Cultured fibroblasts as a model for methionine metabolism in man: Effect of growth and substrates on homocysteine and related metabolites

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Peter Rock
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Dedicated to my family, especially Nicole and Vincent

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Abbreviations

AdoHcy	S-adenosyl-L-homocysteine
AdoMet	S-adenosyl-L-methionine
CBS	Cystathionine β-synthase
Cbl, cbl	Cobalamin (vitamin B_{12})
CH_3	Methyl group
CSF	Cerebrospinal fluid
CTH	Cystathionine γ-lyase
Cys	L-cyst(e)ine
Cys-gly	L-cysteinyl-L-glycine
CVD	Cardiovascular disease
FCS	Foetal calf serum
FR	Folate receptor
GSH	Glutathione (reduced)
GSSG	Glutathione (oxidised)
HCA	L-homocysteic acid
Hcy (L-Hcy), tHcy	L-homocysteine, total L-homocysteine
HH	Hyperhomocystinaemia
LDL	Low-density lipoprotein
MAT	Methionine adenosyltransferase
Met (L-Met)	L-methionine
5-MeTHF	5-methyltetrahydrofolate
MS	Methionine synthase
MTX	Methotrexate
Mw.	Molecular weight
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
N_2O	Nitrous oxide
SOD	Superoxide dismutase
VSMC	Vascular smooth muscle cells

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Contents

Summary

Abnormal homocysteine metabolism has been implicated in a number of common diseases, but mainly cardiovascular disease. Though this association is well established, it remains to be proven whether homocysteine itself is directly responsible. Consideration of the causal mechanism depends on clearly defining the interrelationships between homocysteine and the other metabolites within the transmethylation-transsulphuration pathways, as they may also play an integral role in each disease process. In considering the pathogenesis of sulphydryl compounds, the relationship between intra- and extra-cellular levels is an often underestimated and important regulatory factor. Homocysteine metabolism is regulated by complex mechanisms involving its own export, B vitamins, S-adenosyl-methionine (AdoMet), S-adenosyl-homocysteine (AdoHcy) and amino acids, as well as the proper function of various enzymes. These mechanisms in human cells, and particularly with export studies, have so far been performed in media containing un-physiological substrate levels. Difficulties in attempting to understand these mechanisms are compounded by the use of serum supplemented media, and the need to unravel the metabolic interrelationships by piecing together information from various models.

This work therefore, involved the development and application of a model for methionine metabolism, using human skin fibroblasts cultured in medium containing similar amino acid and vitamin levels to those found in normal human plasma. This required the formulation and production of a basal physiological medium (P-med) that would sustain cell growth, the development and application of methods for metabolite determination, the modification of sample handling and preparation procedures, and improvements to the existing analytical methods. Following the successful completion of this time consuming but indispensable step, it was possible to measure, (i) total, free (non-protein bound) and protein-bound homocysteine, cyst(e)ine, cysteinyl-glycine and glutathione, (ii) 5-methyltetrahydrofolate (5-MeTHF), (iii) AdoMet and AdoHcy, and (iv) free amino acids, in media and/or cells for the first time using conditions more comparable to the physiological situation than those obtained previously.

In a multi-time point study designed to examine changes in media sulphydryl compound levels with log phase and post-confluent cells in basal and supplemented media, homocysteine and glutathione export, cyst(e)ine uptake and media cysteinyl-glycine levels were reproducible in eight cell lines at each time point for each media and growth state. Differences in media sulphydryl compound levels between each media and growth state however, were only subtle. Nevertheless, folate levels accounted for similarities in homocysteine export from log phase cells in each media, and a growth resurgence in post-confluent cells on renewing media accounted for similarities between each media and the post-confluent and log phase cells.

The second part of the work, which included cystathionine β-synthase (CBS)-deficient cells, answered the important question, could the model be used to reliably measure intracellular metabolites? Indeed, total homocysteine, cysteine, cysteinyl-glycine, glutathione, AdoMet and the free amino acids could be measured, 5-MeTHF and AdoHcy could not. This study also highlighted the importance of export in the control of intracellular homocysteine, folate receptors and folate in homocysteine remethylation in post-confluent CBS-deficient cells, transsulphuration in glutathione synthesis, and the unrelated nature of media amino acid concentrations in amino acid transport.

In a final study to evaluate the outcome of methionine and folate on metabolites, supplementation resulted in methionine catabolism and conservation respectively, but was less effective than anticipated and previously shown in commercial media. Although this approach has been extensively applied to a variety of cell types in various commercial media, it may still have scope for examining the effects of additional substrates, for example arginine, taurine and the antioxidant vitamins A, C and E on homocysteine, its export or its modification of low-density lipoprotein.

In conclusion, this thesis added to the existing work by obtaining more comprehensive data on the main metabolite levels in media and in human fibroblasts under similar conditions to better understand the metabolism, and gave support and validation to our knowledge of export from previous studies as applicable to the human physiological situation.

Zusammenfassung

Ein pathologischer Homocystein Stoffwechsel wurde hisher mit einer Reihe von Krankheiten in Verbindung gebracht, hauptsächlich jedoch Herz-Kreislauferkrankungen. Obwohl dieser Zusammenhang gut dokumentiert ist, ist der Beweis bisher noch nicht erbracht, ob Homocystein dafür direkt verantwortlich ist. Für das Verständnis des causalen Mechanismus ist es wichtig, dass die Beziehungen zwischen Homocystein und anderen Stoffwechselprodukten innerhalb der Transmethylierungs-Transsulfurierungs-Abfolge klar definiert sind, da diese auch eine wichtige Rolle in vielen Krankheitsverläufen spielen könnten. Bei der Pathogenese der Sulphuryl-Verbindungen ist der Zusammenhang zwischen intra- und extrazellulären Konzentrationen ein oft unterschätzter regulativer Faktor. Der Homocystein Stoffwechsel wird durch komplexe Mechanismen reguliert, darunter sein eigener Export, B-Vitamine, S-adenosyl-Methionin (AdoMet), S-adenosyl-Homocystein (Ado-Hcy), und Aminosäuren, und auch das korrekte Funktionieren verschiedener Enzyme. Diese Mechanismen in menschlichen Zellen und insbesondere in Export Experimenten, wurden bisher mit Zellmedien durchgeführt, welche unphysiologische Substratlevel aufwiesen. Es ist schwierig, die Mechanismen zu verstehen, da erstens Medien mit Serumzusatz verwendet wurden, und es zweitens nötig war, Zusammenhänge aufzuklären, indem man Informationen aus verschiedenen Modellen zusammentrug.

Diese Arbeit beinhaltet die Entwicklung und Anwendung eines Modells für den Methionin Stoffwechsel. In diesem Modell werden Hautfibroblasten in Medien kultiviert, welche Aminosäuren- und Vitaminkonzentrationen beinhalten, die vergleichbar sind mit normalem menschlichen Plasma sind. Dies bedingte die Formulierung und Produktion eines basalen physiologischen Mediums (P-med), welches folgende Bedingungen erfüllen sollte: Zellwachstum, die Entwicklung und Anwendung von Methoden zur Metabolitendetektion, die Modifizierung der Probenaufbereitung, und die Verbesserungen der bestehenden analytischen Verfahren. Nach dem erfolgreichen Abschluss dieses zeitaufwendigen jedoch unabdingbar notwendigen Schritts, war es möglich, folgende Metaboliten zu messen: (i) Gesamt-, freies (nicht proteingebundenes) und proteingebundenes Homocystein, Cyst(e)in, Cysteinyl-Glycin und Glutathion, (ii) 5-Methyltetrahydrofolat (5-MeTHF), (iii) AdoMet und AdoHcy, und (iv) freie Aminosäuren. Diese Verbindungen wurden in Medium und/oder Zellen unter Bedingungen gemessen, die physiologischen Bedingungen viel näher kommen als bisher.

Es wurden Experimente mit Zellen, die sich in der Log-Phase befanden, und Post-Confluent Zellen sowohl in basalem als auch in Medien mit Zusätzen konzipiert, um Veränderungen in den Konzentrationen der Sulphydryl- Verbindungen zu detektieren. Diese Experimente zeigten, dass der Export von Homocystein und Glutythion, die Aufnahme von Cyst(e)in und die Konzentrationen von Cysteinyl-Glycin in Medium in 8 Zelllinien zu jedem Zeitpunkt der Messung für jedes Medium und jede Wachstumsphase reproduzierbar waren. Die Unterschiede der Konzentrationen der Sulphydryl-Verbindungen in Medium waren zwischen den beiden Medien und zwischen den beiden Wachstumsstadien gering. Dennoch deuteten die Folatkonzentrationen auf gewisse Ähnlichkeiten im Homocystein Export der Zellen in der Log-Phase in beiden Medien hin, und ein Wachstumsschub der Post-Confluent Zellen nach frischer Mediumzugabe zeigte ebenfalls Ähnlichkeiten sowohl zwischen den beiden Medien als auch zwischen den Zellen in den beiden Wachstumsstadien.

Der zweite Teil der Arbeit, bei welchem die Cystathionin β-synthase (CBS) mutierten Zellen eingesetzt wurden, beantwortete die wichtige Frage, ob das entwickelte Modell verwendet werden könnte, um verlässlich intrazelluläre Metaboliten zu messen? In der Tat konnte das Gesamt-Homcystein, Cystein, Cysteinyl-Glycin, Glutathion, AdoMet, und die freien Aminosäuren gemessen werden. 5-MetHF und AdoHcy konnten jedoch nicht gemessen werden. Durch die Experimente konnten die Wichtigkeit folgender Faktoren herausgearbeitet werden: der Export, der vom intrazellulären Homocystein kontrolliert wird, die Folat-Rezeptoren und das Folat bei Homocystein Remethylierungen in Post-Confluent CBS mutierten Zellen, die Transsulphrierungen bei der Glutathion Synthese, und die Unabhängigkeit der Aminosäure-Konzentrationen in Medium vom Aminosäure Transport.

In einem letzten Experiment wurde der der Effekt von Methionin und Folat auf Metaboliten evaluiert. Die Erhöhung der Konzentrationen dieser beiden Verbindungen resultierte sowohl in einer Methionin Konservierung als auch einem Katabolismus. Das Resultat war weniger stark als erwartet und als bisher in kommerziellen Medien gezeigt. Obwohl in vielen Experienten bei einer Vielzahl von Zelltypen in verschiedenen kommerziellen Medien bereits mit demselben Versuchsaufbau gearbeitet wurde, könnten auch hier noch einige zusätzlichen Untersuchungen durchgeführt werden, die die Effekte verschiedener zusätzlicher Substrate, z.B. Arginin, Taurin, und die antioxidativen Vitamine A, C und E auf Homocystein, seinen Export oder seine Modifizierung von Lipoproteinen niedriger Dichte zeigen würden.

Zusammengefasst liefert diese Arbeit umfassende Daten zu den Konzentrationen der Hauptmetaboliten in Zellmedien und in menschlichen Fibroblasten unter ähnlichen Bedingungen, sodass deren Stoffwechsel verständlicher wird. Zugleich hilft sie, unsere Vorstellung vom Export aus früheren Studien auf die menschliche physioliogsiche Situation zu übertragen.

Homocysteine & disease

As early as November 1933, the *New England Journal of Medicine* reported an 8 year-old boy that had died of carotid arteriosclerosis with consequent stroke and death of brain tissue, a condition normally found in the elderly. The cause of this was unknown and although an apparently inherited abnormality, case 19471 was forgotten. The problem was resolved some 32 years later when another child, this time a 9 year-old girl presented with similar abnormalities. Using techniques developed in Belfast, Northern Ireland to study the chemical composition of urine from mentally retarded children, she was found to have high urine levels of homocystine and later discovered to be the boy's niece.

Inborn errors of methionine metabolism, resulting in high levels of homocysteine (hyperhomocystinaemia {HH} and -uria) in body fluids and tissues are associated with severe vascular disease, premature arteriosclerosis, thromboembolism and early death, as well as other abnormalities. So far at least 9 defects have been recognised that can affect a specific reaction within homocysteine metabolism, although the intricate relationship to other metabolites leads to much variation within the various disorders.

Of increasing importance in recent years has been the accumulation of evidence associating a mild to moderate[1] elevation of homocysteine with an increased risk for the development of cardiovascular disease (CVD) (**Figure 1.1**). This is especially important in patients that show no underlying genetic disorder or previous explanatory risk behaviour. Homocysteine as an independent risk factor is claimed to be as important as blood cholesterol, but 40-times more predictive. Between 21-32% of cases of coronary heart, cerebrovascular and peripheral vascular disease have been linked to elevated homocysteine. More specifically, a plasma homocysteine level of 15 μmol/L or more, even in the absence of a previous history of ischaemia, is linked to a 3-times greater chance of death through heart disease, after adjusting for such factors as apolipoprotein levels and blood pressure. It is not surprising that it has now been labelled as the "new cholesterol".

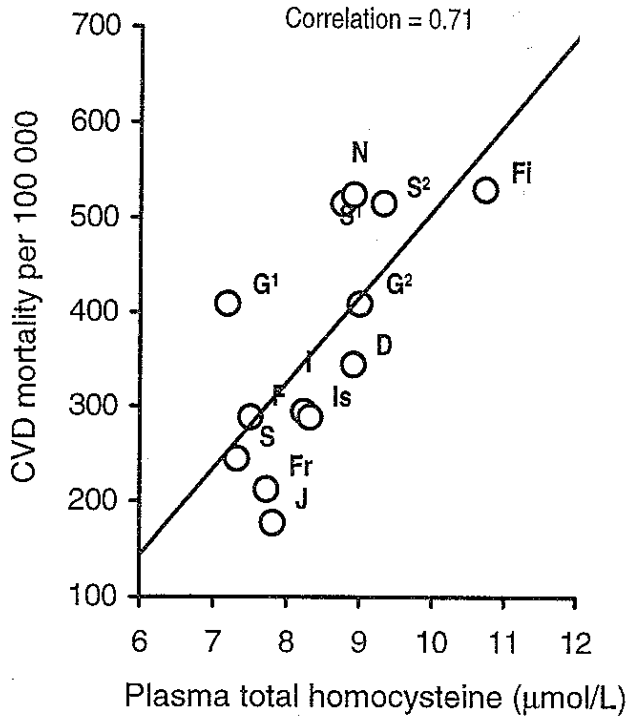
Now homocysteine is accepted as a risk factor for CVD alongside the well established risk factors of gender (being male), advancing age, cigarette smoking, high blood pressure, diabetes, obesity, physical inactivity and abnormal cholesterol levels.

The B vitamins, folate, B_6 and B_{12} all play a role in homocysteine metabolism and it has been proposed that 60-70% of all cases of HH are due to an inadequate status in one or more of these vitamins. Supplementation, either separately or in combination, has proven effective in lowering plasma homocysteine levels, but it is not known whether such therapy will lead to reduced cardiovascular risk in the long-term.

Since the emergence of homocysteine's role in atherogenesis first proposed by McCully (1969), abnormal homocysteine metabolism has been further implicated in a wide range of important disease processes. These include abnormalities in pregnancy (recurrent loss and pre-eclampsia), developmental birth defects (neural tube defects {NTDs}, Down's syndrome and spina bifida), cancer, renal failure, chronic liver, diabetes, hormonal imbalances, HIV infection, autoimmune (rheumatoid arthritis), inflammatory bowel and neurodegenerative (Parkinson's and Alzheimer's) disease. The strength of association is weak for some of these and could be a consequence of the primary cause, possibly involving one or more of the B vitamins associated with the metabolism of homocysteine. Nevertheless, the possibility of homocysteine being a causal agent remains open, as even with CVD where its connection is strong, the exact mechanism of homocysteine's involvement remains to be discerned.

[1] normal = 5-15 μmol/L; mild = 12-15 μmol/L; moderate > 15-30 μmol/L; high > 30-100 μmol/L; severe > 100 μmol/L

Figure 1.1 Homocysteine vs. CVD mortality from the WHO/MONICA Project (after Alfthan *et al.*, 1997)



Legend:

D	Denmark, Glostrup
F	Faroe Islands (Iceland)
Fi	Finland, Kuopio
Fr	France, Toulouse
G¹	Germany, Schleiz
G²	Germany, Cottbus
I	Iceland, Reykjavik
Is	Israel, Tel Aviv
J	Japan, Okinawa
N	Northern Ireland, Belfast
S¹	Scotland, Aberdeen
S²	Scotland, Glasgow
S	Spain, Barcelona

each point represents the mean plasma homocysteine concentration from 20 men

Introduction

Brief overview of homocysteine metabolism

Homocysteine (L-Hcy, Hcy) represents the connection between the ubiquitous methionine (L-Met, Met) or transmethylation cycle and the transsulphuration pathway (**Figure 1.2**). The two pathways only seem to be complete in mammalian liver (Finkelstein, 1990) and homocysteine plays an important role in the recycling of both methionine and folate.

The initiation of these inter-conversions is the formation (reaction **I**) of S-adenosyl-L-methionine (AdoMet) from methionine under the action of methionine adenosyltransferase (MAT, EC 2.5.1.6). AdoMet then participates in transmethylation (reaction **II**) yielding S-adenosyl-L-homocysteine (AdoHcy) which is further metabolised by AdoHcy hydrolase (AHCY, EC 3.3.1.1) (reaction **III**) to homocysteine. Between 33-47% of the available homocysteine is estimated to be remethylated by two alternative reactions back to methionine (Mudd & Poole, 1975). In the first, 5-methyltetrahydrofolate (5-MeTHF) formed from 5, 10-methylenetetrahydrofolate is the methyl donor in the reaction (reaction **IV**) catalysed by 5-methyltetrahydrofolate-homocysteine S-methyltransferase shortened as methionine synthase (MS, EC 2.1.1.13). The second (reaction **V**) is catalysed by betaine-homocysteine S-methyltransferase (BHMT, EC 2.1.1.5) in which a methyl group is donated by betaine.

