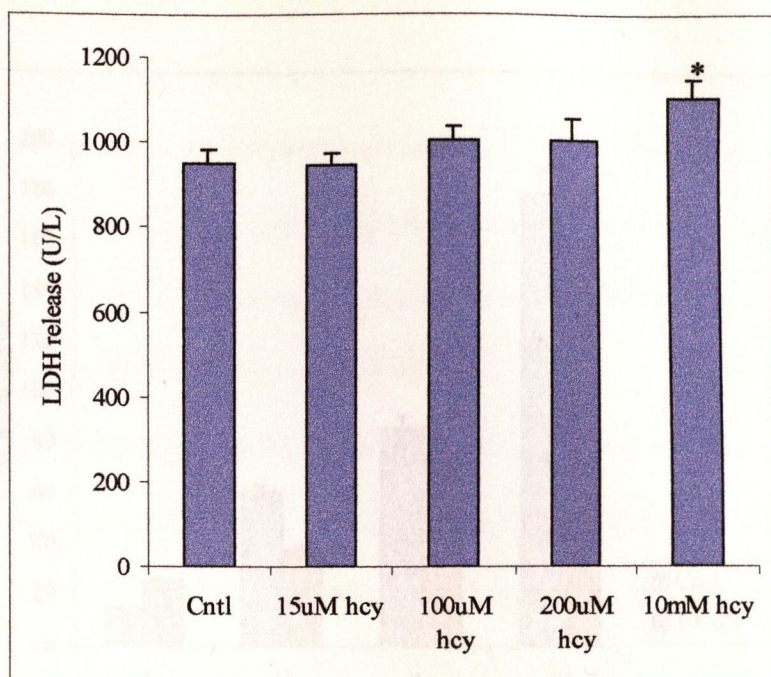


Fig 5.11 Lactate dehydrogenase release by U-937 monocytes exposed to homocysteine.

U-937 cells were cultured as described in section 2.2.3 in the presence of 0, 15, 100, 200 μ M or 10mM homocysteine. After 24hrs, culture media was collected and assayed for LDH release using a LDH kit (Randox, Ireland). Results shown represent the mean $\pm$ SD of triplicate experiments. Using Students paired t-test, there were no significant differences in LDH concentrations, except in the presence of 10mM homocysteine (* $p < 0.05$, $n = 3$). This demonstrates that homocysteine up to 200 μ M was not toxic to the cell cultures.

5.5. DISCUSSION

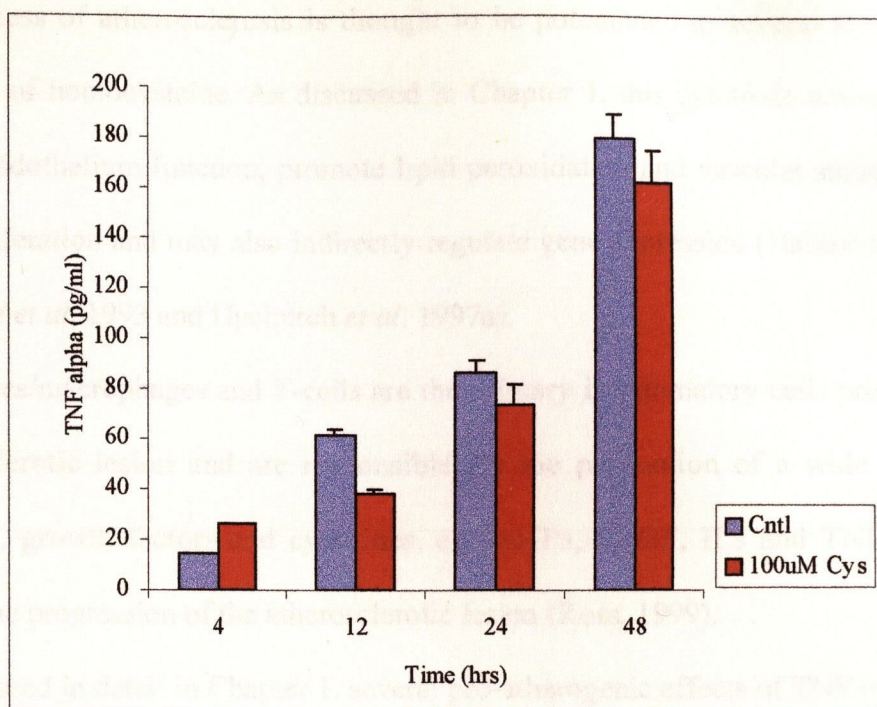


Fig. 5.12 The effect of cysteine on TNF α production by PMA-stimulated U-937 macrophages.

Differentiation of U-937 monocytes to macrophages was induced by PMA (section 5.3.1). Non-adherent cells were removed after 18hrs and fresh media containing 0 or 100 μ M cysteine was added. Media was then collected at the times indicated above and assayed for TNF α release by ELISA, in duplicate. Results shown represent mean $\pm$ SD of three experiments. Cysteine had no significant effect on TNF α production by the macrophages.

5.5. DISCUSSION.

The process of atherosclerosis is thought to be potentiated at several stages by the presence of homocysteine. As discussed in Chapter 1, this cytotoxic amino acid can impair endothelium function, promote lipid peroxidation and vascular smooth muscle cell proliferation and may also indirectly regulate gene expression (Harker *et al*, 1983; Heinecke *et al*, 1993 and Upchurch *et al*, 1997a).

Monocytes/macrophages and T-cells are the primary inflammatory cells present in the atherosclerotic lesion and are responsible for the production of a wide variety of enzymes, growth factors and cytokines, *eg* MMPs, PDGF, ILs and TNF α , which further the progression of the atherosclerotic lesion (Ross, 1999).

As discussed in detail in Chapter 1, several pro-atherogenic effects of TNF α have been demonstrated. TNF α stimulates smooth muscle cell activation and proliferation (Morisaki *et al*, 1993 and Jovinge *et al*, 1997). In proliferating endothelial cells it may cause apoptosis, while in non-proliferating endothelial cells, it has been found to induce a pro-coagulant state and may promote neovascularisation (Leibovich *et al*, 1987; Grau & Lou, 1993 and Toborek *et al*, 1997). TNF α also promotes endothelium-leukocyte interactions by inducing several adhesion molecules, such as ECAM-1, ICAM-1 and VCAM-1 and chemotactic factors for neutrophils and monocytes (Dixit *et al*, 1990; Yoshizumi *et al*, 1992 and Barks *et al*, 1997). In addition, it is believed that through the induction of MMPs, TNF α may promote plaque rupture (Kaartinen *et al*, 1996).

The TNF α promoter contains functional binding sites for the transcription factors NF- κ B and AP-1 (Jovinge *et al*, 1996). Both of these transcription factors are activated by oxidising agents such as hydrogen peroxide (H_2O_2). Oxidation of homocysteine results in the production of several reactive oxygen species, including H_2O_2 and so it is possible that homocysteine might indirectly regulate gene expression (Heinecke *et al*, 1993). Indeed, it has been found to do so in endothelial cells (Kokame *et al*, 1996).

Monocytes, T-lymphocytes, platelets, endothelial cells and smooth muscle cells interact to generate the atherosclerotic lesion. Of these cell types, a role for homocysteine in various aspects of atherosclerosis has been extensively studied, with the exception of macrophages and T-lymphocytes. Monocyte-macrophages are present at every stage of atherogenesis and the advanced atherosclerotic lesion is composed of approximately 80% macrophages (Tipping & Hancock, 1993).

A study into the effects of homocysteine on macrophage function was undertaken with the following points in mind;

- abundance of macrophages in the atherosclerotic lesion,
- importance of TNF α in atherosclerosis,
- the potential regulatory role of homocysteine on gene transcription, via redox-sensitive transcription factors
- lack of data on the effects of homocysteine on macrophage activity.

Specifically, the effect of homocysteine, a potential source of oxidant stress, on the monocyte/macrophage production of TNF α was investigated.

The human cell-lines U-937 and THP-1 were chosen as models of monocytes and macrophages. U-937 and THP-1 cells share many cytochemical and immunological criteria, that allow them to be characterised as monocytic (Tsuchiya *et al*, 1980). The U-937 cell-line was established in 1974 from malignant cells obtained from the pleural effusion of a Caucasian male with diffuse histiocytic lymphoma. It is one of only a few cell-lines still expressing many of the monocytic-like characteristics exhibited by cells of histiocytic origin (Sundström & Nilsson, 1976).

The human monocytic cell-line THP-1 was established in 1980. It was derived from the peripheral blood of a male with acute monocytic leukaemia (Tsuchiya *et al*, 1980).

In their monocytic form, both cell-lines grew as a suspension. Under an inverted microscope, they appeared to be mainly round in shape but some were irregularly

shaped. Small cytoplasmic projections were visible on some cells. These observations were in agreement with descriptions in the literature (Sundström & Nilsson, 1976 and Tsuchiya *et al*, 1980). U-937 and THP-1 cells can be induced to terminal monocytic differentiation by phorbol-esters *eg* PMA (Koren *et al*, 1979 and Tsuchiya *et al*, 1982). As anticipated, in the absence of PMA, the unactivated cells continued to grow as a suspension and produced very low levels of TNF α . Exposure of the U-937 and THP-1 monocytes to PMA (10ng/ml) caused marked alterations in morphology, led to increasing adhesiveness and increased production of TNF α , a cytokine normally produced by activated macrophages (Figs. 5.3a and 5.3b).

In both the U-937 monocytes and THP-1 monocytes, there appeared to be no alterations to TNF α production on exposure to homocysteine for up to 24hrs (section 5.4.3). In addition, homocysteine was not cytotoxic to the cells and did not, of itself, induce adherence.

PMA-induced TNF α production was significantly enhanced by a pre-exposure of U-937 monocytes to 100 μ M homocysteine (section 5.4.4). TNF α levels increased over time in both the homocysteine-treated and control cells. However, both showed a reduction in TNF α at 18hrs, and here the increase in TNF α levels in the homocysteine-treated cells did not reach statistical significance ($p>0.05$, $n=3$) (Fig. 5.4, panel A). This decrease in TNF α output had been previously observed in preliminary experiments, where medium levels of this cytokine rose and fell over the course of a 72hrs exposure to PMA (data not shown) and is corroborated by reports in the literature (Gatanga *et al*, 1991).

In contrast to this enhanced production of TNF α by homocysteine pre-exposure, the rate of PMA-induced cell adherence was significantly lower in the homocysteine-treated cells up to 18hrs but reached the same level of adherence as the control cells by 24hrs (Fig. 5.4, panel B). This was the only instance in the course of these studies,

where the adherence of cells in culture and the appearance of TNF α in the culture medium were not closely correlated. The factors underlying this divergence of effects are not readily understood.

Surprisingly, when U-937 monocytes were cultured in the presence of PMA and homocysteine *simultaneously*, a substantial inhibition of TNF α production resulted ($41.4 \pm 3.0\%$ reduction in response to $100\mu\text{M}$ homocysteine), although no alterations to the progress of differentiation (as indicated by cell adherence) were observed (section 5.4.5). These findings were in stark contrast to those reported in section 5.4.4. In order to further investigate the matter, the effects of homocysteine on macrophage TNF α formation were investigated (section 5.4.6).

Macrophage TNF α production was also substantially inhibited by homocysteine, in both U-937 and THP-1 cells, in a dose-dependent manner (Figs. 5.6 and 5.7).