The transsulphuration pathway is the main catabolic route from methionine via homocysteine and cysteine to inorganic sulphate. Homocysteine is condensed with serine to yield cystathionine in an irreversible reaction (reaction **VI**) catalysed by cystathionine β-synthase (CBS, EC 4.2.1.22). Cystathionine is cleaved to cysteine and α-ketobutyrate (reaction **VII**) by cystathionine γ-lyase (CTH, EC 4.4.1.1). The sulphur atom from methionine is incorporated into the cysteine molecule through the transsulphuration pathway and mainly ends up as inorganic sulphate which is excreted in the urine (reaction **VIII**).

It must be appreciated that extremely dynamic processes control transmethylation, transsulphuration and the associated pathway(s) of folate biosynthesis. Considerable interplay exists between specific metabolites and conversion mechanisms within the whole array of events governing methionine metabolism, even though these pathways are represented as independent sequences. This dynamism is best demonstrated by MS, where the intricate mechanism combines elements of folate and vitamin B_{12} (cobalamin, Cbl/cbl) metabolism with the remethylation of homocysteine. While recognising that all reactions are reversible, in the figures the directions thought to exist under physiological conditions are generally shown.

Transmethylation

Methionine adenosyltransferase

The essential sulphur containing amino acid methionine is converted to AdoMet by MAT (Kotb *et al.*, 1997). The reaction is common to all cells and occurs within the cytosol (Finkelstein, 1998). The conversion involves two intrinsic reactions that occur simultaneously, whereby the adenosyl moiety of adenosine triphosphate (ATP) is transferred to methionine and the remaining tripolyphosphate portion is hydrolysed to inorganic phosphate and pyrophosphate. Two Mg^{2+} ions and one K^{+} ion are necessary for optimal enzyme activity. The enzyme exhibits high substrate specificity for ATP (Hancock, 1966) but not for methionine, since it can also utilise selenomethionine and both their ethyl analogues as substrates (Pan & Tarver, 1967).

Figure 1.2 Metabolism of methionine (after Mudd & Poole, 1975)

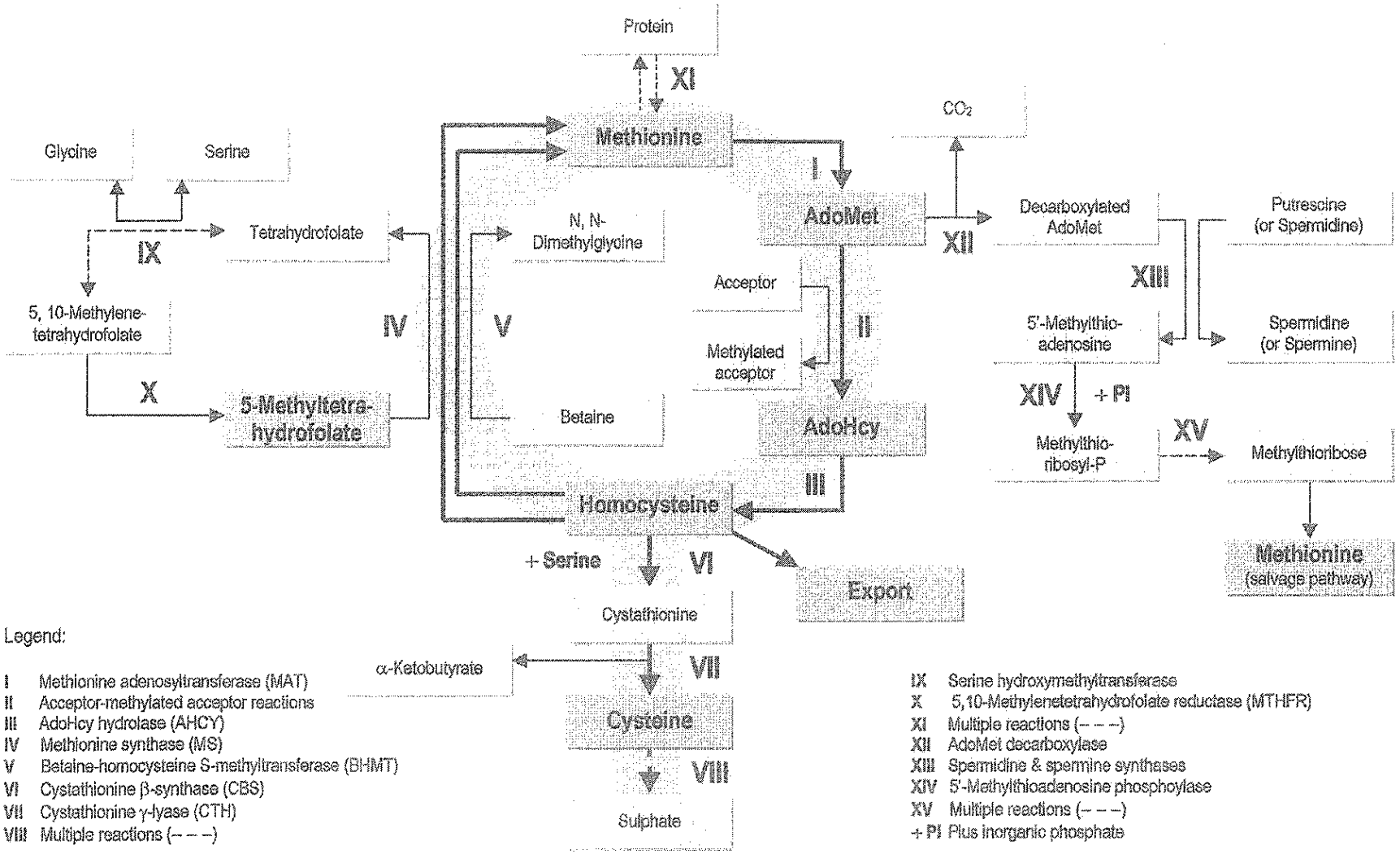
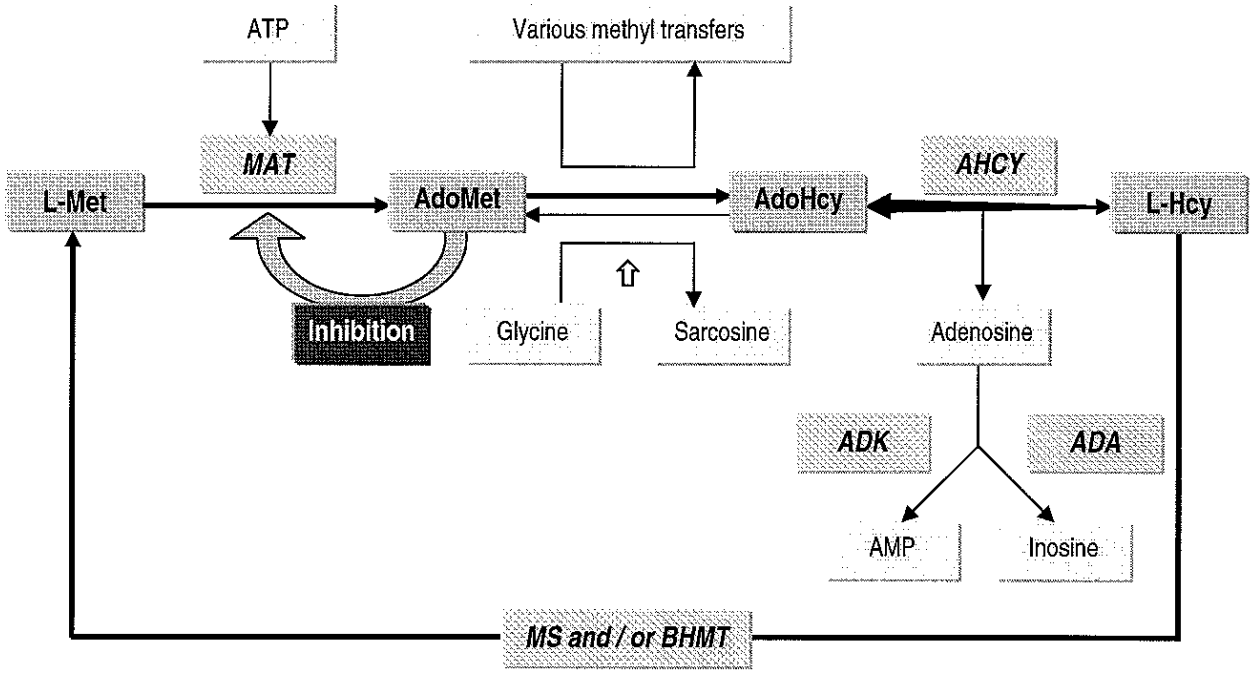


Figure 1.3 Transmethylation pathway



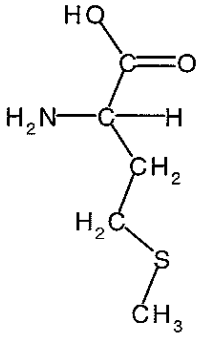
Legend: ⇧ represents the site of inhibition by 5-MeTHF pentaglutamate and reduced folate polyglutamates of glycine N-methyltransferase (GNMT, EC 2.1.1.20); ***ADK*** = adenosine kinase (EC 2.7.1.20); ***ADA*** = adenosine deaminase (EC 3.5.4.4)

Three structurally conserved isozymes of MAT have been identified in mammalian tissues (**Table 1.1**). They are encoded by two related but distinct genes. MAT1A, located on human chromosome 10q22 (Chamberlin *et al.*, 1996), is expressed only in adult liver (mostly parenchymal cells, but also endothelial and Kupffer's cells, Shimizu-Saito *et al.*, 1997) and gives rise to a homotetramer MAT I and a homodimer MAT III composed of catalytic MAT α1-subunits (Alvarez *et al.*, 1994). MAT2A, located on human chromosome 2p11.2 (De La Rosa *et al.*, 1995), is expressed in all tissues but principally extrahepatic (brain, kidney, testis, erythrocytes, lymphocytes) although present in foetal liver and generates MAT II composed of catalytic MAT α2/α2'- and regulatory β-subunits (LeGros *et al.*, 2000).

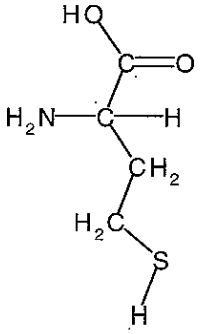
The function of the MAT isozymes have not yet been fully elucidated, but in addition to supplying AdoMet at physiological methionine levels, MAT III provides the unique ability to immediately adapt to high methionine levels after a protein rich meal. It has been suggested that while the bulk of methionine metabolism is undertaken by MAT III, the readily saturable MAT I/II could be involved in AdoMet (and polyamine) synthesis for specialised cellular functions.

Figure 1.4 Major metabolites of transmethylation

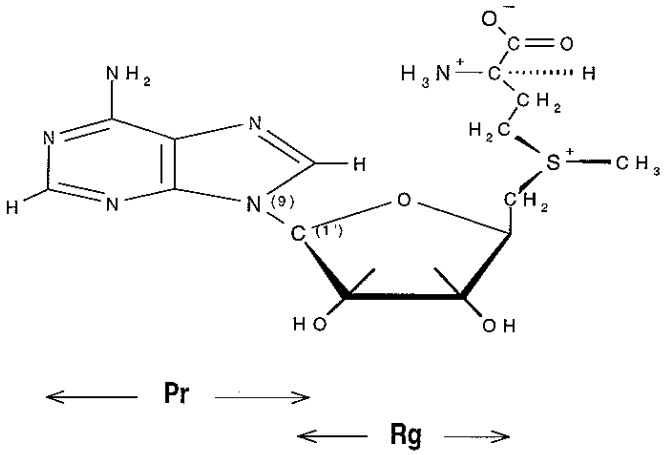
L-Methionine (Met)
(Mw. 149.2)



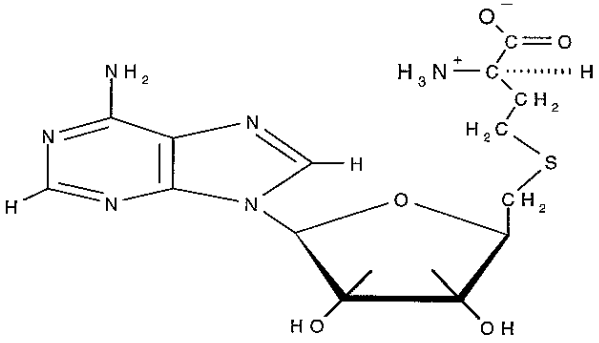
L-Homocysteine (Hcy)
(Mw. 135.2)



S-Adenosyl-L-Methionine (AdoMet)
(Mw. 399.4)



S-Adenosyl-L-Homocysteine (AdoHcy)
(Mw. 384.4)



Legend: AdoMet and AdoHcy are depicted in their anti-conformation at the $N^{(9)}$-$C^{(1')}$ bond, with the ribose group (**Rg**) of the adenosyl moiety pointing away from the purine ring (**Pr**)

Table 1.1 Isozymes of mammalian MAT

MAT isozyme	Genes	Tissue expression	Mw. (kDa) & % of liver total	Activity K_m (µmol/L) †	
				L-Met	ATP
MAT I	MAT1A	Adult liver	174.8 (4x43.7) 15%	20-1000	200
MAT III	MAT1A	Adult liver	87.4 (2x43.7) 80%	120-7000	1000
MAT II	MAT2A	All, but principally extrahepatic and foetal liver ‡	- 5%	4-80	30

† depending on the enzyme purification methods; ‡ also shown in hepatic carcinoma cells

AdoMet

The vitally important methyl donor AdoMet exists as two stereoisomers, one the biologically active form, the other a potent inhibitor of methylases (Borchardt & Wu, 1976). The molecule is highly reactive and unstable in crystalline form or in aqueous solution at room temperature. Under these conditions AdoMet undergoes irreversible conversion to 5'-methylthioadenosine and homoserine lactone. AdoMet is stabilised *in vivo* by macromolecular bonding, possibly by the combined electrostatic interactions of the positively charged adenine ring, sulphonic pole and amino acid group of the molecule.

The involvement of AdoMet in a wide spectrum of biological reactions has been well documented (Fontecave *et al.*, 2004, **Table 1.2**). Of these the conversion of creatinine to creatine is of major quantitative importance. The key to this function is the ability of the highly reactive sulphonium centre of the molecule to donate attached methyl, aliphatic and ribose groups in essential biochemical reactions.

As well as the regulation of MAT and its involvement in a vast number of methyl transfers, AdoMet also acts on reactions within the transsulphuration pathway and folate biosynthesis. For example, Kutzbach & Stokstad (1971) showed that in rat liver AdoMet allosterically inhibits 5, 10-methylenetetrahydrofolate reductase (MTHFR, EC 1.7.99.5) and consequently the resynthesis of methionine by MS. Later Finkelstein *et al.*, (1975) in studies also on rat liver showed that AdoMet also activates CBS and impairs BHMT activity. Based on these findings, Selhub & Miller (1992) proposed the hypothesis that AdoMet acts as a central regulatory "switch", whereby high concentrations favour transsulphuration and low concentrations the conservation of methionine.

In addition, 1% of AdoMet when decarboxylated is the source of the three-carbon moieties required for formation of the polyamines, spermidine and spermine.

Table 1.2 Various methyl & aliphatic group transfer reactions modulated by AdoMet

Substrate or acceptor	Product
Creatinine (guanidinoacetic acid)	Creatine
Cytosine in DNA	5-Methylcytosine involved in mammalian gene activity and cellular differentiation
Dihydroxyphenylalanine (DOPA)	Methyl-DOPA
Glycine	Sarcosine (N-methylglycine)
Haemoglobin	Methylated α and β chains of haemoglobin tetramer
Membrane phospholipid (phosphatidyl-N-monoethyl-ethanolamine) methylated via three successive steps	Phosphatidylcholine
mRNA(s)	N6-Methyladenosine
Noradrenaline	Adrenaline
Phosphatidylethanolamine	Choline
Putrescine (1, 4-diaminobutane) conjugated with an aminopropyl group	Spermidine (spermidine conjugated with an amino-propyl group → spermine)

AdoHcy, glycine N-methyltransferase & homocysteine generation

AdoMet-dependent transmethylases each catalyse a reaction generating AdoHcy and one of a variety of methylated biomolecules. AdoHcy is a potent inhibitor of most, if not all, of these reactions, which include MS and BHMT. Unlike the concentration of AdoMet, which is tightly controlled in most tissues, the concentration of AdoHcy may range more widely leading to substantial significance as a metabolic regulator. It was once thought that with the exception of CBS, which is activated by AdoHcy along with MTHFR, the effect of AdoMet and AdoHcy were opposed. This led to the use of the transmethylation index (the ratio of AdoMet : AdoHcy) to predict the extent of methylation. Inspection of the reaction rate equation for competitive inhibition of a bimolecular reactivation however, led Finkelstein (1998) to suggest that the transmethylation index is an ineffective indicator of methylation. This is supported by data from many tissues (**Table 1.3**) that have vastly different ratios to the 4 : 1 that had been previously determined as the minimum for effective methylation (Cantoni *et al.*, 1979).