The extent of TNF α inhibition however, appeared to be greater in the THP-1 macrophages, *ie* $76.3 \pm 6.4\%$ reduction in THP-1 vs $38.4 \pm 7.7\%$ in U-937 macrophages at $100\mu\text{M}$ homocysteine.

As previously mentioned, TNF α gene expression is controlled at the transcriptional, post-transcriptional and translational levels. The 5' flanking region of the TNF α gene, which affects its transcription, contains several regulatory sites, including AP-2, c-jun/AP-1 and NF- κB binding sites (Nicola, 1994). The signal transduction pathways mediating production of TNF α differ depending on the stimuli and cell type involved. PMA-induced TNF α production by monocytes/macrophages involves activation of protein kinase C and subsequent formation of AP-1/c-jun heterodimers, which activate transcription (Rhoades *et al*, 1992). TNF α production may also be downregulated by a number of factors (Wong & Goeddel, 1989). Jovinge and colleagues have reported that TNF α production by monocytes in the early stages of differentiation was regulated by the presence of oxLDL. At high concentrations ($>15\mu\text{g/ml}$), oxLDL inhibited

expression of TNF α , while at low concentrations ($<8\mu\text{g/ml}$), it was found to upregulate its expression. This is thought to be related to the activity of the redox-sensitive transcription factors, AP-1 and NF- κB (Jovinge *et al*, 1996).

The initial hypothesis underlying this work, was that if homocysteine did regulate PMA-induced TNF α production that it would more than likely do so at the transcriptional level, perhaps by altering expression of the redox-sensitive transcription factor AP-1.

To gain insight into the mechanism of TNF α inhibition by homocysteine, TNF α mRNA expression was assessed by Northern blotting. Hybridisation with radiolabelled GAPDH was used to assess equal loading of RNA on the gel and also to ensure that equal quantities of mRNA were present in each sample. No differences in TNF α mRNA expression by U-937 macrophages were observed in the presence of $100\mu\text{M}$ homocysteine over 24hrs (Fig 5.10). This was despite that fact that TNF α secretion into the culture medium was almost halved at each time point by the presence of homocysteine (section 5.4.7). This suggests that homocysteine does not regulate TNF α expression at the transcriptional level. The only sample to demonstrate reduced expression of TNF α mRNA, was the negative control, *ie* non-PMA-stimulated U-937 monocytes. GAPDH expression was however comparable to the other samples, demonstrating that this reduction in TNF α mRNA did not arise as a result of a lower concentration of RNA in this sample.

If homocysteine does not regulate TNF α mRNA production, how then does it cause decreased secretion of this cytokine? It has been proposed that in the endoplasmic reticulum (ER), aberrant processing of proteins may occur in the presence of high homocysteine as a result of altered redox potential. This could affect proper disulphide bond formation in certain proteins, with subsequent retention of that protein in the ER (Lentz & Sadler, 1993). This view is supported by the studies of Outinen *et al* who

reported that homocysteine alters expression of several genes known to be sensitive to ER stress (Outinen *et al*, 1999).

TNF α is a 157 amino acid acidic polypeptide with an apparent molecular weight of 17kDa, under denaturing conditions. Under native conditions, it exists as a trimer, with cysteines at position 69 and 101 involved in disulphide bridge formation (Aggarwal *et al*, 1985). Therefore it is possible the reduced expression of TNF α observed in the presence of elevated homocysteine is as a result of its aberrant processing and retention in the ER. Alternatively, homocysteine might in some way alter TNF α mRNA stability or inhibit its export from the cell.

There have been no reports to our knowledge directly relating homocysteine and macrophage TNF α expression. Jeong *et al* have found that 3-deazaadenosine, a potent inhibitor of SAH hydrolase inhibits LPS-induced TNF α production by both human and mouse macrophage cell lines (Jeong *et al*, 1996 and 1999). An accumulation of SAH, potentiated by the presence of elevated homocysteine, and related to the transcriptional activity of NF- κ B, is thought to account for this phenomenon (Jeong *et al*, 1999). However, as LPS and PMA are known to function *via* different signal transduction pathways in stimulating TNF α production, these studies cannot be directly compared to those described here.

Many studies on the effects of homocysteine on endothelial and smooth muscle cells have used grossly unphysiological (mM) concentrations of homocysteine. The observations described in this study were seen at relatively low concentrations of homocysteine (15-200 μ M). These concentrations are found in hyperhomocysteinemic (15-100 μ M) and homocystinuric (100-200 μ M) patients.

As homocysteine was found to bring about a decrease in TNF α production by macrophages, it was essential to show that this decrease was not due to any adverse affect homocysteine might have on the ELISA or that it was simply a reflection of cell

death induced by homocysteine. The standard curves derived in the presence of homocysteine illustrate that it has no detrimental effects on the detection of TNF α by this ELISA (Fig. 5.2). By monitoring cell viability by acridine orange staining and cellular stress by LDH release, it was found that homocysteine, up to 200 μ M was not toxic to the cell cultures (Fig. 5.11). These findings regarding the cytotoxicity of homocysteine are in agreement with those reported by Hultberg *et al.* They found that the addition of high concentrations of homocysteine (2000 μ M) to the medium of U-937 cells did not result in any sign of cell damage (Hultberg *et al.*, 1995).

Thus, the reduction of macrophage TNF α production in the face of elevated homocysteine has been demonstrated in two different monocyte-derived macrophage cell-lines and does not arise as a consequence of homocysteine cytotoxicity.

A recent report has suggested that cysteine and not homocysteine is the harmful agent in atherosclerosis as it has been found to be elevated in vascular disease subjects (Jacob *et al.*, 1999). This is more than likely a consequence of hyperhomocysteinemia. Nonetheless, the effect of cysteine on TNF α production was assessed. There was no significant difference in macrophage TNF α expression in the presence of cysteine (Fig. 5.12). This suggests that it is homocysteine, at relatively low levels, that specifically regulates TNF α production.

This study demonstrates that homocysteine is associated with an enhancement of PMA-induced TNF α expression, when monocytes have been exposed to homocysteine prior to the addition of PMA but is associated with a *reduction* in macrophage TNF α production, when added to the cells simultaneously or after the addition of PMA. This suggests that homocysteine has differential effects on TNF α synthesis, depending on the differentiation stage of the monocyte.

How this occurs is not known and can only be speculated upon. Possibly, it is a reflection of the capability of the different cell types to metabolise and dispose of

homocysteine, or to cope with the oxidant stress caused by high levels of homocysteine. That homocysteine may exert cell type- and redox-specific regulation of AP-1 dependent events has been suggested by the findings of Suzuki *et al* (Suzuki *et al*, 2000).

Jovinge *et al* have demonstrated that increased TNF α production by monocyte/macrophages in response to low levels of oxLDL is associated with activation of transcription factor AP-1 (Jovinge *et al*, 1996). They proposed that LDL oxidation resulted in the formation of factors that could enhance monocyte/macrophage release of TNF α . This is perhaps the same mechanism that resulted in the increased TNF α release by PMA-stimulated monocytes that had been exposed to homocysteine, *ie via* production of ROS that induce AP-1 activity, thereby enhancing TNF α production. This enhanced production of TNF α could promote atherogenesis in numerous ways that have been discussed in detail in Chapter 1.

An unexpected finding was the reduction in macrophage PMA-inducible TNF α production - unexpected given the positive effects it had on monocyte TNF α production and given that it is generally an increased TNF α level that is associated with vascular disease (Pesonen, 1994 and Vaddi *et al*, 1994). If this is the case *in vivo*, what are the possible consequences with regards to atherosclerosis?

One of the earliest steps in the process of atherogenesis is the formation of the "fatty streak", a step that requires the surface expression of a scavenger receptor by macrophages. This facilitates the unregulated uptake of modified low density lipoprotein (*eg* oxidised LDL), leading to foam cell formation (Luc & Fruchart, 1991). It has been shown that tumour necrosis factor alpha downregulates THP-1 macrophage scavenger receptor expression by transcriptional and post-transcriptional mechanisms (Hsu *et al*, 1996).

In light of the results found by this study, and in view of the current models of atherosclerosis, the following is proposed. Circulating monocytes or monocytes drawn to the site of inflammation, in the presence of an elevated plasma homocysteine, could lead to enhanced production of tumour necrosis factor on transformation to tissue macrophages and thereby promote atherosclerotic lesion development by inducing smooth muscle cell proliferation and endothelial cell dysfunction as discussed earlier. Thereafter within the vascular wall, macrophages, in the presence of an elevated homocysteine could display a downregulation of TNF α expression. This reduction of TNF α in a localised intravascular area could attenuate its inhibitory effect on macrophage scavenger receptor expression. Together with enhanced lipid peroxidation by homocysteine, this could allow the accumulation of cholesterol within these cells, enhancing foam cell formation and thereby promoting atherogenesis (Heinecke *et al*, 1993 and Hsu *et al*, 1996).

In conclusion, this study has shown that homocysteine may differentially affect monocyte/macrophage TNF expression and suggests a possible *in vivo* mechanism by which homocysteine may promote the atherosclerotic process.

The Effect of Homocysteine on Platelet Function

6.1. INTRODUCTION

Platelets and the coagulation proteins play a major role in the process of atherosclerosis. Many acute cardiovascular events, such as myocardial infarction and stroke arise from thrombotic occlusion of atherosclerotic arteries (Hansson & Loscalzo, 1997).

Vascular thromboembolism accounts for approximately 50% of the vascular events in homocystinuria. As is the case with homocystinemia and atherosclerotic vascular disease,

Chapter 6

an epidemiological relationship has been demonstrated between homocystinemia and deep

vein thrombosis, myocardial infarction, myocardial infarction and stroke (den Heijer *et al.*, 1993 and 1996, Stehouwer *et al.*, 1998). However, a description of the thrombotic mechanism of homocysteine is lacking.

Some evidence suggests that the pro-thrombotic nature of homocysteine may lie in its propensity to increase oxidative stress, thereby contributing to arterial and venous endothelial cell damage (Upchurch *et al.*, 1997a and b). As discussed in section 1.4.1, homocysteine is believed to convert the endothelium from an anti-thrombotic to a pro-thrombotic state, promoting platelet and leucocyte adhesion and aggregation.

Studies on homocysteine and platelet function have provided conflicting results (Datta *et al.*, 1975; McCully & Camacho, 1987 and Rajkumar *et al.*, 1999). In addition, none of the studies that have provided evidence for a pro-aggregatory effect of homocysteine have done so at non-physiological concentrations (Ginsberg *et al.*, 1987). Recent studies in animal models have suggested a pro-aggregatory effect of homocysteine (Ginsberg *et al.*, 1996b).

The Effect of Homocysteine on Platelet Function.

6.1. INTRODUCTION.

Platelets and the coagulation proteins play a major role in the process of atherosclerosis. Many acute cardiovascular events, such as myocardial infarction and stroke arise from thrombotic occlusion of atherosclerotic arteries (Handin & Loscalzo, 1992).

Vascular thromboembolisms account for approximately 50% of the vascular events in homocystinuria. As is the case with homocysteine and atherosclerotic vascular disease, an epidemiological relationship has been demonstrated between homocysteine and deep vein thrombosis, recurrent venous thrombosis, myocardial infarction and stroke (den Heijer *et al*, 1995 and 1996, Stehouwer *et al*, 1998). However, a description of the thrombotic mechanism of homocysteine is lacking.