Table 1.3 AdoMet : AdoHcy in various human & rat tissues

Tissue		AdoMet : AdoHcy	Reference
Human	plasma	3-5 : 1	Capdevila & Wagner, 1998; Loehrer *et al.*, 1998
	whole blood	1 : 1	Cheng *et al.*, 1997
	brain	1-2 : 1	Morrison *et al.*, 1996
Rat	brain	5-11 : 1	Bottiglieri & Hyland, 1994; Gomes-Trolin *et al.*, 1994
	liver	2-8 : 1	She *et al.*, 1994; Svardal *et al.*, 1988
	heart	10 : 1	Eloranta, 1977
	kidney	2-7 : 1	Eloranta, 1977; Svardal *et al.*, 1988
	pancreas	3 : 1	Eloranta, 1977
	spleen	7-77 : 1	Eloranta, 1977; Svardal *et al.*, 1988

Furthermore, it was shown by James *et al.*, (2002) that effective methylation requires the maintenance of low AdoHcy levels, as decreasing AdoMet alone is insufficient to impair the process.

In addition to its conversion of glycine to sarcosine, glycine N-methyltransferase (GNMT) is believed to regulate the ratio of AdoMet : AdoHcy. GNMT is abundant in the cytosol of cells of both liver and pancreas (Yeo & Wagner, 1992), strongly inhibited by 5-MeTHF pentaglutamate or reduced folate polyglutamates and is identical to the major folate binding protein in rat liver. This inhibition by folate seems to represent a metabolic control pathway, linking *de novo* methyl group formation to the methylating activity of the liver.

AdoHcy is subsequently cleaved to homocysteine and adenosine by the action of AdoHcy hydrolase (AHCY), which is widely distributed in mammalian tissues and is cytosol specific (Finkelstein & Harris, 1975; Walker & Duerre, 1975). Although the reaction is reversible and the equilibrium favours the accumulation of AdoHcy and not homocysteine, the rapid removal of homocysteine (and adenosine) by remethylation, transsulphuration or export (see page 24) ensures that hydrolysis predominates.

Homocysteine remethylation by methionine synthase & betaine-homocysteine S-methyltransferase

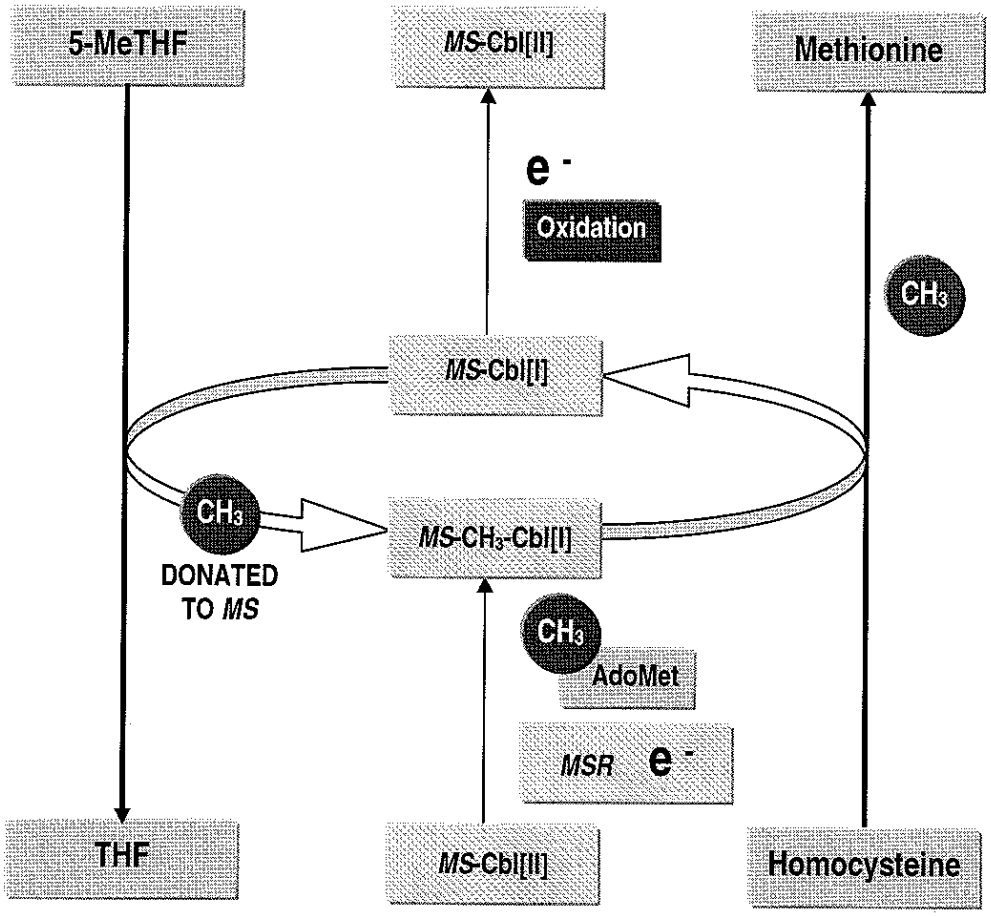
MS

Regeneration of methionine from homocysteine depends on the complex cytoplasmic enzyme MS (**Figure 1.5**). The MTR gene that controls the expression of MS is located on human chromosome 1 (1q42.3-43.8, Chen *et al.*, 1997). The cDNA of MTR is 105 kbp long and encodes a 1265 amino acid residue enzyme with a predicted molecular mass of 141 kDa. The function of MS requires the intermediate generation of enzyme coupled-methylcobalamin (MS-CH_3Cbl[I], where [I] is the valence or oxidation state of cobalt, see page 16 **Figure 1.11**). It transfers a methyl group donated from 5-MeTHF to homocysteine producing methionine, tetrahydofolate (THF) and enzyme bound-cobalamin [I]. The latter is then itself remethylated by 5-MeTHF to repeatedly generate the intermediate and thus perpetuate the cycle. Methylcobalamin is formed by the reductive methylation of cobalamin [II] produced by intracellular vitamin B_{12} metabolism. This reaction is catalysed by methionine synthase reductase (MSR, EC 2.1.1.135). The MTRR gene that controls the expression of MSR is located on human chromosome 5 (5p15.2-15.3, Leclerc *et al.*, 1998). The cDNA of MTRR is 32 kbp long and encodes a 725 amino acid residue enzyme with a predicted molecular mass of 80 kDa. The flavoprotein MSR is a unique member of electron transferases that are collectively known as the ferredoxin-$NADP^+$ reductases or "FNR-family".

BHMT

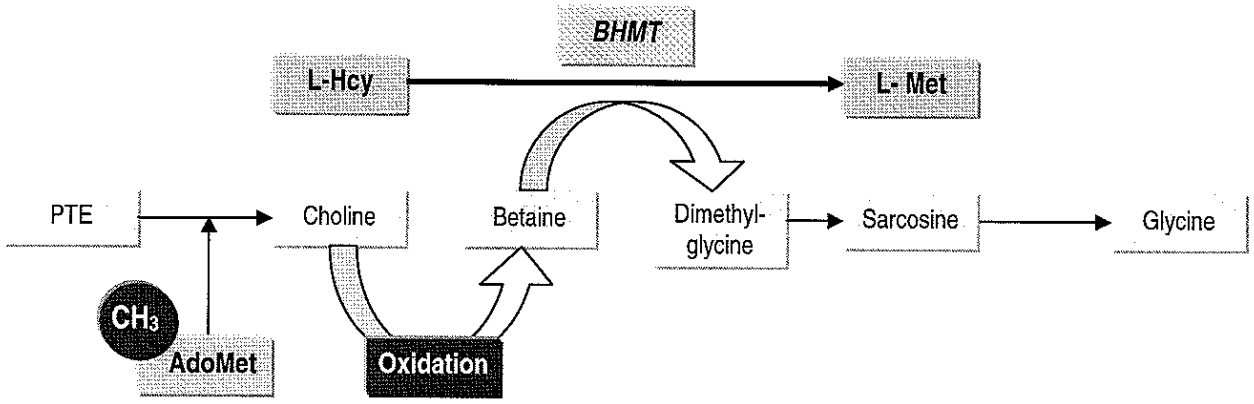
The betaine dependent remethylation of homocysteine is regarded as the secondary methylation pathway of homocysteine back to methionine and AdoMet (**Figure 1.6**). The 815 bp long BHMT gene is located on chromosome 5 (5q13.1-q15) and codes for a 45 kDa subunit. BHMT together with MS belongs to a family of zinc metalloenzymes which possess a characteristic base pair signature motif and utilise thiols or selenols as methyl acceptors (Breksa & Garrow, 1999). The enzyme is part of the pathway of choline oxidation and is abundantly expressed in the liver and kidney, but not in other tissues e.g. intestine (Sowden *et al.*, 1999) or cultured fibroblasts. It has been shown both in rats and chicks that BHMT is induced when methionine is restricted. The liver mRNA content and activity increased 4-fold when rats were fed a methionine deficient, choline rich diet (Park & Garrow, 1999). In contrast, porcine BHMT activity was found to be independent of methionine levels, irrespective of the availability of choline or betaine (Emmert *et al.*, 1998).

Figure 1.5 Remethylation of homocysteine by MS



Legend: CH_3 = methyl group, e^- = electron

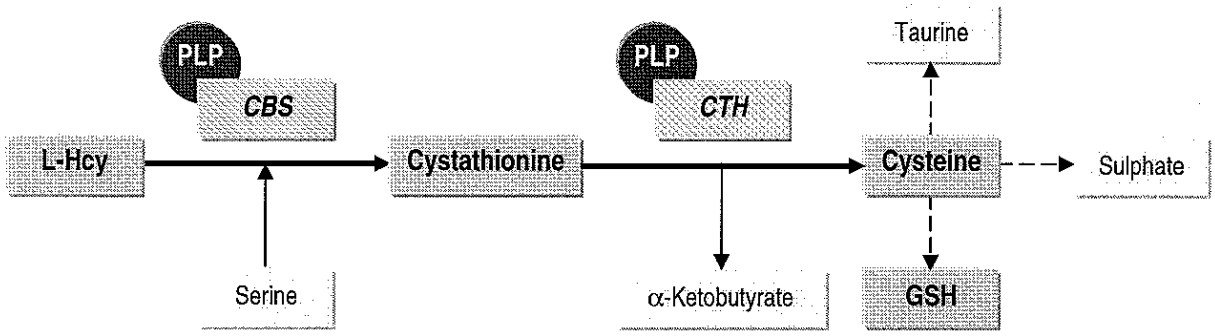
Figure 1.6 Remethylation of homocysteine by BHMT



Legend: PTE = phosphatidylethanolamine, CH_3 = methyl group

Transsulphuration

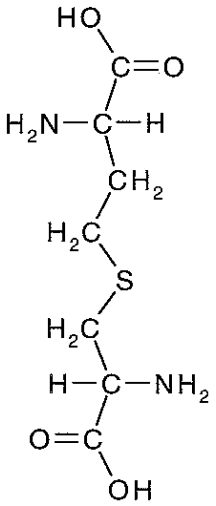
Figure 1.7 Transsulphuration pathway



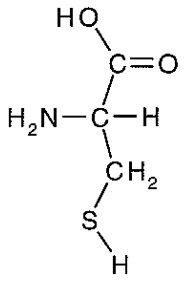
Legend: PLP = pyridoxal 5'-phosphate (vitamin B_6)

Figure 1.8 Major metabolites of transsulphuration

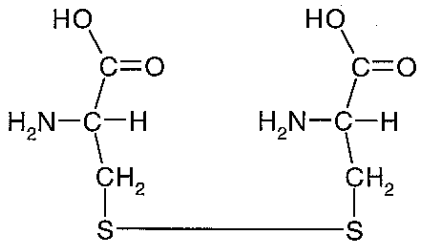
L-(+) Cystathionine [Hcy(Ala)] (Mw. 222.3)



L-Cysteine (Cys) (Mw. 121.2)



L-Cystine [$(Cys)_2$] (Mw. 240.3)



Legend: structure of L-Hcy ($C_4H_9NO_2S$) previously shown (see page 6)

Cystathionine formation & cystathionine β-synthase

The alternative to homocysteine remethylation or export is the condensation with serine to form the thioether cystathionine. The reaction is catalysed by CBS, which has been highly purified from the livers of several vertebrates (Kraus *et al.*, 1978). The 28 kbp long CBS gene (Kraus *et al.*, 1998) is located on chromosome 21 (21q22.3, Munke *et al.*, 1988) and codes both in rats and humans for a 63 kDa subunit (Skovby *et al.*, 1984). The cDNA has been cloned and sequenced and the open reading frame predicts a polypeptide with 551 amino acid residues (Kraus *et al.*, 1998). The active form of the purified enzyme is a homotetramer with each subunit containing a single heme and a pyridoxal 5'-phosphate (PLP) group, the active coenzyme form of vitamin B_6 (Kery *et al.*, 1999). The enzyme has been shown to have different activity in various tissues, the highest being in liver, then pancreas, kidney, adipose tissue, brain, small intestine and spleen, and is not detectable in adrenal tissue, lung, testes and heart (Finkelstein, 1990). In CBS-deficient patients, virtually absent enzyme activity has been demonstrated in liver extracts, cultured fibroblasts and phytohaemagglutinin-stimulated lymphocytes (Mudd *et al.*, 2001). Its activation, regulated by AdoMet, has been shown to occur by the induction of a conformational change in a non-catalytic C-terminal region spanning residues 414-551 (Janosik *et al.*, 2001). As an alternative to the condensation of homocysteine to cysteine, CBS can catalyse the sulphydration of serine using hydrogen sulphide. Although the condensation reaction can be reversed if homocysteine is removed rapidly (Brown & Gordon, 1971), under physiological conditions the equilibrium favours cystathionine formation and serves to divert sulphur in the form of homocysteine from the transmethylation pathway.

Cystathionine cleavage, cystathionine γ-lyase & cysteine

Cystathionine is broken down to cysteine, α-ketobutyrate and ammonia by cystathionine γ-lyase (CTH). The 28 kbp long CTH gene is located on chromosome 1 (1p31.1) and codes for a 44.5 kDa subunit. Both rat (Erickson *et al.*, 1990) and human (Lu Y *et al.*, 1992) cDNAs have been cloned and sequenced, revealing human CTH to have two forms, the shorter of which having an internal deletion of 132 bp. It is not yet known whether the two forms are separate gene products or splice variants. Like that of CBS, the crystalline enzyme is a homotetramer, with each subunit containing a PLP group (Steegborn *et al.*, 1999). CTH is virtually absent from human foetal liver and brain. It appears in the liver during the first few days of the neonatal period. Second trimester foetal kidney contains 60-70% of the mean specific activity of mature kidney (Levonen *et al.*, 2000). Mammalian CTH has several catalytic activities in addition to cystathionine cleavage, including the activity to catalyse cysteine desulphydration generating thiocysteine and pyruvate.

While α-ketobutyrate is decarboxylated and converted to propionyl-CoA, cysteine can be either incorporated into protein, oxidised to sulphinate via cysteine sulphinate by cytochrome-P_{450}-coupled cysteine dioxygenase, enter glutathione synthesis, be converted to hydrogen sulphide and pyruvate by liver desulphurase, or be transaminated to β-mercaptopyruvate which reacts with sulphite producing thiosulphate and pyruvate. The biosynthetic intermediate cysteine sulphinate undergoes decarboxylation and oxidation to produce the bile salt precursor taurine, or transamination to β-sulphinylpyruvate which undergoes desulphuration yielding bisulphite and pyruvate. Sulphite oxidase uses oxygen and water to convert bisulphite to sulphate and hydrogen peroxide. The resultant sulphate is used as a precursor for 3'-phosphoadenosine-5'-phosphosulphate, for the transfer of sulphate to biological molecules such as glycosphingolipid sugars, or excreted in the urine.

Folate

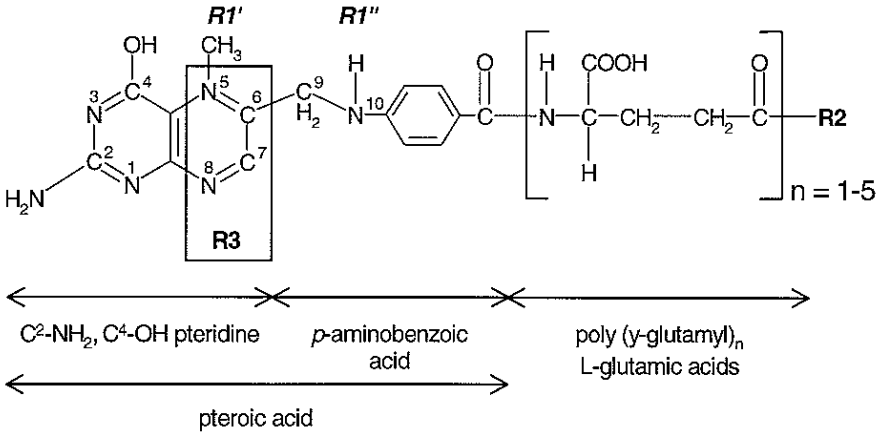
The group of heterocyclic compounds known collectively as the folates stem from the single base structure of 5, 6, 7, 8-tetrahydrofolate. This is composed of a pteridine ring attached to a *p*-aminobenzoate and a polyglutamyl chain. The nature of their chemical diversity emenates from group substitutions at position N^5 of the pteridine ring (***R1'***), N^{10} of the *p*-aminobenzoate (***R1''***), the polyglutamyl chain (R2) and/or other substitutions within the pteridine ring (R3) (**Figure 1.9**).

As well as the remethylation of homocysteine, folates act coenzymatically in the transfer and processing of one-carbon units in mitochondrial metabolism and the synthesis of serine from glycine, nucleotides from purine precursors and indirectly for tRNA, all activities that make them essential for growth and maturation. However, eukaryotes are unable to synthesise folates and require an external source of these compounds.