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Studies on homocysteine and platelet function have provided conflicting results (Davis *et al*, 1975; McCully & Carvalho, 1987 and Rajkumar *et al*, 1999). In addition, some of the studies that have provided evidence for a pro-aggregatory effect of homocysteine have done so at non-physiological concentrations (Graeber *et al*, 1982). Recent studies in animal models have suggested a pro-aggregatory effect of homocysteine (Durand *et al*, 1996b).

With these factors in mind, a study was undertaken to investigate the effect of homocysteine, at concentrations seen in hyperhomocysteinemic/homocystinuric patients, on human platelet aggregation.

6.2.1.1 Platelet preparation

Platelet rich plasma (PRP) was prepared from whole blood (Becton Dickinson, 1986). Blood was drawn into syringes containing one-tenth volume 3.8% (w/v) sodium citrate (anti-coagulant) using a butterfly-type needle (21 gauge). It was ensured that the donors* had not taken any drug that interferes with platelet function such as aspirin, any analogue of it or anti-thrombotics, in the previous 14 days. Blood was then gently mixed with the anticoagulant. Small aliquots of the blood were transferred to 10ml polypropylene tubes and centrifuged at 150g for 10mins. These plasma phases were packed red cells, a thin band of white blood cells (buffy coat) and other cellular platelet rich plasma (PRP). PRP was removed using a sterile pipette pipette and transferred to a small polypropylene separator, taking care not to contaminate the preparation with the buffy coat or the red cells. Following removal of the PRP, the remainder of the buffy coat was spun at high speed (2000g) for 10mins and the supernatant was removed. This supernatant is platelet poor plasma (PPP). PRP can be used for platelet aggregation studies and platelet counts can be determined by platelet count (Becton Dickinson, 1986).

*Donors were selected to be healthy, non-smokers, of age 20-40 years, and of both sexes (n=2).

6.2.1.2 Platelet aggregation assay

Aggregation (expressed as a percentage) was determined using an aggregometer (Biotate Platelet Aggregometer, Biotate), which uses light transmission to measure platelet aggregation. As the platelet suspension is introduced into the aggregometer,

6.2. MATERIALS AND METHODS.

6.2.1. Platelet aggregation in response to homocysteine.

6.2.1.1 Platelet preparation.

Platelet rich plasma (PRP) was prepared from whole blood (McNicol *et al*, 1996). Blood was drawn into syringes containing one-tenth volume 3.8%(w/v) sodium citrate (anti-coagulant) using a butterfly-type needle (21 gauge). It was essential that the donors* had not taken any drug that interferes with platelet function, such as aspirin, any analogue of it or anti-histamines, in the previous 14 days. Blood was then gently mixed with the anticoagulant. 5ml aliquots of the blood were transferred to 15ml polypropylene tubes and centrifuged at 150g for 12mins. Three distinct phases result; packed red cells, a thin band of white blood cells (buffy coat) and straw coloured platelet rich plasma (PRP). PRP was removed using a sterile pasteur pipette and transferred to a fresh polypropylene container, taking care not to contaminate the preparation with the buffy coat or the red cells. Following removal of the PRP, the remainder of the blood sample was re-spun at high speed (2000g) for 10mins and the supernatant was removed. This supernatant is platelet poor plasma (PPP). PRP may be used directly in functional studies. The platelet concentration of the PRP was determined using a cell counter (Sysmex K-1000 haematology analyser).

*Donors were healthy laboratory staff volunteers with an average age of 24.5 ± 0.9 yrs (n=8).

6.2.1.2. Platelet aggregation assay.

Aggregation (expressed as a percentage) was determined using an aggregometer (Biodata Platelet aggregation profiler), which uses light transmission through a stirred platelet suspension as an indication of aggregation. As the platelets aggregate, they fall

to the bottom of the cuvette, thereby allowing an increase in light transmission, which is proportional to the extent of aggregation.

The platelets (as PRP) were incubated at room temperature with tris-buffered saline (TBS) (control) or 20, 50 or 100 μ M homocysteine for 15-60mins. A 10mM homocysteine stock, prepared in tris buffered saline (TBS), was used so that the volumes added to the platelets were kept to a minimum. The volumes of the additions to each sample were identical. Aggregometer cuvette chambers were allowed to reach 37°C. The stirrers were set at 1100rpm. At specified times, 450 μ l of the treated PRP samples were added to the cuvettes and allowed to equilibrate to 37°C. Stir bars were added to each sample and the chart recorder was set at 1cm/min. PPP, at 37°C, was used as a blank. Aggregation (in the absence of agonist) was monitored for approximately 0.5mins to ensure that the baseline was stable. Aggregation was then induced by addition of agonist (ADP) and monitored for 4mins*. Several indices of aggregation may be measured but should correlate, *ie*; maximal rate or maximal extent of aggregation or the extent at a given time after addition of agonist.

*In order to estimate the concentration of agonist needed to induce aggregation, various concentrations of agonist were first added to untreated PRP samples and the response was monitored over 4mins.

6.2.2. Platelet thromboxane synthesis in response to homocysteine.

6.2.2.1. Thromboxane estimation.

Following aggregation assays, PRP samples were snap frozen in liquid nitrogen and stored at -70°C. Thromboxane A₂ synthesis by the sample was assessed by measurement of thromboxane B₂ (TXB₂), the stable derivative of thromboxane A₂, by immunoassay, following the manufacturers instructions (R & D Systems). The assay is

based on the competitive binding of the TXB₂ present in a sample and a fixed amount of alkaline phosphatase-labelled TXB₂, for sites on a rabbit polyclonal antibody.

100µL of sample/standard was assayed in duplicate. During a 2hrs incubation at room temperature, the polyclonal antibody becomes bound to the goat anti-rabbit antibody coated onto the microplate. Following incubation, excess conjugate and unbound sample were removed by washing and a substrate solution (p-nitrophenyl phosphate) was added, to determine the amount of bound enzyme activity. The reaction was stopped by the addition of a trisodium phosphate solution and absorbance at 405nm was measured. Absorbance is inversely proportional to the concentration of TXB₂ in the sample.

6.2.3. Fibrinogen binding in response to homocysteine.

6.2.3.1. Fibrinogen binding assay.

Platelet aggregation involves the binding of fibrinogen to the α IIb/ β ₃ integrin on activated platelets. The fibrinogen binding domains are normally hidden on resting platelets and therefore the exposure of such sites can be used as an index of platelet aggregation.

The expression of the fibrinogen binding domains, and therefore the binding of fibrinogen, was assessed using flow cytometry (FACscan, Beckton Dickinson). Flow cytometry is a method for quantitating components or structural features of cells primarily by optical means. Cells pass, one at a time, through a flow cell with a focussed laser beam. The laser light is then deflected or absorbed, depending on the characteristics of the cell. Typically, cytometers can measure forward and side light scatter and also fluorescent labels bound to cells.

PRP was prepared as described in section 6.2.1.1 and diluted 1:10 with TBS. The fibrinogen binding assay was set up as described below and allowed to proceed in the

dark at room temperature for 30mins. The process was stopped by adding 2mls TBS. This dilutes out the fluorescently labelled fibrinogen. The expression of the fibrinogen binding domains was then assessed using FACS (FACScan, Becton Dickinson). The fluorescence of Oregon green[®] was detected using an excitation wavelength of 488nm and an emission wavelength of 520nm.. A gate was set on the platelet light scatter region. The binding of fibrinogen was determined by analysing 10000 of the particles in this region for Oregon green[®] fluorescence.

	PRP (1:10)	Homocysteine	TBS	Fibrinogen *	ADP
Unactivated platelets	100µl	0, 20, 50, 100µM	Volume depends on concentration of homocysteine†	1µg/µl	None
Activated platelets	100µl	0, 20, 50, 100µM	Volume depends on concentration of homocysteine†	1µg/µl	10µM

* Oregon green[®] fluorescently labelled fibrinogen (Unigene, Oregon, USA) resuspended in 0.1M NaHCO₃, pH 8.3. It is essential to vortex the suspension well before use as fibrinogen forms polymers in storage.

† The total volume in each reaction was 200µl.

Unactivated PRP (TBS) was included as a negative control for fibrinogen binding.

6.3. RESULTS.

6.3.1. Platelet aggregation in response to homocysteine.

Agonist-induced platelet aggregation in response to homocysteine (20, 50 or 100 μ M) or saline (control) was investigated in platelets prepared from eight healthy volunteers. The mean platelet count was $342.1 \pm 57.7 \times 10^3/\mu\text{l}$ ($n=8$) and fell within normal ranges. Homocysteine was added to platelets at concentrations of 0, 20, 50 or 100 μ M. Agonist/ADP-induced platelet aggregation was assayed 15, 30, 45 and 60mins after the addition of homocysteine.

Before this was done, the dose of ADP to be used was determined by monitoring aggregation in response to various concentrations of the agonist. This was necessary as the platelets preparations showed marked differences in their reactivity to ADP. A typical dose response curve is shown in Fig 6.1. At sub-threshold concentrations, (in this case at 0.5, 1 and 1.5 μ M ADP), the agonist triggers expression of platelet receptors causing an initial shape change, then early aggregation. In this early aggregation, the platelets form clumps that allow an increase in light transmission, that appears as the peak seen at 0.5, 1 and 1.5 μ M ADP in Fig 6.1 and is usually reversible, as seen by the return of the aggregatory response to the baseline with time. This early reversible aggregation is known as primary aggregation (McNicol, 1996).

If the stimulus is strong enough (*eg* at 2 μ M in Fig 6.1), irreversible (secondary) aggregation occurs. In this example (Fig. 6.1), 2 μ M ADP supplies sufficient stimulus to release calcium ions that induce the endogenous production of thromboxane and the release of granule contents, that stimulate further and irreversible platelet aggregation.

In each of the platelet preparations studied in these experiments, the concentration of ADP required to bring the platelets into this irreversible phase of aggregation was pre-determined. In the majority of cases, this concentration was between 1.0 and 2.0 μ M ADP.

In these studies, there was a general trend towards increased agonist-induced aggregation in the presence of homocysteine. However, this pro-aggregatory response and the extent to which it occurred varied considerably, as can be seen in Fig 6.2a and 6.2b. Subjects A-D may be broadly classified as a group who demonstrated a pro-aggregatory response to homocysteine (Fig. 6.2a), while subjects E-H demonstrated a lower pro-aggregatory response to homocysteine (Fig 6.2b). The subjects were grouped according to this classification and the mean increases in aggregation were calculated. These are presented in Fig. 6.3. The data clearly show the trend towards increased aggregation in the presence of homocysteine (Fig 6.3, panel A), in a subset of the individuals studied.

Measurement of light transmission through stirred platelet preparations is a relatively crude method of assessing platelet function. For this reason, an increase of at least 40% of the control value was arbitrarily set as an indication of increased aggregation. For example, if agonist-induced aggregation in the control (non-homocysteine treated platelets) was 50%, an aggregation to at least 70% in the presence of homocysteine would be required in order to indicate enhanced aggregation. Of the eight platelet preparations studied, five demonstrated this increase on two instances at least (A, B, C, D and E, Fig 6.2a and b), although subject E tended to have a lower increase in aggregation at most time points in comparison to these four and so was grouped in the 'low-responder' group.

Examples of two different aggregatory profiles are shown in Fig. 6.4. In the first aggregation profile (A), no enhanced platelet aggregation was seen in the presence of homocysteine. This is the 45mins sample of subject H, presented in Fig 6.2b. In the second profile (B), there is a significantly increased platelet aggregation in the presence of 50 μ M homocysteine (3) and to a lesser extent in the presence of 100 μ M homocysteine (4). This is the 45mins sample of subject B, presented in Fig. 6.2a.

6.3.2. Effect of homocysteine on platelet thromboxane synthesis.

Thromboxane synthesis was assessed in a number of the samples on which platelet aggregation was monitored. A typical standard curve for the estimation of thromboxane B₂ is shown in Fig. 6.5. This was in close agreement with that predicted by the manufacturers. The assay has been found to be reproducible, demonstrating an intra-assay coefficient of variation of 3.6% (n=24) and an inter-assay coefficient of variation of 7.7% (n=8) (R & D Systems, Abingdon, UK). Because aggregation assays provide a relatively crude estimation of platelet aggregation, thromboxane synthesis (necessary for secondary aggregation) was assessed. There was a strong correlation between percentage aggregation and thromboxane synthesis (Fig. 6.6). A high percentage aggregation was associated with an increased thromboxane level. Thromboxane synthesis was higher in samples that demonstrated an increased aggregation in the presence of homocysteine compared to controls and did not vary significantly in the samples that did not demonstrate an increased aggregation in the presence of homocysteine. This verifies the aggregation measurements reported in section 6.3.1 and corroborates the finding of an increased aggregation in the presence of homocysteine.

6.3.3. Effect of homocysteine on platelet fibrinogen binding.

Fibrinogen binding, a sensitive index of platelet activation, was assessed by flow cytometry (FACScan, Becton Dickinson).

A light scatter gate was set on the light scatter region of platelets and platelets were thus identified by flow cytometry (Fig 6.7, graph 2 of panels A and B).

The binding of fibrinogen was then determined by analysing 10,000 of these particles for Oregon green[®] fluorescence. Typical results for unactivated (A) and activated (B) platelets are shown in graphs 1 of Fig. 6.7. An increase in fibrinogen binding is manifested as an increase in fluorescence, which appears as a shift in the fluorescent

signal (graphs 1 of Fig. 6.7). This shift is emphasised by overlaying the graphs generated by unactivated and activated platelets, as in Fig. 6.8.

The geometric mean fluorescence of the activated and unactivated platelets (both the control and homocysteine-treated) are presented in Table 6.1. This shows that fibrinogen binding increased following activation of the platelets (*ie* on addition of ADP). However, and this is also demonstrated in Fig 6.9, there was no significant increase in fibrinogen binding by activated platelets in the presence of 20, 50 or 100 μ M homocysteine ($p > 0.05$ in each case, $n=3$).

Fig. 6.1 ADP-induced aggregation, a dose response curve.

Platelets were prepared as described in section 6.2.1.1 and platelet aggregation was measured as described in section 6.2.1.2.

1: 0.3 μ M ADP

2: 1.0 μ M ADP

3: 1.5 μ M ADP

4: 2.0 μ M ADP

An increase in aggregation is seen in response to increasing concentrations of agonist (ADP).

* The four spikes seen over the 10 min period signal the addition of the agonist.

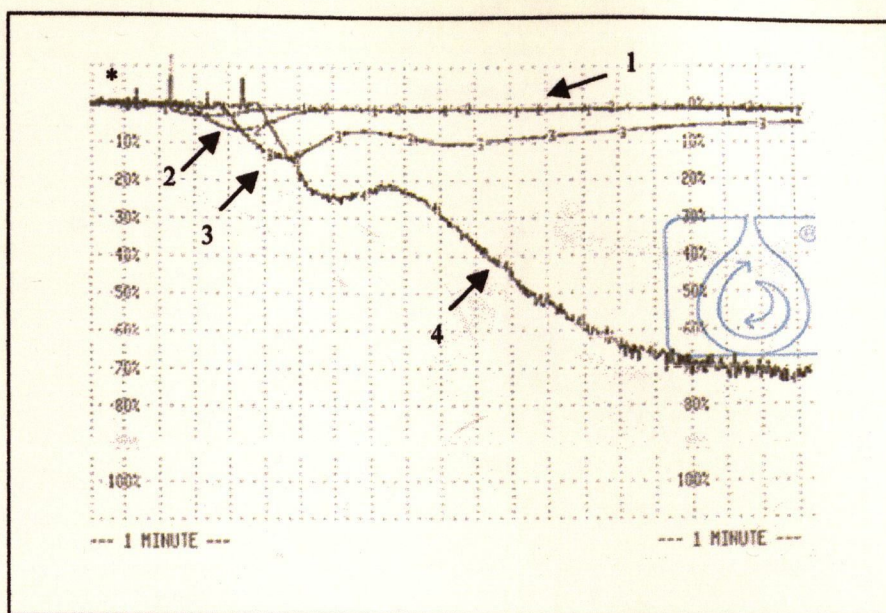


Fig. 6.1 ADP-induced aggregation, a dose response curve.

Platelets were prepared as described in section 6.2.1.1 and platelet aggregation was monitored as described in section 6.2.1.2.

1: 0.5 μ M ADP

2: 1.0 μ M ADP

3: 1.5 μ M ADP

4: 2.0 μ M ADP

An increase in aggregation is seen in response to increasing concentration of agonist (ADP).

* The four spikes seen near this symbol signal the addition of the agonist.

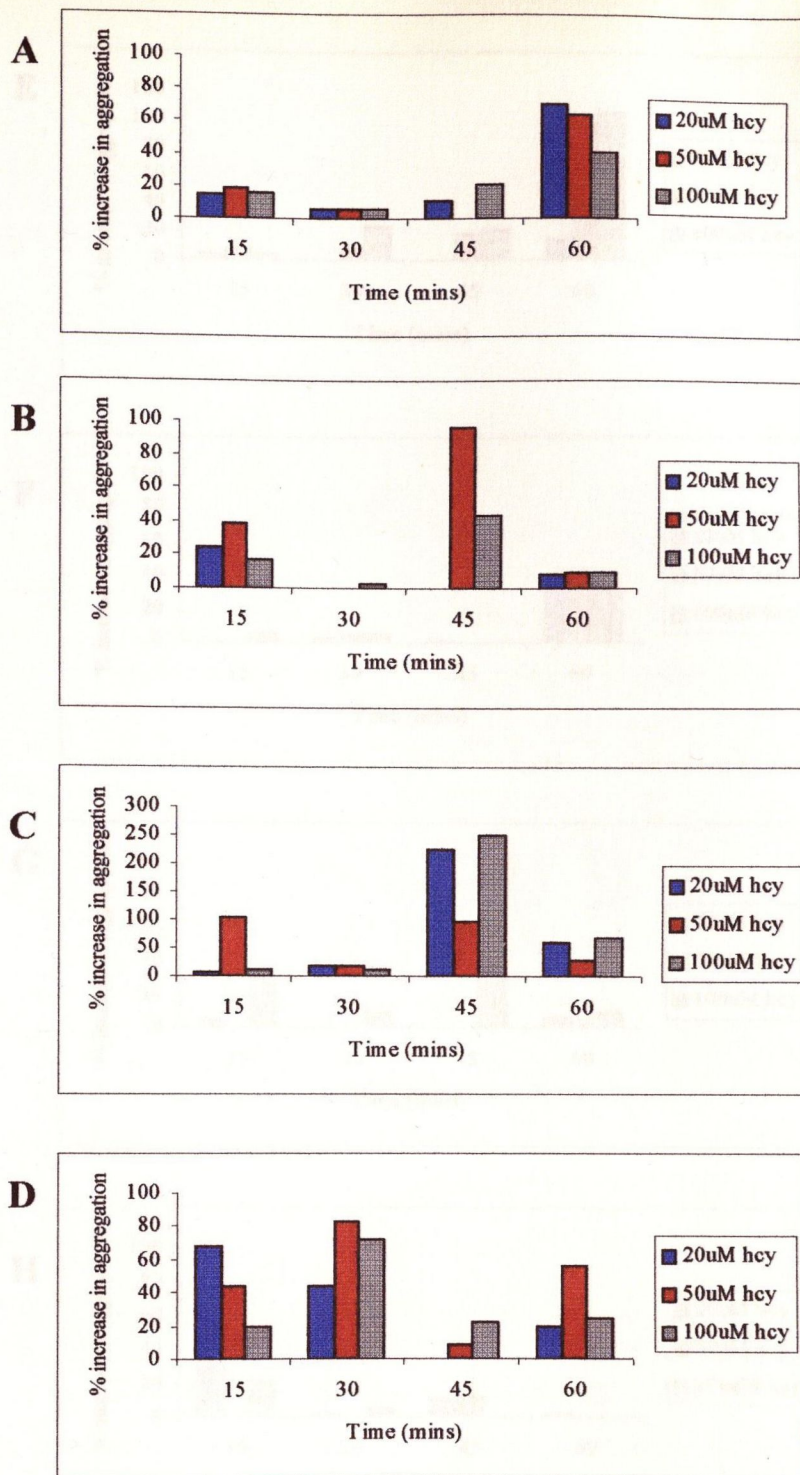


Fig. 6.2a Increase in aggregation (% increase over the control) in the presence of homocysteine.

Platelets were prepared as described in section 6.2.1.1, homocysteine was added at 0, 20, 50 or 100μM concentrations and agonist (ADP)-induced aggregation was assessed 15, 30, 45 and 60mins after the addition of homocysteine to the platelets. Aggregations are expressed above as a *percentage increase* over the aggregation seen in the controls (0μM homocysteine).

A, B, C, and D represent the aggregations for platelet preparations of *four separate individuals*.

Subjects A-D may be broadly classified as a group who demonstrated a marked pro-aggregatory response to homocysteine. Note that in subject C, the scale of the y-axis is 0-300%, not 0-100% as in the other subjects.

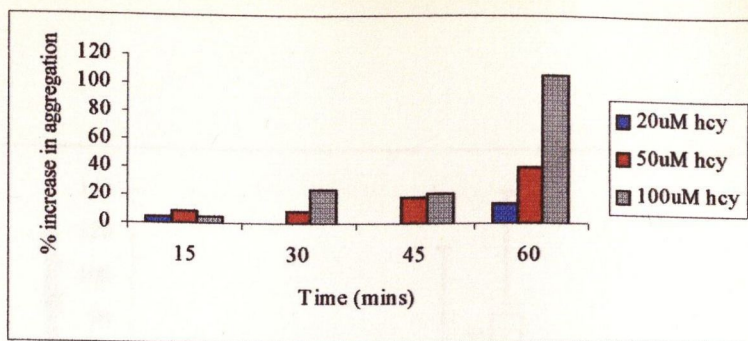
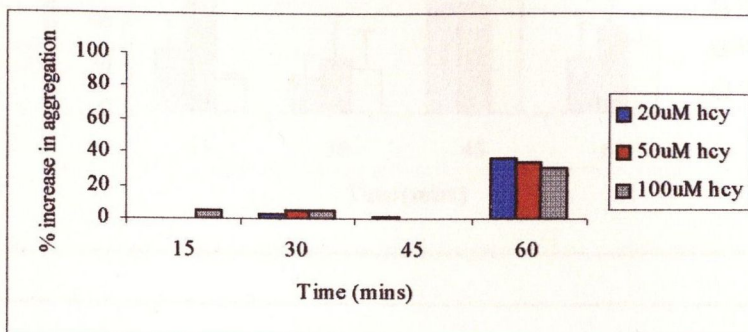
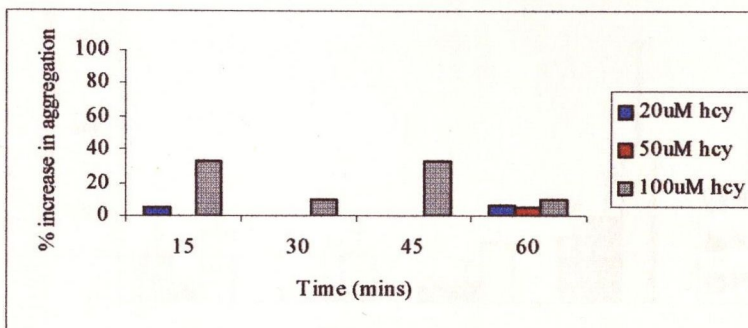
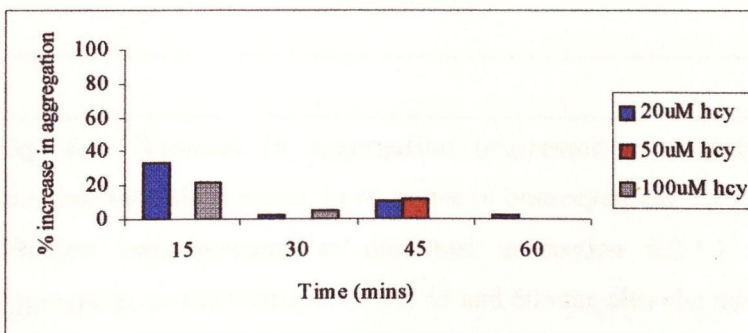
E**F****G****H**

Fig. 6.2b Increase in aggregation (% increase over the control) in the presence of homocysteine.