Food folate largely exists as 5-MeTHF ($5CH_3$-$H_4PteGlu_n$) and formyl-THF (CHO-$H_4PteGlu_n$). At alkaline pH 5-MeTHF is readily, but reversibly oxidised to 5-methyl-5,6-dihydrofolate ($5CH_3$-$5,6$-$H_2PteGlu_n$). The derivative is relatively stable to further oxidation, although at mildly acidic pH under anaerobic conditions, typical of the post-prandial gastric environment, the C^9-N^{10} bond is cleaved to produce tetrahydropteridine and *p*-aminobenzoylglutamic acid. In its oxidised form, 5-MeTHF may constitute as much as 50% of total food folate. Fortunately, ascorbate secreted into the gastric lumen can salvage labile $5CH_3$-$5,6$-$H_2PteGlu$ by reducing it back to acid-stable 5-MeTHF and may be critical for optimising the bioavailability of food folate (Lucock *et al.*, 1995). Dietary folates are absorbed in the proximal jejunum, hydrolysed to folylmonoglutamates by pteroyl-γ-glutamyl hydrolase (EC 3.4.19.9), and converted within the enterocyte into 5-MeTHF, the plasma/transport form of the vitamin (Lucock *et al.*, 1989). Extracellularly, 5-MeTHF exists as a monoglutamate with 90% being free from protein (Branda *et al.*, 1978).

The first step in assimilation of folates by cells is transport into the cytoplasm across the plasma membrane (see page 14). Subsequent to cellular uptake, these compounds are converted by folylpolyglutamate synthetase (FPGS, EC 6.3.2.12) to the polyglutamate form by the addition of several glutamate residues. This process is important for establishing and maintaining a folate pool in the cell, as polyglutamates are better retained and generally have higher affinity for folate-utilising enzymes than monoglutamate forms (Shane, 1989).

Figure 1.9 Chemical structure of 5-MeTHF



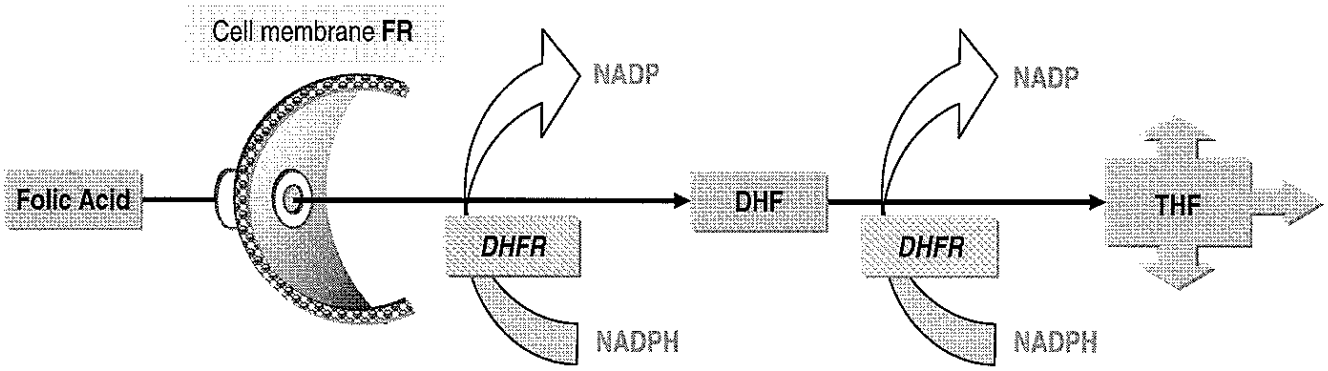
Legend:

Structure of other reduced folates

Coenzyme	Substituent at *R1'*-N^5		Substituent at *R1''*-N^{10}
5-MeTHF	**-CH_3**		**-H**
THF	**-H**		**-H**
5,10-Methylene-THF		**-CH_2-**	
5,10-Methenyl-THF		**=CH^+-**	
*5-Formyl-THF	**-CHO**		**-H**
10-Formyl-THF	**-H**		**-CHO**
10-Formimino-THF	**-H**		**-HCNH**

*Folinic acid

Figure 1.10 Folic acid metabolism



Legend: **FR** = folate receptor; ***DHFR*** = dihydrofolate reductase (EC 1.5.1.3); **DHF** = dihydrofolate; **THF** = tetrahydrofolate

Folate metabolism

Folate metabolism is extremely complex, particularly taking into consideration the various polyglutamate or storage forms of the different one-carbon units and the various oxidation states of the vitamin. Synthesis of the active forms require several enzymes, adequate supplies of riboflavin (vitamin B_2), niacin (vitamin B_3), pyridoxine (vitamin B_6), zinc and serine. It has been estimated that up to 150 different folate coenzymes may exist in nature, a problem that is further compounded by the fact that folate-dependent one-carbon metabolism is compartmentalised, involves an enormous number of low-abundance, difficult-to-measure, highly labile folyl coenzymes, and is subject to genetic variability. As yet, a full understanding of the regulatory mechanisms involved in the partitioning of one-carbon units donated by the various folate coenzymes, into production of methionine, purines, DNA-thymine, formylmethionyl-tRNA and the inter-conversion of serine and glycine is lacking.

Cellular uptake - folate transporters

Multiple membrane transport systems have been identified that mediate the internalisation of folates (Matherly & Goldman, 2003). Each utilises a specific set of proteins that span or are anchored in the membrane and bind folates with high specificity and affinity. For maximal efficiency, proteins are often aggregated by specific agents (Mayor *et al.*, 1994) to form domains enriched in that particular transporter species. Folate preference, temperature dependence and pH can distinguish each transport system. The main known mechanisms are carrier mediated or receptor initiated processes that operate efficiently at physiological pH, but function independently and exhibit distinct properties (**Table 1.4**).

Reduced folate carrier

The reduced folate carrier (RFC) represents the major transport system with the protein belonging to the "Major Facilitator Superfamily" of transport carriers. It exhibits similarity with the 55 kDa human GLUT1 glucose transporter (Dixon *et al.*, 1995) and possesses one or more thiol groups (Smith *et al.*, 1999), but not an ATP-binding region. RFC molecules exhibit tissue- or cell line-specific distribution and their activity depends on the differentiation of the cell. The extent of the transmembrane folate gradient achieved is further

modulated by independent exit pumps that are highly sensitive to the energy balance of the cell. Since an association exists with the co-transport of intracellular anions, a characteristic feature of this process is inhibition by extracellular ions.

Table 1.4 Characteristics of the reduced folate carrier & folate receptor

Transporter	Genes	Mass (kDa)	Direction	Main folate forms	Affinity	
Reduced folate carrier	RFC1	46-58	Bi-	Natural reduced folates	Reduced folates	K_m 1-10 µmol/L
					Folic acid	K_m 200-400 µmol/L
Folate receptor	FR α, β & (γ)	38-40	Uni-	Folic acid	Folic acid †	K_d 1-10 nmol/L
					Reduced folates	K_d 10-300 nmol/L

† together with 5-MeTHF; K_d = dissociation constant

Folate receptor - uptake by potocytosis (Anderson *et al.,* 1992)

An additional transport mechanism is mediated by the folate receptors (FR), which are binding proteins anchored to the cell membrane by glycosylphosphatidylinositol (GPI) residues. After binding folate, which requires energy, chloride anions and a pH above 5.0 (Spinella *et al.,* 1995). The receptors cycle between the membrane and cytoplasm in closed vesicles or open channels formed from membrane invaginations or caveolae. Each cycle delivers a quantity of folate to the caveolae, where it is acidified by a hydrogen pump. At low pH, folate dissociates from the receptor and diffuses through anion carriers (Kamen *et al.,* 1991) in the membrane to the cytoplasm. A protein component of caveolae has been identified and named caveolin-1, which tightly binds cholesterol, is thought to control the closing or pinching off of caveolae to form the transport compartment for potocytosis, and can oligomerise into large protein complexes functioning as domain organisers. As shown in many studies, depletion of cellular cholesterol disrupts domains and impairs the ability of FR to associate with these domains (Varma & Mayor, 1998), to bind folate efficiently (Chang *et al.,* 1992), and be retained within the caveolae before being recycled back to the membrane (Mayor *et al.,* 1998).

The existence of different transport systems raises the question of whether specific folates are channelled to particular cellular enzymes. Although RFC and FR employ diverse mechanisms, folate appears to be delivered to the same intracellular compartment, as the rate and extent of polyglutamylation from RFC or FR uptake are similar. There is also evidence for an influx route that operates optimally at low pH, and of particular importance, is the efflux transport directly coupled to energy metabolism that opposes concentrative transport mediated by RFC at physiological pH.

Transporter distribution & changes in activity

Cell membranes in normal tissues such as choroid plexus, placenta, kidney and thyroid, are rich in folate transporters with uptake an apparent physiological process (Weitman *et al.*, 1992). In brain, the choroid plexus transports reduced folate from blood into cerebrospinal fluid (CSF) against a concentration gradient. Folic acid on the other hand, which the brain cannot use, is removed by blood. High expression of FR in choroid plexus therefore, maintains CSF folate levels within relatively narrow limits which is important in

preventing NTDs (Molloy *et al.*, 1999). Simultaneously, expression of FR in human placenta, maintains a concentrative maternal-to-foetal flux of folate with minimal dependence on maternal dietary intake (Henderson *et al.*, 1995). FR activity in kidney plays an important role in conserving folates to counteract deficiency by regulating urinary excretion and re-absorption across the apical membrane of the proximal tubule (Sikka & McMartin, 1998).

Although a major storage site of folate, mammalian liver (except pig, Villanueva *et al.*, 1998) expresses a relatively low level of FR (da Costa *et al.*, 2000). The liver participates in folate elimination from blood to hepatocytes, from which it is actively excreted into the bile. This process is energy-dependent and sensitive to various bile acids e.g. cholate and taurocholate. The bile acid sensitive carrier in hepatocytes, absent in hepatoma cells, differs from previously described transporters for organic anions (Honscha & Petzinger, 1999).

Folate availability or exposure to chemotherapeutic drugs will cause alterations in transport system activities, provoking an adaptive response in the cell. As observed in numerous studies, these result from RFC1 gene amplification and consequent RFC over-expression (Jansen *et al.*, 1997), structural alteration of RFC increasing its transport of reduced folates and folic acid (Jansen *et al.*, 1998) and increased rates of carrier translocation (van der Laan *et al.*, 1991). Carcinoma cell lines grown in folate-restricted media, contain elevated levels of FR (Mendelsohn *et al.*, 1996). Increased expression of FR is associated with a rearrangement in promotor region producing novel transcripts with enhanced stability (Hsueh & Dolnick, 1993).

Cobalamin & the "methyl-trap" hypothesis

Vitamin B_{12} (**Figure 1.11**) is an octahedral complex consisting of a porphyrin-like macro-ring with a central cobalt ion which binds one of various substrates e.g. CN-, H_2O CH_3- etc. Only two coenzyme forms of vitamin B_{12} are known, methyl (CH_3Cbl) and adenosyl (AdoCbl), and only two dependent reactions have been conclusively established in mammals. These are MS as previously discussed and methylmalonyl-CoA mutase (EC 5.4.99.2), methyl and adenosyl cofactors respectively.

A deficiency in vitamin B_{12} can lead to lowered levels of MS, which results in a functional folate deficiency by leading to "trapping" of an increased proportion of the folate as 5-MeTHF. In addition, as 5-MeTHF is a poor substrate for FPGS compared to THF, there is a decline in the synthesis of polyglutamates and consequently a decrease in the retention of folates by the tissues, which is probably as important physiologically as the direct lack of folates. In fitting with this theory, a significant lowering of holo-MS must occur before vitamin B_{12} can disturb folate metabolism. Methionine can to some extent salvage the adverse effects of "trapping" through AdoMet and its inhibition of MTHFR, therefore preventing the build up of 5-MeTHF (Shane & Stokstad, 1985).

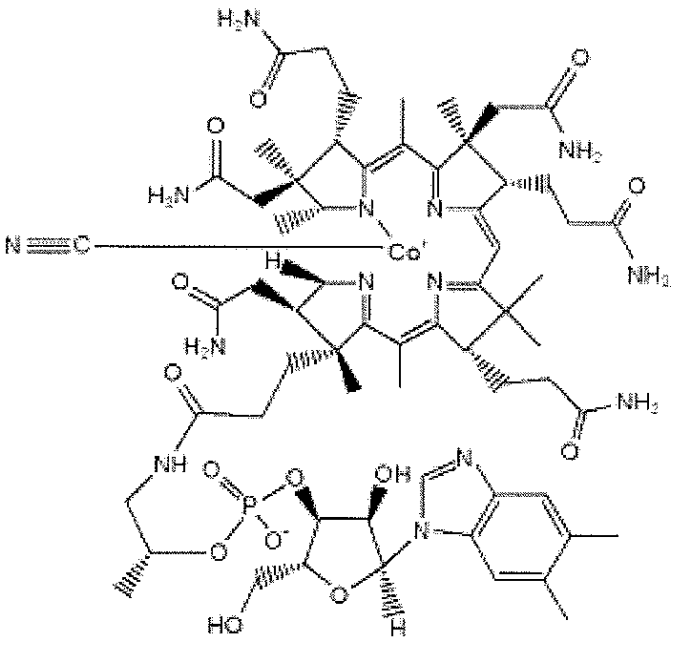


Figure 1.11 Structure of hydroxy-cobalamin

Legend: Co^+ = cobalt

Various forms of homocysteine

As soon as homocysteine is exported from the cell, it will be oxidised to a disulphide with any compound containing a thiol group or undergo a disulphide exchange reaction, both resulting in the formation of homocysteine disulphide. Besides binding to protein, this includes homocysteine itself, cysteine and others. Main disulphides include homocysteine-cysteine disulphide and homocystine (free form) (**Figure 1.12**), and in homocystinuric patients, S-(3-hydroxy-3-carboxy-n-propylthio)-homocysteine and S-(2-hydroxy-3-carboxyethylthio)-homocysteine.

Thiol oxidation and auto-oxidation are extremely complex, and include both enzymatic (mostly governed by non-specific haem-peroxidases) and non-enzymatic reactions, that are further complicated by the involvement of transition metal-ion complexes, nucleophilic attack, thiyl and oxygen radicals. Biological thiols react with molecular oxygen at physiological pH according to the general reaction

$$2RSH + O_2 \rightleftharpoons RSSR + H_2O_2$$

and which is catalysed by copper and cobalt. This is somewhat oversimplified, but the products include the disulphide plus hydrogen peroxide and other reactive oxygen species (De Marco *et al.*, 1971).

The mechanism of oxidation emanates from the concurrent formation of the thiolate anion (RS^-) and the thiyl radical ($RS^\bullet$, from one-electron oxidation). A fundamental property of a biological thiol (being weakly acidic) is the ability of its sulphydryl group hydrogen to dissociate [$RSH \rightleftharpoons RS^- + H^+$]. When the concentration of the conjugate base (RS^-) and the weak acid (RSH) are in equilibrium, the pK_a is equal to the pH of the solution. The pK_a of the sulphydryl group of homocysteine and cysteine are 8.7-10.0 and 8.5 respectively, so that at pH 7.4 < 1% of the free homocysteine and < 10% of the free cysteine molecules are in the thiolate form. Even though the concentration of the homocysteine thiolate anion is low at physiological pH e.g. in culture media, its increased nucleophilicity with respect to the cysteine thiolate anion is likely to result in enhanced thiol/disulphide exchange activity. A similar mechanism occurs with protein albumin (or mercaptalbumin) cysteine residues, where the homocysteine thiolate anion attacks the protein-bound cysteine, resulting in displaced cysteine in favour of homocysteine.

The main conjugative reactions in cellular systems revolve around interactions of these with thiolate anions and molecular oxygen, but can also include reactions with superoxide, hydrogen peroxide and thiol. The steady-state concentration of thiyl radicals however, in comparison to that of thiol or oxygen, mean that reactions of the nature

$$[RS^\bullet + RS^- \rightleftharpoons RSSR^{\bullet-}] \text{ and } [RS^\bullet + O_2 \rightarrow RSOO^\bullet] \text{ predominate over } [RS^\bullet + RS^\bullet \rightleftharpoons RSSR]$$

since thiyl radicals react more rapidly with thiolate anions than with the protonated forms and explaining the pH-dependence of the overall thiol oxidation. Disulphide formation and oxygen consumption both increase with pH in the range 5-9. This dependence of activity coincides with the deprotonation of thiols. The formed disulphide radical anion is relatively stable and a powerful reductant. It reduces molecular oxygen [$RSSR^{\bullet-} + O_2 \rightarrow RSSR + O_2^{\bullet-}$] producing superoxide radicals that disproportionate to molecular oxygen and hydrogen peroxide [$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$].

Homocysteine thiolactone

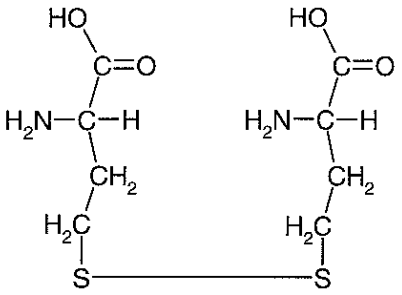
Another form of homocysteine, in addition to those normally encountered, is the intramolecular cyclic thioester homocysteine thiolactone (HCTL) (**Figure 1.12**). Its formation by methionyl-tRNA synthetase (MetRS, EC 6.1.1.10) is the result of a universal error-editing reaction that prevents the translational incorporation of homocysteine into proteins (Jakubowski & Goldman, 1993). All cells (human endothelial, fibroblasts and mouse 3T3 fibroblasts) have the ability to produce HCTL, but especially those that have aberrant transmethylation and/or transsulphuration (CBS and MS deficient, HeLa, human breast tumour and oncogenically transformed mouse RAG), or those that have been exposed to an antifolate drug (aminopterin or MTX).

Because HCTL forms at the active site of MetRS, the rate of synthesis depends on the homocysteine : methionine ratio, with high methionine levels being inhibitory in rodent and human cells. Under normal metabolic conditions therefore, synthesis in human cells is low. The conversion of the endogenous homocysteine to HCTL was only 0.2 and 0.7% in normal and CBS-deficient fibroblasts respectively, increasing to 2.7 and 10% respectively in the presence of aminopterin (Jakubowski, 1997).

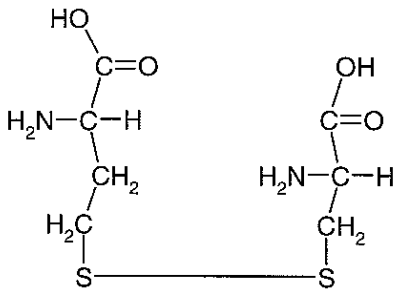
Due to its mostly neutral character at physiological pH (pK = 7.1, Anderson & Packer, 1974), HCTL is rapidly eliminated from cells by diffusion through the cell membrane appearing in the culture medium (Jakubowski, 1999), where its half-life is < 1 hour (Dudman *et al.*, 1991).

Figure 1.12 Structure of homocystine, homocysteine-cysteine disulphide & homocysteine thiolactone

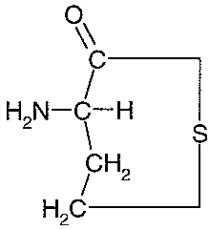
L-Homocystine [$(Hcy)_2$]
(Mw. 268.3)



L-Homocystine-L-cysteine mixed disulphide (I) (Mw. 254.3)



L-Homocysteine thiolactone (HCTL)
(Mw. 135.2)



Sources of homocysteine

The dietary intake of homocysteine in comparison to that of methionine is very small. Due to the nature of its metabolism, assimilation from the point of intestinal absorption to specific cellular uptake is probably redundant. The 40% of the homocysteinyl moiety that is remethylated, cycles between homocysteine and methionine between 1.5-1.9 times in an individual (Mudd & Poole, 1975), and providing there is an adequate supply of methionine in the diet, the homocysteine pool will be constantly replenished.

Distribution

Much information exists on levels of total homocysteine (tHcy)[1] in human plasma and serum in various healthy and disease groups (**Table 1.5**). In contrast, such information is sparse for CSF and urine, as well as for tissues, especially in relation to pathological changes seen in the homocystinurias, although some data on erythrocytes is available. In relation to other tissues, the use of *in vitro* cultures and animal models has provided some information, although its interpretation and extrapolation to human subjects needs care.

Animal studies have shown clear differences in homocysteine levels in various tissues. In mice, plasma concentrations of free and total homocysteine were found to be 1.17 μmol/L (Ueland *et al.*, 1984) and 3.0 μmol/L (Watanabe *et al.*, 1995), and total homocysteine ranged from 0.5-6.0 μmol/kg (wet weight) in both mouse and rat tissues. Liver and kidney contained the highest levels in both animals, with less in brain, heart, lung and spleen (Ueland *et al.*, 1984). The regional distribution of total free homocysteine has also been investigated in the brains of mice, rats, guinea pigs and rabbits. Large interspecies variation was apparent but cerebellum contained the highest amount in all species, the concentrations being 4-10 μmol/kg in the rabbit, 6.4 μmol/kg in the rat and 1.0 μmol/kg in both the mouse and guinea pig. In the rabbit, most of the homocysteine was located specifically in the cerebellar white matter, the total surpassing that of liver. In a similar study, levels of free homocysteine in frozen sections ranged from 5.1 μmol/kg in rat cerebellum to 4.0 μmol/kg in liver, with less found in other tissues.

In human CSF, the level of homocysteine is much lower than that of plasma, but nevertheless shown to be elevated in some cases of CBS deficiency (Surtees *et al.*, 1997), in cancer patients receiving methotrexate (MTX) (Quinn *et al.*, 1997), in patients with fibromyalgia and chronic fatigue syndrome (Regland *et al.*, 1997) and in nonketotic hyperglycaemia (Van Hove *et al.*, 1998). In CBS patients, a mean level of 1.2 μmol/L was detected (control < 0.1 μmol/L) and in cancer patients receiving a seven day treatment of MTX, the average concentration was 0.814 μmol/L (SEM±0.215) (control 0.210 μmol/L {SEM±0.028}). Additionally, in cancer patients on MTX, the concentration of homocysteate or homocysteic acid (quisqualate, HCA), a neurotoxic analogue of glutamate that is normally undetectable in CSF, was found to be 119.1 (SEM±32.0) μmol/L and suggested to be linked to neurotoxicity of MTX.

In the livers of CBS patients, homocysteine levels of 13.0-61.8 μmol/100 g have been reported (Tada *et al.*, 1970).

[1] thiol (including homocysteine) — compound with free -SH group (RSH); homocysteine — thiol (sylphydryl) compound (HcyH); homocystine — symmetrical disulphide (Hcy-Hcy); homocysteine-cysteine mixed disulphide (Hcy-Cys); homocysteine-RSH mixed disulphide (Hcy-SR); Hcy-HcyH & Hcy-SR (i.e. thiol status not specified); free Hcy — non-protein-bound Hcy (fHcy); bound Hcy — protein-bound Hcy (bHcy); total Hcy — fHcy+bHcy (tHcy) (Mudd *et al.*, 2000)

Table 1.5 Homocysteine levels in humans (after Fowler, 2001)

Tissue	Free (μmol/L)	Protein-bound (μmol/L)	Total (μmol/L)	Reference
Plasma				
Normal	1.94 ±0.46		6.18 ±1.19	Araki & Sako, 1987
Normal	(♂) 2.27 ±0.48	(♂) 6.5 ±1.35		Refsum *et al.*, 1985
Normal	(♀) 1.95 ±0.56	(♀) 7.3 ±2.26		"
Normal	2.06 ±0.33		9.0 ±1.1	Andersson *et al.*, 1992
Normal			11 ±2	Suliman *et al.*, 1999
Uremic patients			39 ±10	"
CSF				
Normal			0.46 ±0.13	Hayland & Bottiglieri, 1992
Normal			< 0.1	Surtees *et al.*, 1997
Normal			0.02-0.08 (n = 7)	Van Hove *et al.*, 1998
CBS deficiency			1.18 (0.7-1.99) †	Surtees *et al.*, 1997
NKH			0.10-0.28 (n = 4)	Van Hove *et al.*, 1998
Erythrocytes				
Normal			8.2 ±1.2 (n = 11)	Perna *et al.*, 1995
Normal			0.8 ±1.05	Ubbink *et al.*, 1992
Normal			3.9 ±0.6	Suliman *et al.*, 1999
Uremic patients			32.0 ±6	Perna *et al.*, 1995
Uremic patients			5.6 ±1.9	Suliman *et al.*, 1999
Urine				
Normal			3.5-9.5	Refsum *et al.*, 1985
Normal			7.2 (1.4-24.7) (0.2-3.67) ‡	Stabler *et al.*, 1987 "

† 95% confidence limits; ‡ μmol/L/mmol creatinine; NKH = nonketotic hyperglycinaemia

Protein binding

An important factor in the distribution of homocysteine is its ability to form mixed disulphides with protein cysteine residues. The *in vivo* binding capacity of plasma proteins for homocysteine is not known although reports suggest that it rarely exceeds 150 μmol/L (Mansoor *et al.*, 1993a & 1993b). Levels of plasma free homocysteine (thiol and disulphide forms) and protein-bound have been determined for both men and women and are, 2.27 (SEM±0.11) and 6.51 (SEM±0.32) μmol/L for men and 1.95 (SEM±0.13) and 7.29 (SEM±0.65) μmol/L for women. This equates to a free fraction of 23.5% and a bound fraction 76.5% (Refsum *et al.*, 1985). Similar values were reported by Araki & Sako (1987) who found a total combined free fraction of 1.94 μmol/L (0.23 μmol/L thiol plus 1.71 μmol/L disulphide forms) and a protein-bound fraction of 4.01 μmol/L, i.e. 31.4% free and 64.9% bound.

The extent of protein binding in tissues seems to be less than that in plasma. In rat tissues, bound homocysteine accounted for 40% of the total in liver and 25% in spleen, but only 5% in cerebellum (Svardal *et al.*, 1986). This again reflects the variability of metabolism within the various tissues. Cleavage of disulphide bonds (e.g. transdehydrogenation by glutathione) could provide an ample supply of homocysteine for methylation in times of methionine restriction. The question still remains however, as to whether the free and bound forms of homocysteine are equally available for metabolism.

Homocysteine loading & renal clearance

Administration of homocysteine has been investigated in healthy subjects, in those with HH due to nutritional factors or agents interfering with remethylation and in renal failure. Oral loading studies have shown homocysteine is well absorbed, with an estimated bioavailability of 0.53 (Guttormsen *et al.*, 1993), producing both a transient HH and hypermethioninaemia with peak homocysteine levels 10-fold higher than those observed after an equivalent dose of methionine.

There is no doubt that the function of the kidney in the urinary excretion of tissue derived plasma homocysteine is vital for the maintenance of its homeostasis. Clearance studies in healthy subjects have indicated that intracellularly produced homocysteine is exported into the plasma at a rate of 50 μmol/L/h which is only 5-10% of the total formed. Only 2% of the plasma homocysteine was excreted in the urine unchanged, demonstrating that most homocysteine is metabolised. In patients with moderate HH, Brattstrom *et al.*, (1992) showed that the elimination of homocysteine related to creatinine was significantly increased and strongly correlated to both the plasma concentration, and to levels of folate, vitamin B_6 and B_{12}, and is partly related to renal function. In contrast, Guttormsen *et al.*, (1996) and Refsum *et al.*, (1998), found normal clearance of homocysteine (6.5 ±3.0% unchanged in the urine) in folate and cobalamin deficient subjects, suggesting that elevated homocysteine levels were due to higher tissue export, and that MS function is not an important determinant of homocysteine elimination. In chronic renal failure however, HH was found to be due to reduced clearance (Massy, 1996; Bostom & Culleton, 1999).

Homocysteine disposition in relation to kidney function has also been addressed by several comparative studies of humans and rodents. In normal healthy individuals with a mean fasting total homocysteine of 10.8 μmol/L, no net uptake by the kidney was found (Van Guldener *et al.*, 1998). In rats however, with physiological homocysteine concentrations, 15% of the arterial plasma homocysteine was removed on passage through the kidney, renal uptake was 85% of the filtered load and urinary exretion was < 2%. During acute HH produced by either nitrous oxide (N_2O) inactivation of MS or continuous infusion of homocysteine, renal uptake was increased 3-4-fold respectively, being equivalent to 50% of the infused dose, while urinary excretion remained negligible. This provides strong evidence that the rat kidney has a significant capacity for metabolising acute elevations in plasma homocysteine, the role of remethylation in renal homocysteine metabolism is very limited and that a lack of uptake cannot be the cause of HH in patients with renal failure. The factors responsible for the differences between rats and humans are not clear, although it is known that human but not rat kidney contains BHMT (Finkelstein, 1990).

The underlying pathophysiological mechanism by which renal function is linked to plasma homocysteine remains unclear. The main proposals are either impaired homocysteine disposal at a so far unidentified site in the kidney, or impaired extra-renal metabolism through inhibition of MS or CBS by uremic toxins.

Transport of homocysteine & related compounds

The transport of homocyst(e)ine as thiol, disulphide or as mixed disulphide(s) is critically important to its tissue disposition and together with protein binding, may have a significant impact on the various disease processes pertaining to it. Unfortunately, most *in vitro* studies have used physiologically inappropriate concentrations and/or forms of homocysteine in the examination of its transport. This problem is compounded by its measurement as mainly tHcy after reduction. Thus, the role(s) of the different oxidised and reduced forms have received little attention. The following is a brief review of the current understanding of "homocysteine" transport, with **Table 1.6** providing a summary of the last 14 years of *in vitro* work on its uptake and export.

Hcy	Cell line	Complete medium		Modulation		Effect on Hcy	Reference
		Basal	Serum (v/v)	Agent	Concentration		
Uptake	HUVEC	M199	20 % hiFCS	L-Amino acids & D-serine *p*-Chloromercuribenzene sulphonate	0.5-2.5 mmol/L 0.05-0.10 mmol/L	↓ ↑	1
Export	Murine fibroblast (C3H/10T½ MCA Cl 8 & C3H/10T½ MCA Cl 16*) Murine lymphoma (R 1.1 WT)	BME RPMI-1640	10 % hiFCS 10 % hiFCS	L-Buthionine-(S,R)-sulphoximine Cysteamine 3-Deazaadenosine 3-Deazaaristeromycin	20-50 µmol/L 300 µmol/L 30-300 µmol/L 30-300 µmol/L	- ↑ ↓ ↓	2
"	Human acute mylogenous leukaemia (KG-1a) Human glioma (GaMg) Human lymphocyte Human promylocytic leukaemia (HL-60) HSF: CBS $^{-}$; MS $^{-}$ (cblG & cblE) Rat primary hepatocyte Rat hepatoma (MH1C1) C3H/10T½ MCA Cl 8 C3H/10T½ MCA Cl 16 R 1.1 WT	EMEM EMEM – L-Met RPMI-1640 RPMI-1640 – L-Met	2.5 % FCS 10 % FCS 10 % hiFCS 10 % hiFCS	Folic acid L-Met 5-MeTHF Nitrous oxide	2.3 µmol/L 15-1000 µmol/L 3-2300 µmol/L + or –	↓ ↑ ↓ ↑	3
"	C3H/10T½ MCA Cl 8 C3H/10T½ MCA Cl 16 R 1.1 WT	BME	10 % hiFCS	5-Formyl-THF Methotrexate 5-MeTHF	Up to 10 µmol/L Up to 0.03 µmol/L Up to 10 µmol/L	↓ ↑ ↓	4
"	Human glioma (GaMg)	DMEM	10 % hiFCS	Methotrexate Nitrous oxide	Up to 1 µmol/L + or –	↑ ↑	5
"	HUVEC: normal; CBS $^{+}$ & CBS $^{-}$	M199	10 % HS + 10 % NCS	Vitamin B_{12} (as CH_3Cbl) Vitamin B_6 (as PLP) Flavin adenine dinucleotide Folic acid Folinic acid L-Met 5-MeTHF	Up to 100 µmol/L Up to 100 µmol/L Up to 0.75 µmol/L Up to 10 µmol/L Up to 1 µmol/L Up to 200 µmol/L Up to 1 µmol/L	- - - ↓ ↓ ↑ ↓	6
"	Endothelial (ECV 304); HeLa; HepG2	RPMI-1640	10 % FCS	Copper ions FCS	1-100 µmol/L Up to 10 % (v/v)	↑ ↑	7
"	Rat hepatocyte	Williams' E	10 % FCS	Hydrazine	4-12 mmol/L	↑	8
"	HRPTEC	EMEM	10 % hiFCS	Cyclosporine A L-Met	200 or 800 ng/ml High & low	- ↑	9

HUVEC = human umbilical vein endothelial cells; * = chemically transformed; HSF = human skin fibroblasts; HRPTEC = human renal proximal tubule endothelial cells; hi = heat-inactivated; HS = human serum; NCS = new-born calf serum; **1**= Edwadh *et al.*, 1990; **2** = Djurhuus *et al.*, 1989 & 1990; **3** = Christensen *et al.*, 1991-1994; **4** = Refsum *et al.*, 1991; **5** = Fiskerstrand *et al.*, 1994, 1997a & 1997b; **6** = van der Molen *et al.*, 1996 & 1997; **7** = Hultberg *et al.*, 1998a & 1998c; **8** = Kenyon *et al.*, 1999; **9** = Ignatescu *et al.*, 2001

Uptake

Neurological receptors - NMDA & GABA

Some of the principal studies involved the action of L-, D- and DL-HCA, with the concentration range of 0.62 to 2.5 mmol/L, on rat brain slices and cortex cells. Uptake was found to be stereoselective and coupled with increased influx of calcium into the non-inulin space (Cox *et al.*, 1976 & 1977; Berdichevsky *et al.*, 1983).

Further studies on various neuronal tissues involved a series of sulphur containing amino acids (SAAs) including L- and D-HCA, L- and D-homocysteine sulphinate and L-homocysteine in receptor binding assays designed to specifically label, (i) the AA1/N-methyl-D-aspartate (NMDA), AA2/homocysteic acid and AA3/kainate receptor recognition sites, (ii) a calcium chloride-dependent site, and/or (iii) a glutamate uptake site. Of these, L-HCA and D-homocysteine sulphinate had affinities most selective for the NMDA binding site, causing excitotoxicity by sodium efflux (Pullan *et al.*, 1987), a phenomenon which has also been shown with L-HCA with NMDA receptors at the Schaffer collateral-CA1 synapse in rat hyppocampus (Ito *et al.*, 1991) and dissociated cortical neurons from embryonic Wistar rats (Gortz *et al.*, 2004). In conjunction, the D-enantiomers of SAAs with shorter or equal chain length to glutamate were found to be particularly effective at completely inhibiting its vesicular uptake (Dunlop *et al.*, 1991a). Recent findings have confirmed that homocysteine also interacts with NMDA receptors in vascular smooth muscle cells (VSMC) and related its action to a signal cascade involving phospholipids and protein kinase C (Dalton *et al.*, 1997). Whether these interactions play a specific role in signal transduction mechanisms remains to be established. It has been suggested that because of the endogenous nature of L-HCA, it could function as a neurotransmitter at the NMDA receptor (Olney *et al.*, 1987).