Platelets were prepared as described in section 6.2.1.1, homocysteine was added at 0, 20, 50 or 100 μ M concentrations and agonist (ADP)-induced aggregation was assessed 15, 30, 45 and 60mins after the addition of homocysteine to the platelets. Aggregations are expressed above as a *percentage increase* over the aggregation seen in the controls (0 μ M homocysteine).

E, F, G, and H represent the aggregations for platelet preparations of *another four separate individuals*. Subjects E-H may be broadly classified as a group who demonstrated a low pro-aggregatory response to homocysteine.

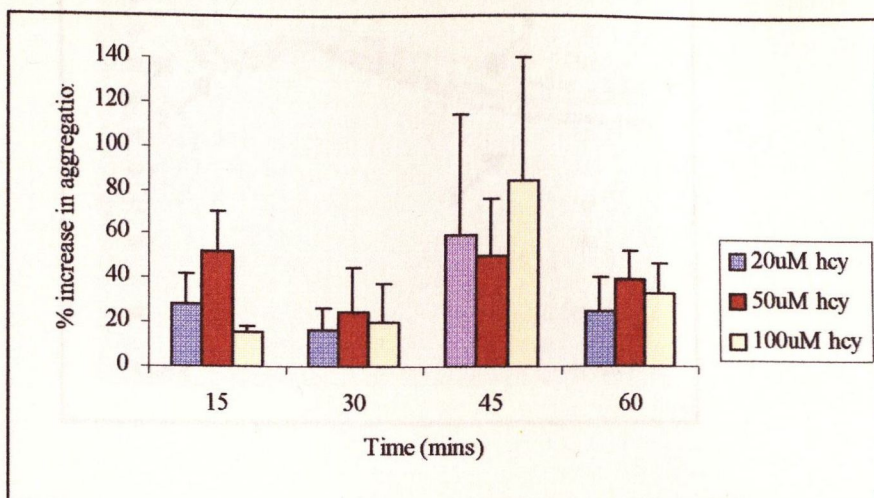
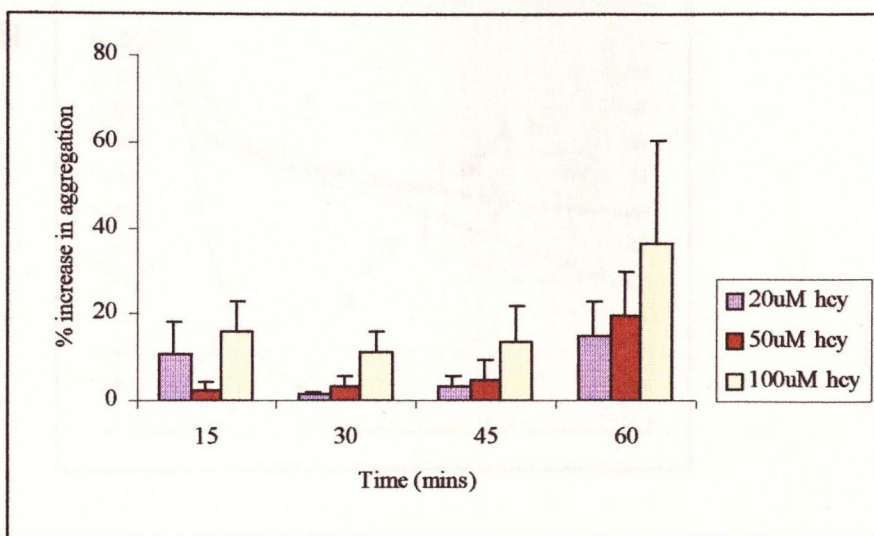
A**B**

Fig. 6.3 Increase in aggregation (expressed as a percentage increase over the control) in response to homocysteine.

Platelets were prepared as described in section 6.2.1.1. platelet aggregation was assessed at 15, 30, 45 and 60mins after the addition of homocysteine at the concentrations indicated above. The data represent the mean $\pm$ SEM (n=4).

A: This represents the mean increase in aggregation of the four subjects (A-D) who were classified as showing a pro-aggregatory response to homocysteine.

B: This represents the mean increase in aggregation of the four subjects (E-H) who were classified as showing little pro-aggregatory response to homocysteine.

Note that the scale of the y-axis is different in A and B.

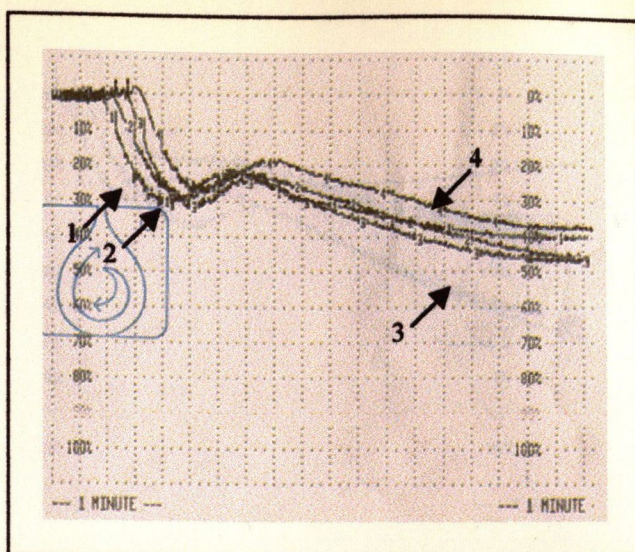
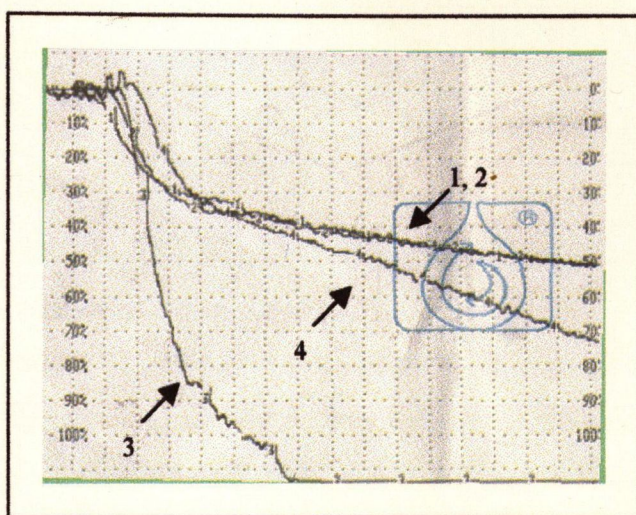
A**B**

Fig. 6.4 Agonist-induced platelet aggregation for two separate platelet preparations, 45mins after the addition of homocysteine.

A: The presence of homocysteine does not alter the rate or extent of platelet aggregation (subject H in Fig 6.2b).

B: The presence of 50 and 100 μ M homocysteine caused significant increases in ADP-induced aggregation (subject B, in Fig 6.2a).

% Aggregation at 4mins

	A	B
1: Control (no homocysteine)	40%	50%
2: 20 μ M homocysteine	45%	50%
3: 50 μ M homocysteine	47%	100%
4: 100 μ M homocysteine	38%	71%

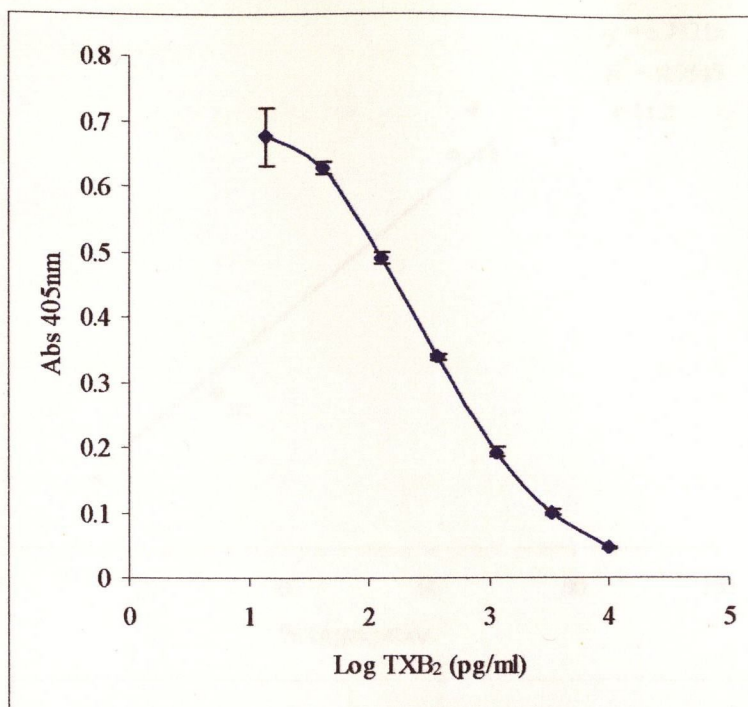


Fig. 6.5 Typical standard curve for the estimation of thromboxane B₂ concentration, by immunoassay.

The assay was carried out as described in section 6.2.2. All samples and standards were assayed in duplicate. The error bars shown represent the range of values found in duplicate analyses. Internal controls, such as non-specific binding, total activity and blank all fell within the expected ranges.

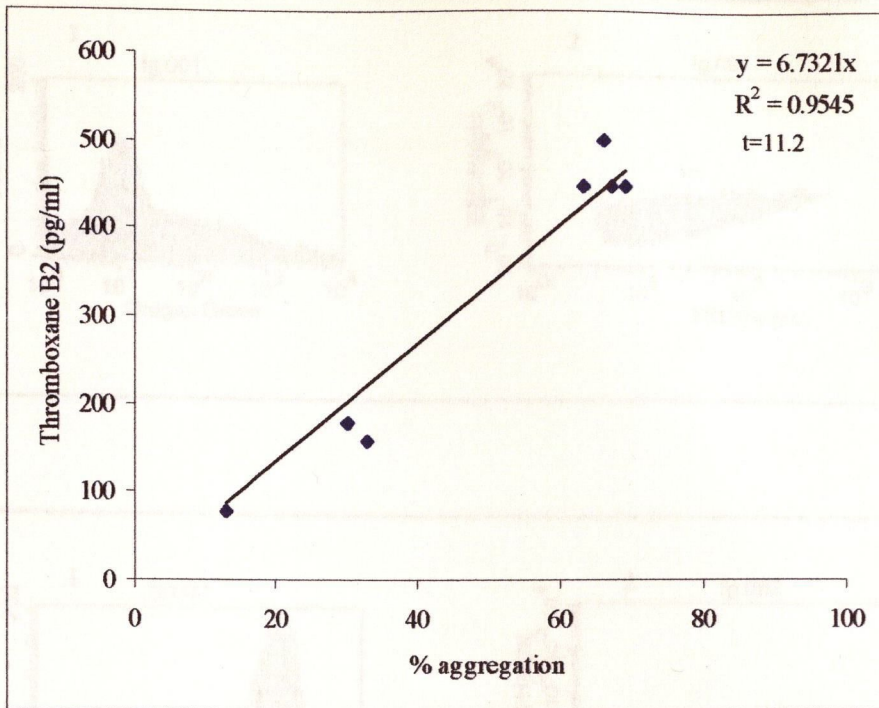


Fig. 6.6 Correlation between thromboxane synthesis and platelet aggregation.

The above graph represents the aggregations and thromboxane levels of a single individual. There was a significant positive correlation between levels of thromboxane B₂ (section 6.2.2) and agonist-induced aggregation (section 6.2.1) ($p < 0.05$). This verified the findings of the aggregation assays.

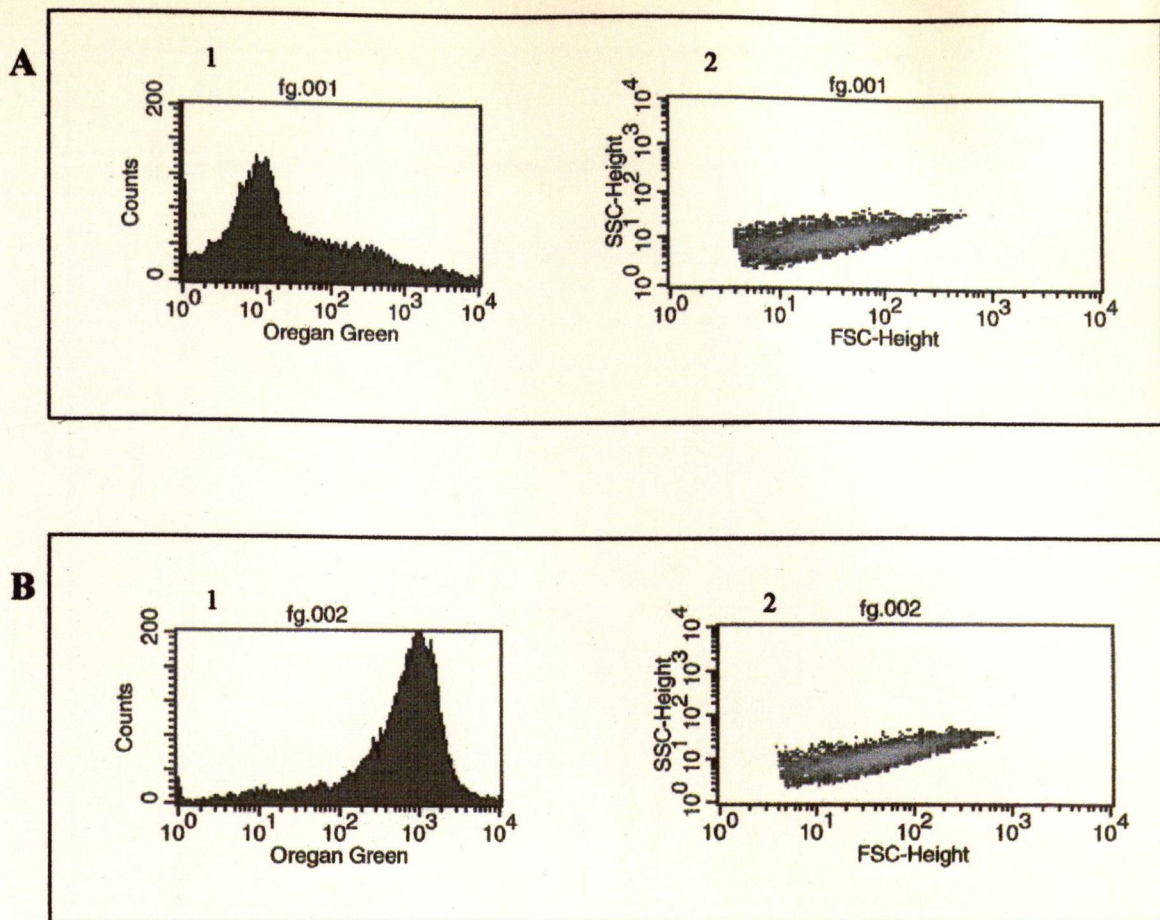


Fig. 6.7 FACS analysis of platelet activation.

A: Unactivated platelets.

B: Activated platelets.

On the left (1) in panels A and B, the graph (counts vs Oregon green®) is an indication of the concentration of the fluorescent label (Oregon green® fibrinogen). On the right (2) of panels A and B, is a graph of forward light scatter vs side light scatter. By selecting a light scatter gate on the platelet light scatter region, platelet identification was accomplished.

The shift of the peak from left to right in graphs 1 of panel A and B, indicate that the addition of agonist has activated the platelets and induced fibrinogen binding. The geometric mean fluorescence of unactivated platelets in this case was 56.4, while that of the activated platelets was 1050.9, thereby indicating the fibrinogen binding of the activated platelets.

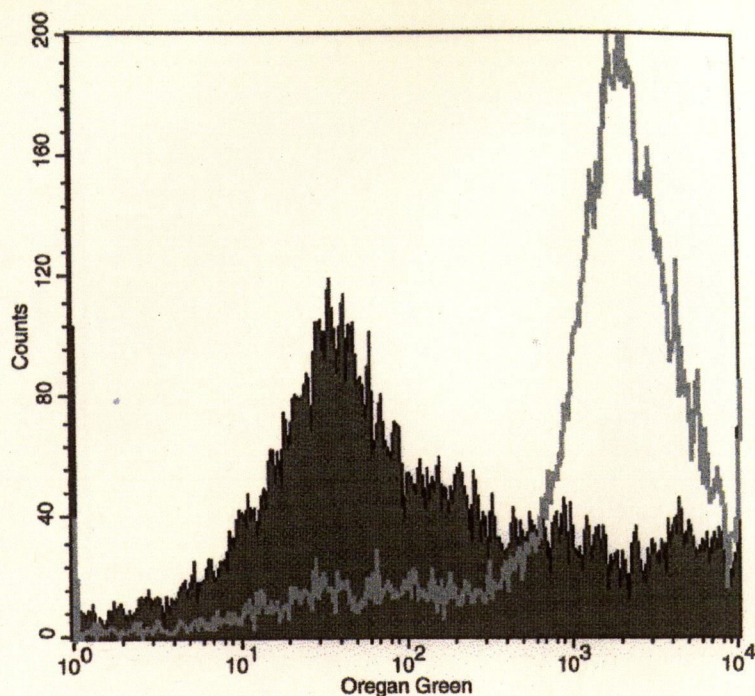


Fig 6.8 FACS analysis of fibrinogen binding by unactivated and activated platelets.

This emphasises the shift in the fluorescent signal seen in graphs 1 of panel A and B in Fig 6.7. The darker area represents the signal generated by unactivated platelets (no fibrinogen binding), while the lighter coloured area represents the activated platelets that have bound fibrinogen.

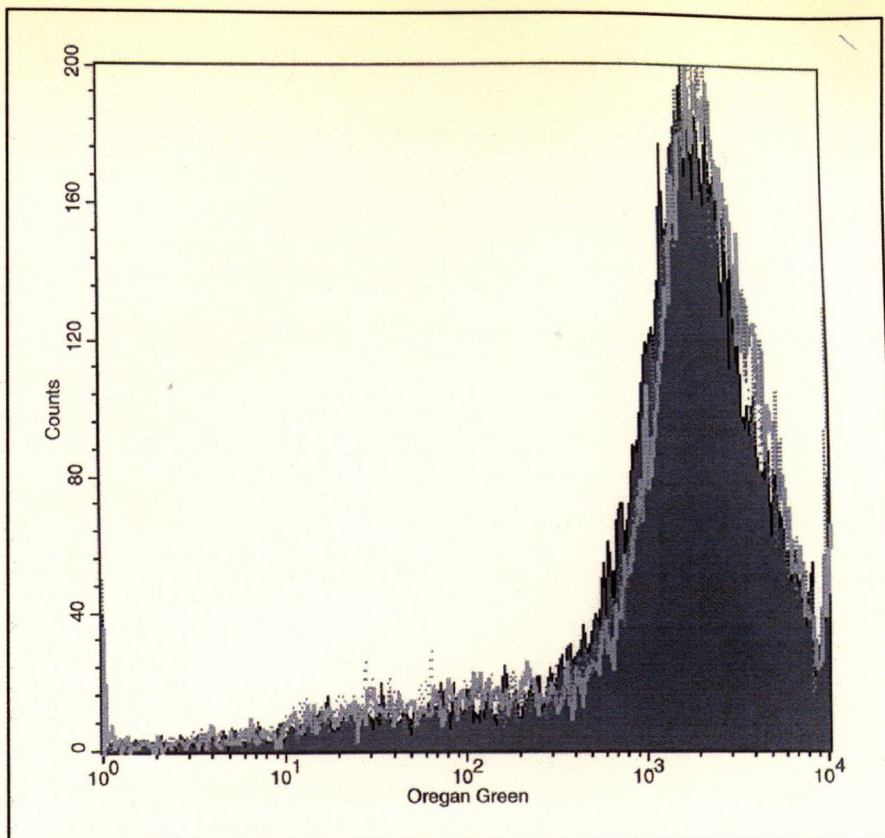


Fig. 6.9. FACS analysis of fibrinogen binding by activated platelets in the presence of 0, 20, 50 or 100 μ M homocysteine.

The dark area represents the fluorescence of the control (0 μ M hcy), while the black line represents that of the 20 μ M sample, the grey line that of the 50 μ M sample and the navy line (quite difficult to see) represents that platelets exposed 100 μ M homocysteine. This shows that there was no significant increase in fluorescence and therefore fibrinogen binding by the activated platelets exposed to 20, 50 or 100 μ M homocysteine.

6.4. DISCUSSION

Epidemiological evidence points to a role for homocysteine in atherosclerosis and arterial and venous thrombosis (Stamler et al, 1992). However, the precise mechanisms responsible for these associations are still under investigation.

	Control (no hcy)	20µM hcy	50µM hcy	100µM hcy
Unactivated	73.7 ± 64.3	72.5 ± 60.4	75.7 ± 62.2	75.5 ± 63.9
Activated	879.0 ± 391.1	855.0 ± 397.4	884.7 ± 381.6	872.2 ± 383.9

Evidence from animal studies is limited at best, indicating that homocysteine may also adversely affect platelet function. In models of hyperhomocysteinemia by methionine

Table 6.1 Platelet fibrinogen binding in the presence of homocysteine.

Fibrinogen binding was assessed by FACS as described in section 6.2.3. Results shown represent the mean ± SD of the fluorescence of unactivated and activated platelets (n=3). Fibrinogen binding (fluorescence) increased upon activation of the platelets (*ie* the addition of agonist (ADP)). However, no additional increase in fibrinogen binding was seen in the presence of homocysteine.