Derivatives of homocysteine have been shown to selectively inhibit the import of taurine and γ-aminobutyrate (GABA) into synaptosomes, cultured neurons and astrocytes. Both L-homocysteine and L-homocystine prevented high affinity uptake of taurine into synaptosomes and astrocytes, but only L-homocystine inhibited GABA uptake into astrocytes, unless the concentration exceeded 5 mmol/L (Allen *et al.*, 1986). It has been subsequently shown that L-homocysteine sulphinate and L-HCA cause release of GABA through two distinct mechanisms (receptor-mediated and depolarisation-induced) comprising first activation of NMDA receptors, followed by a calcium-dependent component (Dunlop *et al.*, 1991b).

Direct toxicity

Many studies investigating the toxic effects of homocysteine have provided indirect evidence of uptake, e.g. inhibition of muscimol binding to calf synaptic membranes (Griffiths *et al.*, 1983), seizures in rats (Kubova *et al.*, 1995), alteration in neural crest and neural tube development in chicken embryos (Rosenquist *et al.*, 1999), and genotoxicity in various tissues including liver stellate, VSMC (Garcia-Tevijano *et al.*, 2001) and lymphocytes (Crott & Fenech, 2001).

Studies on DL-homocysteine by Lewis *et al.*, and Taguchi & Chanarin (1978), demonstrated its ability to inhibit [^{3}H]-L-histidine uptake in S37 ascites tumour cells, and to reduce the uptake of both [^{3}H]-deoxyuridine and [^{3}H]-thymidine in human normoblastic and megaloblastic bone marrow cells, though this second finding could be attributed to membrane damage.

Large decreases in cellular NAD^+ levels occurred in response to homocysteine toxicity to human umbilical vein endothelial cells (HUVEC). This was accompanied by the production of single-stranded DNA and a lack of deoxyuridine uptake (Blundell *et al.*, 1996).

Wang *et al.*, (1997) showed inhibition of DNA synthesis in vascular endothelial cells with specific alterations at the level of carboxylation of p21ras. The concentration of homocysteine used was 10-50 μmol/L, implying uptake into cells at levels seen in patients with vascular disease.

Lash & Anders (1989) used isolated rat renal proximal tubules to show S-conjugates could be nephrotoxic, with 30% of S-(1,2-dichlorovinyl)-L-homocysteine uptake being sodium-dependent. The probenicid-sensitive organic anion transport system (sodium-dependent) and system L (sodium-independent)

were both responsible, although system L played a greater role in S-(1,2-dichlorovinyl)-L-homocysteine uptake.

Direct apoptosis has been demonstrated by homocysteine sulphinic acid and HCA in primary cultured rat immature (embryonic) neurons. Because these cells do not express the receptor channel, both derivatives were thought to cause cell death by inhibiting cystine uptake, leading to glutathione depletion, an effect increased by N-acetylcysteine but exacerbated by the γ-glutamyl-cysteine synthetase inhibitor butathionine sulphoxamine (BSO) (Tsukamoto, 1998). Homocysteine has been found to cause lysis of erythrocytes at concentrations which are much higher than those seen in mild-moderate HH (15-30 μmol/L), and then only in the presence of activated polymorphnuclear leucocytes (PMNL) and when the ratio of PMNL : erythrocytes is sufficiently high (Olinescu *et al.*, 1996).

Amino acid transporters

Direct cellular entry of [^{35}S]-L-homocystine has been demonstrated by high and low affinity systems in isolated rat renal cortical tubules. Arginine, lysine and cystine inhibited homocystine uptake by the high affinity (K_m 0.17 mmol/L) system, but had little or no effect on the low affinity (K_m 7.65 mmol/L) system. Homocystine prevented entry of arginine, lysine and cystine, but only cystine with both systems, suggesting that the high affinity system is shared with cystine and the dibasic amino acids. Imported homocystine was mainly converted to transsulphuration metabolites, predominantly cystathionine (Foreman *et al.*, 1982). In fibroblasts, L-HCA caused a potent reduction in intracellular and extracellular cysteine levels, instigated by competitive interaction with a transporter which is convincingly similar to the ASC/L system described by Christensen *et al.*, (1967) (Bannai & Ishii, 1982).

"Homocysteine (redox) receptor"

Having shown that homocystine induces both cell proliferation and collagen expression in a dose- and time-dependent manner in human VSMC, Tyagi (1998) went on to investigate the possibility of a "homocysteine (redox) receptor". Sodium dodecyl sulphate-polyacrylamide gel electrophoresis fluorography identified a 25-40 kDa membrane protein, to which a fluorescamine-primary amine labelled homocystine conjugate bound and subsequently appeared in the cytosol. N-acetylcysteine and homocystine, but not homocysteine disrupted binding, suggesting a disulphide nature in the interaction between homocystine and receptor.

In summary

The emerging picture of homocysteine uptake, concluded on the inhibitory effects of a number of amino acids, seems to be through, (i) uptake of HCA by the NMDA-glutamate-gated calcium ion channel receptors for neuronal and neuronal associated VSMC, and (ii) uptake of the L- and D-enantiomers of homocysteine and homocystine by the ASC/L-sodium-dependent-independent system for endothelial, fibroblast and renal cells.

Export

The concept of homocysteine export was established by Ueland *et al.*, (1984), although the actual molecular mechanism remains to be fully elucidated. Their observations on the levels of homocysteine in the tissues of mice and rats in comparison to those in the plasma (see page 20), led them to postulate that export exists to ensure low intracellular levels which may be critical for maintaining vital cellular functions. Extracellular homocysteine is therefore likely to be a measure of the balance between homocysteine formation and utilisation. Additional studies, which have predominantly involved the use of cultured cells in a variety of

conditions (**Table 1.6**), have helped to further characterise the nature of the export process. The main features are summarised as follows and described in more detail under the respective headings.

① Malignant (L-Met-dependent) vs. non-malignant cells —effect of methotrexate on remethylation
Homocysteine export is MTX-dependent and probably reflects an intracellular deficiency of 5-MeTHF required for remethylation.

—effect of methionine supplementation on homocysteine export
Homocysteine export is likely to be a function of intracellular concentration, although a direct enhancement by methionine could not be discounted.

—effect of nitrous oxide on remethylation
Depending on the cell type, exposure to nitrous oxide increases homocysteine export through inactivation of MS.

② Studies in CBS-deficient & normal cells
CBS-deficient endothelial cells have normal endothelial function and homocysteine export, and the high plasma homocysteine levels seen in CBS-deficient patients thought to cause endothelial damage, are probably due to export from the liver and kidney.

③ Influence of copper ions & FCS on homocysteine re-usage
Endothelial cells have a lower re-usage requirement for exported homocysteine, whereas HeLa cells have a higher re-usage requirement and therefore appear to export less homocysteine. Copper ions increase homocysteine export from HeLa cells. A positive correlation exists between FCS levels and homocysteine export.

④ AdoHcy hydrolase inhibitors
Inhibition of the catabolism of AdoHcy causes a decrease in homocysteine export.

⑤ Links to glutathione
Homocysteine causes an increase in glutathione levels.

⑥ Links to nitric oxide - explaining hydrazine toxicity
Reducing agent hydrazine increases homocysteine export probably through inhibition of MS.

Malignant (L-Met-dependent) vs. non-malignant cells —effect of methotrexate on remethylation

The disposition of endogenous homocysteine was investigated in both non-malignant and transformed mouse fibroblasts after MTX exposure by Ueland *et al.*, (1986). Both cell lines exported homocysteine into the extracellular medium with only small amounts being retained within the cells. Homocysteine efflux from the malignant cells was markedly increased after MTX (0.5-10 μmol/L) exposure, but not in the presence of the MTX "rescue" agent folinic acid (a stable precursor of 5-MeTHF). Furthermore, increased homocysteine export was not associated with the intracellular build up of AdoHcy, indicating that the level of cellular homocysteine was kept below the level required for inhibition or reversal of the AdoHcy hydrolase reaction. In contrast, high concentrations (> 10 μmol/L) of MTX were required to increase homocysteine efflux from non-malignant cells. MTX-dependent homocysteine export probably reflects an intracellular deficiency of 5-MeTHF required for remethylation.

This work was expanded by Refsum *et al.*, (1991) and Fiskerstrand *et al.*, (1997), to include murine lymphoma cells and two variants of a human glioma cell line, additionally exposed to N_2O which irreversibly inactivates MS through cob(I)alamin. Folinic acid or 5-MeTHF added to the fibroblasts not exposed to MTX

reduced homocysteine egress, whereas it was unaffected with the lymphoma cells. In contrast to that reported by Ueland *et al.*, (1986), all cells exposed to MTX were rescued by thymidine and hypoxanthine. In fibroblasts, non-toxic and toxic conditions of MTX in the presence of thymidine and hypoxanthine enhanced homocysteine export. MTX did not increase homocysteine egress from lymphoma cells, and in the absence of rescue, it declined in proportion to the inhibition of cell growth. With the glioma cells, after a 3 hour exposure to concentrations of MTX (+ thymidine + hypoxanthine) up to 1 μmol/L, 5-MeTHF was reduced by 50%, with the consequence that MS activity and the level of cellular methylcobalamin declined, and homocysteine export increased. Additionally, with the glioma cells, the effect of MTX on methionine salvage and methylcobalamin levels was more pronounced than that of N_2O.

—effect of methionine supplementation on homocysteine export

Supraphysiological levels of methionine (15-1000 μmol/L) have also been investigated in relation to their enhancement of homocysteine export from proliferating, quiescent, non-transformed and transformed cells. Homocysteine export from growing cells was greatest during the early to mid-exponential phase and then decreased as a function of cell density, probably due to a higher intracellular metabolism. The export rate was higher with phytohaemagglutanin-stimulated than non-stimulated lymphocytes, and with growing compared with fibroblasts at confluence, but highest from hepatocytes. The effect of methionine on homocysteine export ranged from no stimulation to marked enhancement, depending on the cell type investigated, with three different response patterns being distinguished. These were as follows, (i) little or no increase with quiescent fibroblasts, growing murine lymphoma cells and human lymphocytes, (ii) a 3-8-fold increase in proliferating hepatoma cells and benign and transformed fibroblasts, and (iii) a 15-fold increase in non-transformed primary hepatocytes in stationary culture. This study concluded that homocysteine export was likely to be a function of intracellular concentration, although a direct enhancement by methionine could not be discounted (Christensen *et al.*, 1991).

—effect of nitrous oxide on remethylation

Christensen *et al.*, (1992) also investigated homocysteine remethylation during N_2O exposure, using two murine (fibroblast and T-lymphoma R 1.1 WT) and four human (two glioma GaMg, promyelocytic leukaemia HL-60 and acute myelogenous leukaemia KG-1a) cell lines grown in media containing various concentrations of folates. When cultured in a standard medium (2.3 μmol/L folic acid), MS was inactivated by 55-85% in each cell line. The rate and extent of this inactivation were markedly reduced when the cells were transferred and cultured in a medium containing a low level (10 nmol/L) of 5-MeTHF. If 5-MeTHF was increased to the original concentration of folic acid however, this stimulation of MS was reversed. MS inactivation was associated with a marked enhancement of homocysteine export from the murine fibroblasts, and a moderate increase from the two human glioma cell lines. In contrast, in the three leukaemic cell lines, the homocysteine export was unaffected by N_2O exposure indicating that inhibition of MS does not lead to higher homocysteine production.

Similar studies on normal and CBS-deficient fibroblasts in medium containing from physiological to very high concentrations (15-100 μmol/L) of methionine (conditions favouring conservation or catabolism) have highlighted the importance of MS and CBS activity on homocysteine export. In the normal cells, both the rate and extent of MS inactivation were reduced by increasing methionine concentrations in the culture medium. In cells not exposed to N_2O, methionine increased homocysteine export in a dose-dependent manner, but neither N_2O nor methionine significantly affected the amount of AdoMet or folate in these cells. In the CBS-deficient cells, high medium methionine partly protected MS as previously found in the normal fibroblasts. The homocysteine export rate was 0.2-0.6 nmol/h/10^6 cells at low methionine levels but increased 2-3-fold at high methionine levels. As with the normal cells, N_2O increased the export at low methionine concentrations, so that it was essentially independent of the extracellular level of methionine (Christensen & Ueland, 1993; Christensen *et al.*, 1994).

Studies in CBS-deficient & normal cells

Homocysteine export has also been studied in normal and CBS-deficient HUVEC. Under standard culture conditions tHcy concentrations increased incrementally after 24, 48 and 72 hours (approximately 2.5 μmol/L homocysteine every 24 hours). Several markers of endothelial function e.g. von Willebrand factor, tissue plasminogen activator and its inhibitor also increased with the progression of time. Folic acid supplementation reduced homocysteine export in a dose-dependent manner, and this effect was 10-fold greater with 5-MeTHF and folinic acid. A 50% reduction in homocysteine export was seen with 10-30 nmol/L 5-MeTHF and this fell to almost zero with 100-300 nmol/L. The addition of vitamin B_6, B_{12} or flavin adenine dinucleotide had no effect on export. The similarity in the results obtained with both normal and CBS-deficient cells showed that the latter had normal endothelial function and homocysteine export, and that the very high plasma homocysteine levels seen in CBS-deficient patients that are thought to cause endothelial damage, are not native to endothelium, but probably arise from export from tissues such as the liver and kidney (van der Molen *et al.*, 1996 & 1997).

Homocysteine re-usage, copper ions & FCS

The ratio between intracellular and extracellular homocysteine is at least in part a measure of the net flux across a cell membrane. This not only includes the ability of a cell to export homocysteine, but also its ability to reuse that which reaches the surrounding medium. It has been shown that the intracellular homocysteine concentration of endothelial cells is more influenced by the external level than that from intracellular production. In a comparison between endothelial, HeLa and hepatoma cell lines under basal conditions, endothelial cells exported more homocysteine, but more importantly, had a lower re-usage requirement for that released. HeLa cells on the other hand, exported homocysteine continuously, a process that was increased by copper ions, but exhibited a higher re-usage requirement, the net flux being therefore lower. Additionally, a positive correlation was demonstrated between the amount of FCS (0-10%) in the culture medium and the total level of external homocysteine (Hultberg *et al.*, 1998a & 1998c).

AdoHcy hydrolase inhibitiors

Homocysteine export has been shown to be inhibited by 3-deazaaristeromycin and D-eritadenine (both potent inhibitors of AdoHcy hydrolase) in a manner that closely parallels the inhibitory effect on AdoHcy catabolism suggesting that homocysteine export may be coupled to its formation from AdoHcy. In isolated rat hepatocytes, the accumulation of AdoHcy exceeded the inhibition of homocysteine export, whereas in the non-transformed and malignant mouse embryo fibroblasts, the accumulation of AdoHcy equalled the inhibition of homocysteine export. Inhibition of AdoHcy catabolism was associated with an increase in both the free- and protein-bound homocysteine in the hepatocytes, whereas depletion of intracellular homocysteine occurred in the fibroblasts. Conclusions were, that homocysteine is exported into the extracellular medium in proportion to its formation from AdoHcy, and that two pools (exportable and a greater non-exportable) of homocysteine exist in liver cells (Svardal *et al.*, 1986).

Links to glutathione

Examining the link between homocysteine metabolism and glutathione synthesis (see pages 28 & 29) has also provided insight into the export mechanism. Homocysteine has been shown to increase glutathione levels in confluent murine embryo fibroblasts (Djurhuus *et al.*, 1989), an effect that was greater with several other thiols and disulphides including cysteamine, mercaptoethanol and dithioerythritol in dividing cells. However, in non-dividing cells this effect was only seen with homocysteine. The possible role of homocysteine and other thiols in mediating glutathione levels raises the question whether glutathione is in some way regulating homocysteine. This was examined by depleting cellular glutathione to approximately 5% of controls with 20 μmol/L BSO, an irreversible inhibitor of γ-glutamyl-cysteine synthetase, and measuring homocysteine

export from non-transformed murine fibroblasts. During logarithmic growth, fibroblasts showed decreased but similar export to untreated cells which indicated that glutathione depletion did not influence homocysteine formation, and BSO did not interfere with AdoMet-dependent transmethylation, since homocysteine formation and export are a measure of overall transmethylation in extrahepatic tissue (Iizasa & Carson, 1985). It was concluded that homocysteine does regulate glutathione, albeit unspecifically, by a mechanism that is still unknown (Djurhuus *et al.,* 1990).

Links to nitric oxide - explaining hydrazine toxicity

The reducing agent hydrazine (4-12 mmol/L) has been shown to affect both MS and the levels of homocysteine, cysteine, taurine and glutathione in isolated rat hepatocyte suspensions and monolayers. Incubation with hydrazine up to a concentration of 12 mmol/l showed toxicity (measured by lactate dehydrogenase leakage) only after 24 hours. MS activity was reduced by 45-55% by 8-12 mmol/L hydrazine after 3 hours in cells in suspension, and by 65-80% by 12 mmol/L hydrazine after 24 hours in cell-monolayers. MS inactivation was not due to nitric oxide (NO) production, since the NO synthase inhibitor Nomega-nitro-L-arginine failed to protect against both hydrazine-induced ATP and glutathione loss, and a reduction in urea synthesis at 24 hours. In this system hydrazine led to increased homocysteine export at a concentration of 6 mmol/L, whereas total taurine content was increased at 12 mmol/L. Thus, hydrazine was found to have several important and possibly deleterious effects on certain parts of the sulphur amino acid pathway (Kenyon *et al.*, 1999).

Homocysteine & redox status

Ueland *et al.,* (1996) summarised current knowledge of this aspect as follows, reduced, oxidised and protein-bound forms of plasma homocysteine, cysteine and cysteinyl-glycine (see page 29) interact through redox and disulphide exchange reactions, comprising a dynamic system referred to as the redox thiol status. Increased homocysteine export results in changes in this status by increased protein binding of homocysteine in favour of cysteine. When binding reaches saturation, continued export further increases the ratio of reduced : total homocysteine that in turn causes parallel changes in the proportionate balance of the other aminothiols. These dynamics have been observed in both chronic HH due to cobalamin deficiency and acute HH induced by methionine or homocysteine loading. In addition, altered status has been seen in patients with vascular disease (low reduced : total for cysteine), renal failure (low reduced : total for aminothiols) and HIV infection (high reduced homocysteine), which suggests a primary imbalance in the pro-oxidant and antioxidant processes in these patients. Due to the importance of extracellular antioxidant defence to both the redox thiol status and the processes involved in the development of vascular disease, no discussion would be complete without first mentioning glutathione.