6.4. DISCUSSION.

Epidemiological evidence points to a role for homocysteine in atherosclerosis and arterial and venous thrombosis (Stehouwer *et al*, 1998). However the precise mechanisms responsible for these associations are still under investigation. Homocysteine, as discussed in detail in chapter 1, is thought to adversely effect the normally anti-thrombotic nature of the endothelium. Its effects include an inhibition of von Willebrand factor secretion, induction of tissue factor, a decrease in the bioavailability of nitric oxide and an inhibition of endothelial cell regeneration (Fryer *et al*, 1993; Lentz & Sadler, 1993; Bellamy *et al*, 1998 and Lee & Wang, 1999).

Evidence from several studies by Durand *et al*, indicate that homocysteine may also adversely affect platelet function. Induction of hyperhomocysteinemia by methionine loading or folate-deficient diet in rats was associated with an enhancement of platelet aggregation, an increase in thromboxane synthesis and an increase in macrophage tissue factor activity (Durand *et al*, 1996a and 1997a). In addition, an abnormally high thromboxane biosynthesis in homocystinuric patients, suggestive of platelet activation, has been reported (Di Minno *et al*, 1993).

In an effort to clarify the role of modestly elevated levels of homocysteine on human platelet function, this study was carried out.

Platelets were prepared by the standard procedure described in section 6.2.1.1. Many drugs are known to interfere with platelet function, *eg* by inhibiting platelet aggregation. For this reason, blood for PRP preparation, was taken only from donors who had not ingested these drugs *eg* aspirin, in the previous 14 days. Following the preparation of platelet rich plasma, the number of platelets and the presence of any excess of contaminating cells (*eg* RBC or leukocytes) was assessed using a cell counter. In all cases the number of WBC did not exceed 0.1×10^3 cells/ μ l while RBC were never

in excess of 0.01×10^6 cells/ μ l. The average number of platelets was $342.1 \pm 57.1 \times 10^3$ cells/ μ l and was within the expected range (McNicol, 1996).

The effect of homocysteine on agonist-induced aggregation was examined. Platelet aggregation may be measured by several methods including the measurement of light transmission and fibrinogen binding.

Several agonists can be used to induce platelet aggregation. These include ADP, adrenaline and collagen. These all act by calcium mobilisation, an essential step in platelet aggregation, but appear to act *via* slightly different pathways that are not yet completely understood. ADP in low doses produces a 'primary wave' of reversible aggregation but in higher doses appears to mobilise enough calcium to cause irreversible aggregation that appears as a 'secondary wave'. Thus the response to ADP is characteristically bi-phasic (Kay, 1988). This is apparent in Fig 6.1, where 2μ M ADP, the agonist used in these studies caused sufficient stimulation for irreversible aggregation.

In the absence of agonist, homocysteine did not induce aggregation. To ensure that homocysteine was not cytotoxic to the platelets, PRP samples that had been incubated with homocysteine (20, 50 and 100μ M) for up to one hour were counted by the Sysmex K-1000 haematology analyser. There was no difference in cell numbers between the homocysteine-treated and non-treated PRP samples (data not shown). ADP-induced platelet aggregation in the presence of homocysteine was assessed in eight individual platelet preparations. These individual platelet preparations were from eight separate donors who were healthy laboratory staff, with an average age of 24yrs. There was considerable variation in the aggregatory response to homocysteine, the magnitude of which is apparent in Figs. 6.2a and 6.2b. There was however overall, a general trend towards increased aggregation in the presence of homocysteine (Fig. 6.3, panel A). Within this wide spread of data, a general pattern emerged. The pro-aggregatory effect

of homocysteine could be broken into two groups, *ie* those who demonstrated a relatively pronounced increase in aggregation in the presence of homocysteine and those who did not (Figs. 6.2a and 6.2b).

The reasons behind these wildly varying inter-individual responses to homocysteine are unknown. The plasma homocysteine levels of the donors were not measured in these experiments but because the volunteers were of the same background as the control subjects who participated in the study described in Chapter 5, it is unlikely that their plasma homocysteine was abnormally elevated. Regardless, if their plasma homocysteine was elevated, it was unlikely to be no more than a few micromolar higher than normal and with the addition of homocysteine at 20, 50 and 100 μ M to the PRP, any physiological elevation would be masked.

As mentioned earlier, enhanced aggregation did not occur at any one particular homocysteine dose or exposure time, suggesting that the effect is very much dependent on the individual platelet preparation and may be influenced by the environment of the platelets, *ie* the plasma. Several investigators have suggested that the pro-thrombotic activity of homocysteine may be mediated by increasing levels of oxLDL, that would in turn lead to an increase in platelet arachidonate metabolism (Di Minno *et al*, 1983 and Durand *et al*, 1997b). The variations in aggregation in the presence of homocysteine in this study may be caused by the presence of other factors in the plasma that amplify the pro-aggregatory activity of homocysteine *eg* lipid peroxides or transition metal ions. This is supported by the observation in preliminary experiments (data not shown) that homocysteine failed to have any effect on platelet aggregation when washed platelets were used. Washed platelets are suspended in a citrate buffer and therefore are free of any of biological components present in the normal platelet environment. Furthermore, the largest increases in aggregation were generally seen 45-60mins after the addition of

homocysteine, suggesting its interaction with some other factor/process *eg* lipid peroxidation.

The calcium released by agonists causes aggregation by several actions, one of which is the production of thromboxane A₂, via the prostaglandin pathway (Kay, 1988). TXA₂ is itself an aggregating agent. Through positive feedback, it amplifies the aggregatory response leading to irreversible aggregation. Thus thromboxane levels may be measured as a reflection of aggregation. TXA₂, however has a half-life of only 37 seconds under physiological conditions and so its synthesis is usually monitored by measurement of thromboxane B₂ (TXB₂), its stable derivative.

In an effort to validate the results of the aggregation assays, thromboxane synthesis was measured in several of the samples used in the aggregation assays. TXB₂ was found to positively correlate with the extent of aggregation (Fig. 6.6), confirming the findings of the aggregation assays. The finding of an increased thromboxane synthesis in the samples that also demonstrated an increased aggregation in the presence of homocysteine are in agreement with the finding of Graeber *et al*, who found an increased thromboxane synthesis in PRP pre-incubated with 1mM homocysteine (Graeber *et al*, 1982).

It has been suggested that if homocysteine does indeed have a pro-aggregatory effect it does so *via* oxidative stress. Hydrogen peroxide is one by-product of homocysteine oxidation and has been found to increase agonist-stimulated platelet aggregation (Heinecke *et al*, 1993 and Naseem & Bruckdorfer, 1999). In addition, free radicals (which may be produced during homocysteine oxidation) are known to interact with the arachidonic acid cascade causing activation of that pathway, while lipid peroxides (which may also be produced during homocysteine oxidation) inhibit prostacyclin synthetase and therefore selectively augment production of thromboxane A₂, a potent platelet aggregant. It is possible therefore that homocysteine, through enhanced

formation of free radicals and lipid peroxides, might enhance platelet activity (Jennings *et al*, 1991). This proposal is supported by the work of Durand *et al*, who have found that experimentally-induced hyperhomocysteinemia in rats is associated with platelet hyperactivity and an increase in oxidative stress characterised by an increase in the concentrations of lipid peroxidation products (Durand *et al*, 1996a and b). Furthermore, cellular glutathione (GSH), which protects against platelet aggregation, may be reduced in the presence of oxidative stress, such as that seen in the presence of an elevated plasma homocysteine (Cho *et al*, 1997).

That homocysteine appeared to have a pro-aggregatory role in PRP but not in washed platelets suggested a physiological role for homocysteine in platelet aggregation, possibly through some indirect mechanism involving oxidative stress as discussed above.

Glycoprotein (GP) IIb/IIIa is a platelet-specific integrin that mediates the interaction between platelets and fibrinogen in aggregation and adhesion. Following platelet activation (*eg* upon the binding of agonist to platelet membrane receptors), GP IIb/IIIa undergoes a conformational change resulting in its conversion from a low to a high affinity receptor for fibrinogen. Therefore, the binding of fibrinogen may be used to assess platelet aggregation. In this study, FACS analysis was used to determine if homocysteine had any effect on platelet fibrinogen binding.

FACS can distinguish between lymphocytes, monocytes, neutrophils and platelets, as these cells differ from one another in size, shape, cytoplasmic granularity and therefore light scatter. For instance, forward light scatter (an indication of particle size) is lower for lymphocytes than for monocytes. Side light scatter (an indication of granularity) is again lower for lymphocytes than for monocytes. With this knowledge it is possible to focus on a subset of cells within a heterogeneous population using a process called 'gating'. By setting up a 'gate', the cells of interest may be regionalised and thus studied.

This is illustrated in Fig. 6.7, graphs 2, panel A and B. In this case, where PRP is the sample and not for example whole blood, there is little interference from other cell types. In PRP, platelets are the predominant cell type, with leukocytes present at about 0.05% and red blood cells present at about 3%.

In the absence of agonist the platelets bind fibrinogen poorly or not at all. However, on addition of ADP (agonist), the platelets become activated, and bind fibrinogen. This is seen as a shift in the peak from left to right in Fig. 6.7, graphs 1, panel A and B, and a concurrent increase in fluorescence. This shift is particularly apparent when these graphs (*ie*, activated and unactivated platelets) are merged (Fig. 6.8). This peak shift may also be expressed as a measurement of Oregon green[®] fluorescence.

The mean fluorescence of the activated and unactivated samples is shown in Table 6.1. Addition of the agonist was seen to cause an increase in this value (73.7 ± 64.3 vs 879.0 ± 371.1 , (mean $\pm$ SD), $n=3$), therefore implying an increase, as expected, in fibrinogen binding. There was however, no additional increase in fibrinogen binding in the presence of homocysteine at 20, 50 or $100\mu\text{M}$ ($p > 0.05$, $n=3$) (Table 6.1 and Fig. 6.9). Following on from the aggregation and thromboxane synthesis data, this was surprising.

There are several possible explanations for this apparent discrepancy. Due to factors dictated to by the requirements of the FACS analysis system, it was not possible to monitor fibrinogen binding using the exact conditions used in the aggregation and thromboxane experiments.

It is possible that the dilution of the PRP (1:10) with TBS resulted in a dilution of the factor(s) alluded to above that in conjunction with homocysteine contribute to oxidative stress and therefore promote platelet dysfunction.

It should also be noted that a relatively high dose of ADP is necessary for this assay ($10\mu\text{M}$), while between 1 and $2\mu\text{M}$ was used in the aggregation and thromboxane

synthesis studies. It is possible, that the platelets have reached 100% aggregation and therefore the presence of homocysteine could not in any way enhance this. Also the agonist was added at the same time as homocysteine. In studying the effect of hydrogen peroxide on platelet aggregation, it has been found that several factors effect the outcome of the experiments. These include the type of platelets used in the experiment eg PRP or washed platelets, the order in which the peroxide was presented to the platelets relative to the agonist, the concentration of the agonist and indeed the actual agonist used (Naseem & Bruckdorfer, 1999). It is possible that the same phenomena are at work here. Another factor to be considered is the heterogeneity of the aggregatory response to homocysteine. Because FACS is a sensitive analytical method, fibrinogen binding in the presence of homocysteine was assessed in just three separate experiments, while the aggregation assays were carried out eight times.