Glutathione - form & function

Reduced glutathione (γ-glutamyl-cysteinylglycine, GSH) (**Figure 1.13**) is a ubiquitous and mainly cytosolic molecule that functions as both an antioxidant, antitoxin and enzyme cofactor, as well as having a regulatory effect on the synthesis of AdoMet in the liver and possibly central nervous system (Castagna *et al.,* 1995). In normal human plasma its mean concentration was found to be 4.5 (SEM±0.7) μmol/L (Hagenfeldt *et al.*, 1978) and in tissues between 0.1-10 mmol/L (Kosower, 1976). Tissue levels were highest in liver (up to 10 mmol/L), followed by spleen, kidney, lens (3.5 mmol/L at age 20 with lower levels of 1.75 mmol/L at age 65, Harding, 1970), erythrocytes (2.1-2.72 mmol/L) and leucocytes, but undetectable in urine. Glutathione is

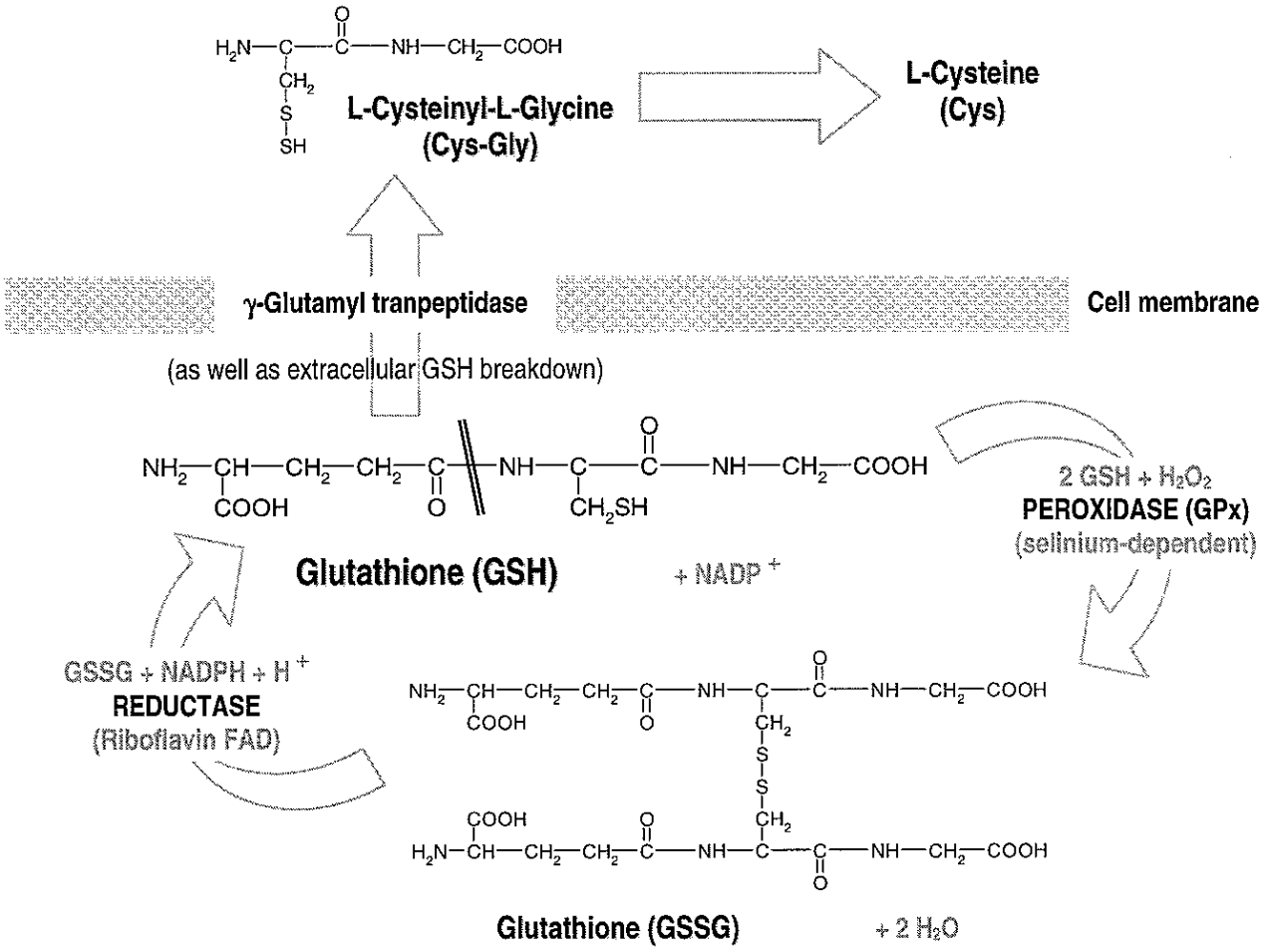
present at 100-fold higher concentrations than its oxidised form glutathione disulphide (GSSG) in tissues, including erythrocytes.

Elevated intracellular concentrations combined with a potent electron-donating capacity characterise its strong antioxidant action and enzyme cofactor properties, and regulate cell activity through a complex thiol-exchange system. Its function include the synthesis and repair of DNA and proteins, lymphocyte replication and function etc., as well as serving as the γ-glutamyl donor for cyst(e)ine, glutamine, methionine and other neutral amino acid transport.

Glutathione synthesis involves two closely linked enzymatic reactions that utilise ATP and certain non-essential amino acids as substrates. First, cysteine and glutamate are combined by γ-glutamyl-cysteinyl synthetase and second, glutathione synthetase combines γ-glutamyl-cysteine with glycine to generate glutathione. Synthesis is controlled by feedback inhibition and otherwise cysteine levels are rate-limiting. Glutathione recycling is catalysed by glutathione reductase, which uses reducing equivalents from NADPH to reconvert GSSG to two molecules of GSH. Inherited enzyme defects of the γ-glutamyl cycle have been described and include glutathione synthetase, γ-glutamyl-cysteine synthetase, γ-glutamyl transpeptidase, 5-oxoprolinase and cysteinyl-glycinase deficiencies (Griffith, 1999).

Glutathione status is a highly sensitive indicator of cell function and viability, with progressive intracellular loss leading to cell death. In humans, glutathione depletion has been linked to a number of disease states including atherosclerosis, cancer, liver cirrhosis, diabetes, pulmonary disease (idiopathic fibrosis and respiratory stress syndrome), metal storage disorders (Wilson's disease), HIV infection, Crohn's, Parkinson's and Alzheimer's disease. Conditions similar to those associated with moderate HH.

Figure 1.13 Relationship between GSH, GSSG, cysteinyl-glycine & cysteine



The link between homocysteine & cardiovascular disease - mechanisms of action

Accumulating evidence suggests that mild to moderate HH leads to the development and progression of CVD. Though countless studies have investigated its damaging action, no one single mechanism has yet been identified. This is not really surprising, as homocysteine has been shown to have a number of diverse effects ranging from altering gene expression to its modification of large macromolecules. The main mechanisms though, appear to involve endothelial dysfunction and peroxidation of low-density lipoprotein (LDL) by free radicals generated during its oxidation. Homocysteine also stimulates the proliferation of VSMC and inhibits the growth of vascular endothelial cells. Elevated levels may also promote thrombosis by increased generation of thrombin. Other possible mechanisms for homocysteine-mediated atherogenesis include altered DNA methylation and regulatory membrane proteins, decreased bio-availability of nitric oxide, increased elastolysis and collagen accumulation, over-stimulation of NMDA receptors and excessive adhesion of monocytes and neutrophils to endothelium. As the starting point for these adverse effects seems to be hydrogen peroxide, emphasis will be given to this and the association of homocysteine with GSH.

Thiol compounds, metal ions & low-density lipoprotein peroxidation

Metal ions are known to exhibit high-affinities for thiol groups and may severely disturb the metabolic function of cells. Starkebaum & Harlan (1986) first showed in a cell-free system that oxygen-dependent oxidation of homocysteine (500 μmol/L) generated hydrogen peroxide. This was catalysed by copper ions (1-50 μmol/L), ceruloplasmin and normal human serum in a dose dependent manner. Copper ions have also been shown to increase protein-binding of thiol compounds, an effect that was greater with homocysteine than with other thiol compounds, increase homocysteine export by possibly interacting with AdoHcy hydrolase, and unlike cadmium, mercury and silver ions, reduce intracellular cysteine, inhibiting GSH synthesis and release (Hultberg *et al.,* 1998a, 1998b & 1998c). The main distinguishing characteristic of CVD is atherosclerosis or the accumulation of macrophages/monocytes (mϕ), VSMC and LDL in the intima of the arterial wall (Ross, 1986). Homocysteine has been shown to induce over-expression of the gene controlling MCP-1 (a potent chemokine that stimulates mϕ migration) by altering their intracellular redox status (Zhang *et al.,* 2003). In addition, LDL peroxidation by lipoxygenases in endothelial cells, mϕ, fibroblasts and VSMC, as well as by thiol compounds by free radical generation catalysed by transition metals, leads to its recognition by scavenger receptors (acetyl-LDL receptors) and increased mϕ and VSMC uptake. Since VSMC oxidise LDL in a superoxide ($O_2^{\bullet -}$)-dependent mechanism that requires cysteine and a redox-active transition metal, Heinecke *et al.,* (1993) investigated whether a similar mechanism leads to cholesterol accumulation in macrophages. In a cell-free system, L-cysteine, L- & D-homocysteine and GSH all modified LDL in a process that required either copper or oxidised insoluble trivalent ferric ($iron^{3+}$) ions. Superoxide dismutase (SOD) inhibited modification of LDL by cysteine-copper, but had little effect on homocysteine- or glutathione-copper modification. Moreover, only cysteine-copper modified LDL was degraded by human monocyte-derived macrophages, a process blocked by SOD and therefore involving $O_2^{\bullet -}$ but not peroxide or the hydroxyl radical. This suggests that the VSMC and macrophage oxidising mechanisms are indeed similar, and that thiols also promote lipid peroxidation by $O_2(^{\bullet -})$-independent mechanisms. Thiol-induced LDL oxidation has also been investigated *in vitro* by Lynch & Frei (1997). Cysteine (25 μmol/L), DL-homocysteine (1 mmol/L) and GSH (1 mmol/L) inhibited copper-dependent, but facilitated $iron^{3+}$-dependent oxidation of LDL in a dose-dependent manner. Inhibition of copper-dependent oxidation was greatest with GSH and lowest with cysteine, with the sequence reversed for $iron^{3+}$-dependent oxidation. Cystine and homocystine were found to have no effect, but both GSSG (0.5 mmol/L) and methionine (1 mmol/L) inhibited copper-dependent oxidation but had no effect on $iron^{3+}$-dependent oxidation. This suggests that while $iron^{3+}$-dependent oxidation requires a free (reduced) thiol group, copper-dependent oxidation is thiol-independent. Increased lipid peroxidation and atherosclerosis have been demonstrated in rabbits fed high methionine diets. Supplementation for 6-9 months resulted in significant increases in plasma and aortic thiobarbituric acid reactive substance levels and aortic antioxidant

enzyme activities, although plasma antioxidant activity was decreased. In erythrocytes, SOD activity increased, catalase remained normal and glutathione peroxidase (GPx) decreased. Histological examination of aortas showed typical atherosclerotic changes, such as intimal thickening, deposition of cholesterol and calcification (Toborek *et al.*, 1995).

Mitochondrial alterations caused by homocysteine

Homocysteine (1 mmol/L) in the presence of copper ions (4 μmol/L) has been shown to significantly decrease mitochondrial RNA levels, cause gross morphological changes in mitochondrial ultra-structure and inhibit both cell growth and mitochondrial respiration. These could be prevented however, by precursors of GSH, GSH itself, pre-exposure to heat shock proteins or catalase, but not free radical scavengers. It was concluded that homocysteine and hydrogen peroxide act synergistically to cause mitochondrial damage (Austin *et al.*, 1998).

Thiol/S-nitosothiol levels & nitric oxide

Nitrosation of homocysteine prevents the generation of hydrogen peroxide. Stimulation of endothelial cells to produce NO therefore, should also detoxify homocysteine by forming the S-nitroso-thiol (RSNO), S-nitroso-homocysteine. In studies by Upchurch *et al.*, (1997), bovine aortic endothelial cells (BAEC) were incubated with cysteine, homocysteine or GSH (0-5 mmol/L) in the presence of arginine (1 mmol/L) and the NO agonist bradykinin (1 μmol/L). Results demonstrated that only homocysteine was able to increase RSNO in a time- and dose-dependent manner. Northern blot analysis also showed concomitant increases in endothelial nitric oxide synthase (eNOS) transcription by 58% and eNOS activity by 78%. Pre-incubation with homocysteine followed by bradykinin showed similar increases, suggesting *de novo* synthesis had occurred. Further studies lead to the discovery that a dose-dependent decrease in nitrite-plus-nitrate (NOx) concentrations took place during homocysteine treatment of BAEC. This was independent of eNOS transcription, protein levels and eNOS activity, suggesting homocysteine affects NO bioavailability but not production. In parallel, homocysteine (250 μmol/L) was additionally shown to impair the activity of the intracellular isoform of GPx by 81%. Taken together, these findings may represent a novel mechanism whereby homocysteine renders endothelial-derived NO more susceptible to oxidative inactivation through impairment of intracellular antioxidant enzymes. Homocysteine has been shown to also increase NO synthesis (measured as nitrite) in IL-1β-induced VSMC in a dose-dependent manner. Cysteine, GSH and hydrogen peroxide also increased nitrite accumulation, but co-incubation with catalase or SOD markedly reduced homocysteine-induced nitrite accumulation (Ikeda *et al.*, 1999). Levels of N-dansyl-S-nitroso-homocysteine (NDSN-homocysteine), a fluorescent probe of intracellular thiols/S-nitrosothiols, were directly proportional to intracellular GSH levels in fibroblasts. Cells preloaded with NDSN-homocysteine were also sensitive to S-nitroso-GSH uptake as intracellular fluorescence decreased as a function of time upon exposure to extracellular S-nitroso-GSH (Ramachandran *et al.*, 1999).

Effect of homocysteine on GSH synthesis

Homocysteine was found to be weakly mitogenic in VSMC by interruption of their GSH synthesis. Although concentrations used were high (250-500 μmol/L), even in relation to CBS deficiency, homocysteine caused decreased cytosolic GPx activity and enhanced SOD activity, but had no effect on catalase (Nishio & Watanabe, 1997). In addition to its unspecific regulation of glutathione synthesis, homocysteine and methionine unlike cystathionine, could not replace cysteine in the synthesis of GSH in astroglial cultures (Kranich *et al.*, 1998). In isolated hepatocytes, cystathionine, methionine, the thioether analogues of methionine, cystine and homocysteine, together with the S-adenosyl derivatives, all inhibited reduced GSH efflux. The thioethers were found to interact in a competitive and specific fashion with the sinusoidal GSH transporter, without themselves being transported in the process (Fernandez-Checa *et al.*, 1996).

AdoMet & AdoHcy

Distribution

The content of AdoMet in mammalian tissues is dependent on the organ in question and the availability of methionine. Levels of AdoMet and Adohcy have been determined in many tissues from a variety of species, though most extensively in the rat (**Table 1.7**). Rats fed a standard diet containing 0.3% methionine had liver AdoMet and AdoHcy concentrations of 141 ±27 nmol/g and 20 ±27 nmol/g respectively. In contrast to AdoHcy, the level of AdoMet remained unchanged with increased dietary methionine up to 2.0%. Only when methionine intake increased to 3.0% was a 5-fold increase in AdoMet observed (Finkelstein & Martin, 1986).

At least two distinct metabolic pools of AdoMet have been identified in rat liver. Using selective labelling with [methyl-^{14}C]-methionine and [methyl-^{3}H]-methionine over long and short periods, both a stable and labile pool were distinguished. Subsequent subcellular fractionation revealed that the "labile" pool existed in the cytosol and the "stable" pool in the mitochondria (Farooqui *et al.*, 1983). As mitochondria lack MAT, intracellular segregation of AdoMet also supports the assumption that some form of uptake must occur across the mitochondrial membrane. In fact 30% of the total hepatic AdoMet resides in the mitochondria and it has been susequently shown that both methyl group incorporation into phospholipids and carrier-mediated upake do occur (Horne *et al.*, 1997).

Transport & uptake

The finding that AdoMet exists as complexes with proteins lead Svardal & Ueland (1987) to examine whether this was also true for AdoHcy. Not surprisingly it was shown that 30-50% of AdoHcy in rat hepatocytes is associated with proteins, and under physiological conditions this is restricted to the microsomal fraction, with free AdoHcy being cytosolic. Additionally, AdoHcy binding was found to be saturable, with both the free and bound forms representing kinetically distinct pools.

At physiological concentrations, AdoMet seems to be retained in the cell of production (Hoffman *et al.*, 1980). Any extracellular uptake does not lead to an increase in the intracellular pool but is limited to membrane phospholipid binding of methyl groups forming mainly phophatidylcholine (Duerre & Gordon, 1989). Uptake has been demonstrated by erythrocytes and hepatocytes (Zappia *et al.*, 1978), although the latter has been disputed (Bontemps & Van Den Berghe, 1998).