Aggregation (by light transmission) was monitored up to 4mins after the addition of agonist to the platelets. In FACS analysis, fibrinogen binding, (a marker for aggregation) was assessed 30mins after the addition of the agonist. It is possible that the homocysteine causes an *early* enhancement of platelet aggregation and that by monitoring fibrinogen binding at a late stage in aggregation the pro-aggregatory effect of homocysteine is underestimated.

For these reasons, the fibrinogen binding experiment does not allow conclusions to be drawn on the potential role of homocysteine in platelet aggregation.

In total, these data comprising: i) a trend towards increased aggregation and ii) a trend towards increased thromboxane synthesis in the presence of homocysteine, at concentrations seen in hyperhomocysteinemic subjects, suggest that a possible platelet pro-aggregatory role for this metabolite might be further considered.

7.1 General discussion.

Recent epidemiological studies have shown that moderately elevated plasma/serum levels of homocysteine are highly prevalent in the general population and are associated with an increased risk of fatal and non-fatal cardiovascular disease, independent of other classical risk factors. This association is usually consistent, strong, dose-related and biologically plausible (Ekelboom, 1999).

The basis of the first part of this thesis (Chapters 3 and 4) was to devise a strategy that would allow an investigation into the possible cellular cause for the common finding of an elevated plasma homocysteine in cardiovascular disease patients.

Chapter 7

Discussion.

Chapter 3 describes a system that was designed to allow the formation and fate of homocysteine in the cell to be followed. In order to maximise uptake of radiolabelled methionine (the tracer used in this study), and to increase the relative flux through the methionine cycle PHA-stimulated lymphocytes were used. PHA, by increasing the metabolic activity in the cells, led to an increase in protein synthesis by the cells and induced increased activity of both the remethylation and transsulfuration pathways (Fig. 3.5).

In order to quantitate the amount of radiolabel in methionine and each of its metabolic derivatives, a process that would allow their separation was required. A two-dimensional thin layer chromatographic system was successfully designed that was highly reproducible and allowed the subsequent quantitation of the amount of radioactivity in each metabolite by scintillation counting (section 3.3.3). Using this strategy (described in Chapter 7) methionine metabolic profiles were obtained so that the activity of the pathways involved in cellular homocysteine disposal could be assessed.

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Chapter 4 describes the metabolic profiles of nine control subjects and seven vascular disease subjects. The control subjects demonstrated activity of both the remethylation and transsulphuration pathways and could be divided into two groups, those that had a relatively high flux through the transsulphuration pathway and those that showed a lower flux. Nonetheless, none of the nine control subjects showed an accumulation of the radiolabel in homocysteine.

The vascular disease subjects presented profiles that deviated in several ways from the controls. As such they could not be easily grouped and are presented individually (Figs. 4.8 – 4.14). In part, nutritional and genetic factors could be used to explain the labelling patterns seen. Four of the vascular disease group were mildly hyperhomocysteinemic. Three of these individuals (CM4, HK5, EB7) demonstrated a trend towards increased labelling of homocysteine compared to the controls, while in the fourth subject (TF6), almost all of the label accumulated in homocysteine. Of the remaining vascular disease subjects, only one presented with an abnormal profile (MF1) as will be discussed later but nonetheless did not demonstrate any accumulation of the label in homocysteine.

It was found that the presence of a particular polymorphism and/or nutritional deficiency was not enough to precipitate an accumulation of the label in homocysteine. The presence of the TT genotype did not necessarily have adverse effects on the remethylation pathway. The results suggest that this may be related to folate and/or B₁₂ status. Of the three vascular disease subjects who had the TT polymorphism, only one, MF1, demonstrated a decreased activity in the remethylation pathway. As the primary difference between this subject and the other two TT homozygotes was a deficiency in B₁₂, it is possible that MTHFR TT genotype alone was not responsible for the reduced activity in the remethylation pathway, but that it was compounded by the cofactor deficiency. Nonetheless, it appears that in this individual at least (MF1), this reduced activity was not sufficient to induce the accumulation of homocysteine, as a

compensatory increase in the activity of the transsulphuration pathway adequately disposed of the homocysteine. This phenomenon has been previously reported in the literature (Mudd, 1995). Two other hyperhomocysteinemic vascular disease subjects showed that despite the presence of the TT polymorphism (JC2 and HK5), the remethylation pathway could work at levels comparable to the controls (Figs. 4.9 and 4.12). Their profiles suggested that defective transsulphuration was the probable cause of their elevated homocysteine.

A reduction in the recycling of homocysteine through the remethylation pathway could be inferred from the profile of the subject CM4, who was deficient in folate.

Of the four hyperhomocysteinemic subjects, the profiles suggested that in one case (HK5), defective transsulphuration and in another two (CM4 and TF6) defective remethylation were the contributing factors. In the fourth (EB7) subject, the profile was comparable to that of the controls and an explanation for the increased plasma homocysteine could not be determined.

The profiles of methionine metabolism in PHA-stimulated cells was therefore successful to the extent that it brought anomalies to light. It is in attempting to rationalise these altered metabolic profiles, with respect to the presence of certain polymorphisms or nutritional deficiencies that the drawbacks of the system become apparent.

Although some accumulation of intracellular homocysteine was apparent in three of the four subjects, they were small accumulations (the extent of the accumulation of the label in homocysteine (>80%) in subject TF6, is considered to be more of an exception than the normal). It is possible that homocysteine is exported from the cell or becomes protein bound. However, the fact that so much label did accumulate in subject TF6's cells is indicative of the fact that accumulated homocysteine can remain in the cell, without the need to be exported. It may also need to be pointed out that subject TF6

may also be described as an 'outlier' clinically, as he was the youngest vascular disease subject studied and had suffered a major stroke at the age of 23yrs.

Another alternative is that the lack of accumulation of the label in the cells of CM4, HK5 and EB7 is a fault of the system. The activated state of the cells, where methionine is driven into protein synthesis, might mean that the methionine metabolic pathway is not stressed and as such does not have the opportunity to accumulate homocysteine.

It must also be stressed that this is only a small study, where just four hyperhomocysteinemic subjects were assessed. In addition, these subjects could all be classified as *mildly* hyperhomocysteinemic and any cellular accumulation of homocysteine might be beyond the sensitivities of this assay.

As a means of investigating the role of certain polymorphisms in homocysteine metabolism, in particular the TT genotype, the system may not be suitable, as the presence of folate in the media may mask the *in vivo* effects of such a polymorphism. However, the D919G and I278T polymorphisms are thought not to be as sensitive to cofactor concentrations, so their influence on methionine metabolism would be expected to be noticed.

Although the system used provided information with regard to the activity of the methionine metabolic pathways, it was quite a labour intensive and time consuming assay. Following the completion of this study, preliminary experiments on the labelling of methionine and its metabolic derivatives, in whole blood, indicated that labelling occurs rapidly. By using the same strategy in whole blood samples a number of problems could be circumvented. The system would be significantly faster and less labour-intensive. In addition, there would be no exogenous supply of vitamins and so the profiles generated might be a better reflection on the *in vivo* situation and may be more suited to studying the effects of the TT genotype on homocysteine remethylation.

While the epidemiological evidence supporting a role for homocysteine in atherosclerosis and thrombosis is convincing, the molecular mechanisms by which homocysteine initiates and/or aggravates these processes remain unclear. In order to investigate if homocysteine might play a role in atherosclerosis by affecting macrophage function, the effect of homocysteine on the production of the pro-atherogenic cytokine, tumour necrosis factor alpha was investigated (Chapter 5). The monocytic cell-lines U-937 and THP-1 were used as models of monocytes, while their mitogen-stimulated counterparts were used as models of macrophages.

It was found that homocysteine had differential effects on mitogen-induced TNF α production, depending on the maturation state of the cell. These cells produce negligible amounts of TNF α unless stimulated. Homocysteine itself was not capable of inducing synthesis but when the monocytes were exposed to homocysteine for 24hrs before being stimulated by PMA, TNF α production was enhanced (Fig. 5.4). However, when homocysteine was added with or after the addition of the mitogen, TNF α production was significantly reduced (Fig 5.5 - 5.7). This reduction did not appear to be controlled at the transcriptional level as no differences in TNF α mRNA expression, between homocysteine treated and non-treated U-937 macrophages, could be seen when assessed by Northern blotting (Fig. 5.10).

Homocysteine-mediated differential effects on TNF α production raise several questions with regard to the *in vivo* situation. It is important to stress that these observations were made at concentrations of homocysteine that are found in hyperhomocysteinemic individuals and may represent a previously undescribed pro-atherogenic mechanism of homocysteine.

The enhancement of mitogen-stimulated TNF α production by a homocysteine pre-exposure was not investigated at the mRNA level. It is possible that this increased production was as a result of an upregulation of TNF α transcription. If this was the

case, the activity of the transcription factor AP-1 could be of particular relevance. The finding that a reduction in TNF α production was not associated with a reduction of the TNF α transcript, may reflect an effect at the translational or processing stages and warrants further investigation.

Oxidant stress is thought to activate a number of transcription factors and therefore indirectly regulate gene expression (Jovinge *et al*, 1996). It is possible the increase in TNF α production, following the U-937 monocytes' pre-exposure to homocysteine was as a result of enhanced activity of the transcription factor AP-1, in response to oxidant stress in the form of an elevated homocysteine. Because TNF α levels may also be regulated by other factors in plasma *eg* oxLDL, and also because homocysteine is thought to encourage lipid peroxidation, it may be of interest to study the effects of homocysteine on monocyte/macrophage TNF α production in conjunction with other factors, such as LDL. It might also be interesting to see if circulating TNF α is higher in hyperhomocysteinemic subject, than in those with normal homocysteine levels.

The results of the investigation into the effects of homocysteine on platelet aggregation are presented in Chapter 6. Of eight separate experiments, homocysteine was associated with an increase in aggregation in four. This finding was supported by the finding of an increased thromboxane synthesis by the samples that demonstrated the pro-aggregatory effect of homocysteine. The inconsistencies in the aggregation responses to homocysteine may be a reflection of inter-individual variation. Such variation may be due to other plasma factors that interact with or independently of homocysteine and alter platelet reactivity, *eg* oxLDL. Nonetheless, the trend towards increased aggregation in the presence of an elevated homocysteine suggests that this may reflect the *in vivo* situation, at least in some individuals.

The fibrinogen binding assay did not substantiate these results but, as discussed in section 6.4, this may be a fault of the assay as it was carried out, rather than of a lack of effect. Although not consistent, the overall data pointed to a possible role for homocysteine in enhancing platelet aggregation.

Platelet adhesion to damaged endothelium is one of the first steps in the formation of the atherosclerotic plaque. It is possible that platelet activation by homocysteine may be manifested not only by an increased propensity towards aggregation but also to an increased propensity towards platelet adhesion. In order to clarify the role of homocysteine on platelet reactivity, further studies should be carried out and an attempt should be made to measure other factors such as lipid levels and markers for oxidant stress (eg TBARS) to see if these factors regulate the effect of homocysteine.

In conclusion, this body of work has succeeded in designing a system that through some changes may be made amenable to large-scale studies on the cellular biochemical basis for hyperhomocysteinemia. Such studies may help to clarify the interaction between genetic and nutritional factors and the contribution of the remethylation and transsulphuration pathways to the accumulation of homocysteine.

The work regarding the effect of homocysteine on TNF α production and homocysteine-mediated enhancement of platelet aggregation has provided evidence for a biologically plausible atherothrombotic mechanism, especially in view of the fact that these effects were seen at physiologically attainable levels of homocysteine.

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