It remains clear, that any uptake is dependent on high external concentration (Pezzoli *et al.*, 1978), e.g. 200 µmol/L in one study (Bontemps & Van Den Berghe, 1997). When administered in high doses, either orally or by intravenous injection, increased levels of AdoMet are observed in the kidney, liver and brain (Finkelstein, 1994). Concentration of AdoMet in the CSF and brain would suggest it has the ability to cross the blood-brain barrier (Bottiglieri & Hyland, 1994).

Table 1.7 Levels of AdoMet & AdoHcy in various tissues (after Fowler, 2001)

	AdoMet		AdoHcy		Reference
Human CSF control	0.214 µmol/L (n=27) 1				Surtees *et al.*, 1997
	0.161 ±0.03 µmol/L 2				Bottiglieri, 1990
Human plasma	60 ±3 nmol/L		24.4 ±1.1 nmol/L		Loehrer *et al.*, 1998
	103 ±9.9 nmol/L		22.7 ±3.1 nmol/L		Capdevila & Wagner, 1998
Human RBC 3	3.5 ±0.5		1.3 ±0.5		Oden & Clarke, 1983
Human whole blood	0.68 ±0.03 µmol/L		0.48 µmol/L		Cheng *et al.*, 1997
	1.8 ±0.3 µmol/L				Loehrer *et al.*, 1996
Cultured lymphoblasts	98 nmol/10^9 cells		-		German *et al.*, 1983
Rat cultured hepatocytes					Farooqui *et al.*, 1983
Nucleus (mean)	3.2 nmol/2x10^8 cells		-		
Mitochondria (mean)	2.44 nmol/2x10^8 cells		-		
Human brain	**Control**	**Alzheimer's**	**Control**	**Alzheimer's**	Morrison *et al.*, 1996
Frontal cortex 4	0.95	0.25	0.45	0.2	
Occipital cortex 4	1.15	0.3	0.65	0.3	
Temporal cortex 4	1.2	0.25	0.7	0.15	
Putamen 4	0.75	0.15	0.6	0.25	
Hyppocampus 4	1.5	0.6	0.65	0.2	
Rat brain (19-22 months)	**nmol/g wet wt.**		**nmol/g wet wt.**		Gomes-Trolin *et al.*, 1994
Cortex	23.0 ±1.9		2.67 ±0.11		
Striatum	18.9 ±1.2		4.10 ±0.23		
Midbrain	26.5 ±2.2		2.53 ±0.2		
Hypothalamus	21.6 ±0.9		2.58 ±0.28		
Brainstem	20.5 ±1.6		2.10 ±0.02		
Cerebellum	21.6 ±1.0		2.28 ±0.17		
Rat perfused liver	45 ±13 nmol/g		8 ±6 nmol/g		Hoffman *et al.*, 1980
Rat tissues					
Liver					
(standard diet) 5	141		20		Finkelstein, 1990
(excess methionine) 5	673		427		"
Liver 5	60-90		10-15 (adult)		Hoffman *et al.*, 1979
	67.5 ±1.1		43.8 ±3.2		Eloranta, 1977
	83.6 ±11.4		12.9 ±7.0		Finkelstein *et al.*, 1982
	139.6 ±16.3		17.5 ±2.9		Svardal *et al.*, 1988
	95.6 ±11.4		46.7 ±2.4		She *et al.*, 1994
	63.4 ±7.3		25.3 ±2.6		Bottiglieri & Hyland, 1994
Adrenals 5	51.5		16.1		Eloranta, 1977
Brain 5	25.4 ±0.9		3.4 ±1.2		"
	26.7 ±3.4		3.7 ±0.7		Finkelstein *et al.*, 1982
Brain cortex 5	18.9 ±1.74		2.4 ±0.32		Bottiglieri & Hyland, 1994
Heart 5	38.5		3.9		Eloranta, 1977
Kidney 5	47.2		22.5 ±3.4		"
	53.5 ±8.1		9.9 ±1.4		Finkelstein *et al.*, 1982
	81.0 ±12.0		11.3 ±3.4		Svardal *et al.*, 1988
Pancreas 5	39.8		11.4		Eloranta, 1977
Skeletal muscle 5	22.7 ±2.1		4.7 ±0.7		"
Spleen 5	42.2		6.4		"
	76.5 ±14.9		1.0 ±0.4		Svardal *et al.*, 1988

1 95% confidence limit; 0.19-0.24 µmol/L

2 CSF values in control subjects and patients with psychiatric and neurological disorders

3 concentration given in µmol/L packed RBC

4 values converted to nmol/g from protein concentration by Gomes-Trolin *et al.*, (1996)

5 nmol/g

Inborn errors of metabolism

The terms hyperhomocystinaemia (-uria), hypermethioninaemia (-uria) and cystathioninuria refer to unspecific disease entities that may result from a variety of causes and are unrelated to a single defined genetic defect (**Table 1.8**). Consideration will only be given to defects causing hyperhomocystinaemia (-uria), i.e. CBS, MTHFR, MAT and MS deficiencies, although CTH and GNMT deficiencies also occur. As mentioned previously there are a whole range of non-genetic causes of elevated homocysteine and these are summarised in **Table 1.9**. It must be noted that usually homocysteine levels are considerably lower in the latter than those found in the genetic defects although some overlap exists.

Table 1.8 Genetic defects causing hyperhomocystin-aemia (-uria), hypermethionin-aemia (-uria) & cystathioninuria

Biochemical abnormality	Causal genetic defects
Hyperhomocystin-aemia (-uria)	(i) CBS deficiency (ii) MTHFR deficiency (iii) isolated functional MS deficiency (cblE & G) (iv) MS deficiency combined with MMA-CoA mutase deficiency (cblC, D & F) (v) defective absorption of cobalamin
Hypermethionin-aemia (-uria)	(i) MAT deficiency (ii) CBS deficiency (iii) secondary to liver disease e.g. hereditary tyrosinaemia I
Cystathioninuria	(i) CTH deficiency (ii) MTHFR deficiency (iii) impaired activity of MS

Cystathionine β-synthase deficiency & "the pyridoxine effect"

The first case of CBS deficiency was reported in 1962 and has been subsequently found to be the most prevalent inborn error of methionine metabolism. It has an autosomal recessive mode of inheritance and gives rise to classical homocystinuria. This manifests as an accumulation in the plasma and urine, of homocystine (> 200 μmol/L in plasma and > 1 mmol in urine/day), methionine (as high as 2 mmol/L in plasma) and a variety of metabolites derived from these two compounds. Hypermethioninemia is specific for CBS deficiency, as methionine levels are normal to low in other genetic causes of homocystinuria. Plasma levels of cysteine and cystathionine are low, as is the relative percentage of sulphur excreted as inorganic sulphate. The most frequent clinical abnormalities are ectopia lentis, osteoporosis, mental retardation and arterial and venous thromboemboli although the risk of development increases with age and can vary considerably depending on disease severity. The world wide frequency is reported to be 1 in 339,500 cases, but varies from 1 in 65,000 in Ireland to 1 in 900,000 in Japan (Yap & Naughten, 1998; Yap, 2003).

Table 1.9 Possible causes of mild-moderate hyperhomocystin-aemia (-uria) (after Angst, 1997)

	Reference
Age/gender	
Increased Hcy with:	
❑ age	Kang *et al.*, 1986
❑ being male	Jacobsen *et al.*, 1994
❑ postmenopausal status	Andersson *et al.*, 1992
Decreased Hcy with:	
❑ pregnancy	
Renal function	
❑ positive correlation of Hcy with creatinine	Brattstrom *et al.*, 1992
Nutritional (deficiencies)	
❑ folate	Kang & Wong, 1987
❑ vitamin B_6	Ubbink *et al.*, 1993
❑ vitamin B_{12}	Brattstrom *et al.*, 1988
❑ zinc	Duerre & Wallwork, 1986
Disease states	
❑ cancer, acute lymphocytic leukaemia (ALL)	Refsum *et al.*, 1991
❑ chronic renal failure	Kinsell *et al.*, 1947
❑ diabetes mellitus (reduced renal function)	Hultberg *et al.*, 1991
❑ hyperthyroidism	Parrot *et al.*, 1995
❑ liver (disturbed methionine metabolism)	Chen *et al.*, 1969
❑ psoriasis (lower folate levels)	Refsum *et al.*, 1989
Medication	
Increased Hcy with:	
❑ 6-azaribine triacetate (vitamin B_6 antagonist)	Drell & Welch, 1989
❑ carbamazepine (disturbed folate metabolism)	Ueland & Refsum, 1989
❑ colestipol plus niacin	
❑ isonicotinic acid hydroxide	
❑ methotrexate (5-MeTHF depletion)	Ackland & Schilsky, 1987
❑ nitrous oxide (MS inactivation)	Nunn, 1987
❑ oral contraceptives (deficient vitamin B_6)	Thomson & Tucker, 1986
❑ phenytoin (disturbed folate metabolism)	Billings, 1984
Decreased Hcy with:	
❑ penicillamine (stable cysteine analogue)	Ueland & Refsum, 1989

Following cloning of the CBS gene, 551 homocystinuric alleles have been analysed revealing 131 mutations, most being missense or private mutations Two relatively common mutations have been observed, T833C and G919A (designated I278T-{pyridoxine-responsive} and G307S-{pyridoxine-nonresponsive} according to the amino acid substitutions). I278T accounts for 25% and G307S for 16% of all mutations (Miles & Kraus, 2004). In Ireland, G307S accounts for greater than 70% of all alleles, whereas in mainland Europe I278T accounts for approximately 45% of them.

Barber & Spaeth (1967 & 1969) reported on several cases of CBS deficiency that responded to very high doses (250-500 mg/day) of pyridoxine with decreases in methionine levels to normal and virtual elimination of homocystine from the plasma and urine. Not all CBS-deficient patients are responsive to vitamin B_6. In one study out of 272 patients, 40.5% were responsive, 47.4% nonresponsive, and 12.1% had intermediate or questionable response. There is ample evidence to suggest that the induced response was not

due to the correction of a pre-existing vitamin deficiency, or to alleviation of a defect in conversion of pyridoxine to PLP. Folate depletion may explain the apparent failure to respond to pyridoxine in some patients.

The effects of pyridoxine are not uniform, and some patients continue to show elevations in plasma homocysteine, although responsiveness appears to be constant in sibships. There is evidence that responsiveness or nonresponsiveness may also be determined by the specific properties of the mutant enzyme. Thus, there is a strong correlation between the presence of detected residual activity of CBS (in liver extracts, cultured fibroblasts and phytohaemagglutinin-stimulated lymphocytes) and clinical responsiveness to vitamin B_6. Quantitatively, the residual activities of CBS in extracts of fibroblasts cultured from responsive patients have varied from 0.1-10% of mean control values, but in liver, between 1-31% of mean control values. When the same patients were receiving large dietary intake of pyridoxine (250-1200 mg/day), the specific activities of CBS in liver extracts were enhanced 1.3-4.5-fold.

The molecular properties of mutant CBS crucial in conferring vitamin B_6 responsiveness remain unclear. The presence of residual activity is not sufficient, as in cases of non-responsiveness with detectable residual activities. With enzymes from responsive patients, restoration of near-normal activity by high concentrations of PLP *in vitro* such as would take place if the mutations affected only the K_m for this cofactor, have usually not occurred. Some of the largest observed enhancements have occurred in non-responsive patients. It has been suggested that for a patient to be vitamin B_6 responsive, not only must some residual activity by present, but the affinity of this mutant enzyme for cofactor must not be overly impaired (Mudd *et al.*, 2001; Meier *et al.*, 2003).

5, 10-methylenetetrahydrofolate reductase deficiency

Although less frequently observed than CBS, MTHFR deficiency is the most common congenital defect in mammalian folic acid metabolism. The MTHFR gene is located on chromosome 1 (1q36.3). To date, 33 mutations in the MTHFR gene have been identified (Sibani *et al.*, 2003). The severity of the disorder varies considerably and generally correlates with the presence or absence of residual enzyme activity. Anaemia in these patients is rarely observed, although during short-term folate restriction, patient erythrocytes exhibit a higher mean cell volume similar to that of routinely encountered folate derived megaloblasic anaemia and a higher mean cell haemoglobin concentration. Most patients are unresponsive to folate therapy alone and mortality often occurs in early childhood. Exceptional patients are known who respond specifically to high dose folate, and a single riboflavin responsive patient has been reported (Fowler *et al.*, 1990).

Methionine adenosyltransferase deficiency

First reported in 1974, MAT deficiency is characterised by reduced MAT I/III activity caused by mutations in MAT1A resulting in most cases to increased methionine, methionine sulphoxides, but normal AdoMet levels in tissues and body fluids. Of a total of 30 patients described, 27 were symptom-free, indicating a benign disorder, although two subjects have been reported on that developed neurological abnormalities and brain demyelination attributed to decreased AdoMet levels (Chamberlain *et al.*, 2000; Surtees *et al.*, 1991). Except in these cases, where administration of AdoMet is advisable, specialised treatment is not usually undertaken (Andria *et al.*, 2000).

Methionine synthase deficiency & impaired (methyl)cobalamin synthesis

MS deficiency occurs as either an isolated enzyme defect (of either apo-MS itself or MSR) or combined with methylmalonyl-CoA mutase deficiency (Rosenblatt & Cooper, 1990; Suormala *et al.,* 2004). It leads to elevated levels of homocysteine in both plasma and urine, low plasma methionine and reduced plasma methylcobalamin. Involvement of methylmalonyl-CoA mutase additionally leads to increased methylmalonic acid in the plasma and urine, with reduced formation of both methylcobalamin and adenosylcobalamin. To date, 9 classes of cobalamin disorders, delineated cblA-cblH by complementation analysis (somatic cell hybridisation studies) have been recognised. All of these disorders are thought to be autosomal recessive in inheritence.

cblA, B & H	methylmalonic aciduria (MMA) without homocystinuria or megaloblastic anaemia
cblC, D & F	homocystinuria and MMA
cblE & G	homocystinuria without MMA (cblE MSR deficiency; cblG MS deficiency)

Clinical symptoms in both forms of MS deficiency are predominantly psychomotor retardation, lethargy, failure to thrive and megaloblastic anaemia (Banerjee & Matthews, 1990). In addition, within combined defects of methylmalonyl-CoA mutase, there can be acute episodes of metabolic decompensation (Fenton & Rosenberg, 1995). Though the majority of patients present in infancy or early childhood, some are not diagnosed until adolescence or later (cblC & G) with mainly neurological symptoms (Rosenblatt, 2001). For some of these disorders, prenatal diagnosis and therapy with cobalamin during pregnancy have been attempted. In infants and older children, intramuscular injection of cobalamin usually corrects biochemical changes and most clinical symptoms, but severe neurological problems appear to be irreversible.

Theoretical aspects of cell culture

Cells

In the selection of the types of cells which are suitable for the study of export and metabolism, a number of factors must be considered including the cell type, as well as the characteristics of cell culture dynamics.

Type of cells - normal

The term "normal" cell(s) is difficult to define precisely, but for primary cultures implies that the cells were derived from a normal, healthy tissue or organ, or organ fragment, not a tumour or other pathological lesion. Following sub-culture or passage and the generation of cell lines, normal cells commonly exhibit a distinctive set of properties. This is by no means the case with all normal cells, but includes:

(i) a diploid chromosomal complement (diploid karyotype);
(ii) anchorage (substrate)- and density-dependent inhibition of growth;
(iii) a finite life-span (number of cell doublings attainable);
(iv) altered characteristics with increased *in vitro* growth; and
(v) the lack of formation of tumours following injection into immuno-compromised mice.

- transformed

Transformed or neoplastic cells produced by chemical, irradiated, genetic or spontaneous means on the other hand, lack the above features and exhibit the following characteristics:

(i) a reduced requirement for serum or factors for optimal growth;
(ii) a shorter population doubling time;
(iii) increased colony formation on soft agar; and
(iv) a general increase in cloning efficiency.

It should be noted that not all continuous cell lines (e.g. McCoy, MDCK, 3T3 and Vero)[1] display alterations in growth control attributed to cellular transformation. The terms immortalised and transformed are therefore, not synonymous. Consideration must also be given to the fact that though normal and transformed cells are heterogeneous in their nature, cloned populations of normal cells will also come to exhibit this to a certain degree. Clonal culture does not imply absolute homogeneity and long-term stability of characteristics within the cell population.

Fibroblasts

Monolayer cultures of human fibroblasts are now well-established tools for the study of inborn errors of metabolism in man. This probably reflects the ease at which mesenchymally or mesodermally derived cells (fibroblasts, endothelium and myoblasts) can be cultured in comparison to epithelium, neurones or endocrine tissue. Fibroblasts from primary cultures however, are more representative of the cell types in the tissue from which they came, and in the expression of its specific properties. They offer therefore, not only large amounts of consistent material for prolonged use, but within limitations due to comparing single cells with tissues in the body, also a close approximation in terms of metabolism to the *in vivo* situation.

[1] McCoy = mouse fibroblasts; MDCK = Madin Darby canine kindey cells; 3T3 = Swiss mouse embryo fibroblasts; Vero = normal adult African green monkey fibroblasts

Culture dynamics - density-dependence, ageing & senescence

The majority of normal cells exhibit a marked reduction in their net proliferative activity when all the available surface for attachment is occupied i.e. confluence. Data from cell counts has shown that the decline in growth rate culminating in quiescence, is a smooth and not a step-wise process. In most cases this is an effect of population density and not cell-to-cell contact, although this cannot be ruled out entirely. Adult human skin and 3T3 fibroblasts, in contrast to those of human foetal lung, kidney and salivary gland, do become density inhibited at confluence, although this inhibition can also occur earlier. Either way, it is thought to reflect diminished nutrient supply through decreased substrate uptake (the "boundary effect", Stoker, 1973) or, an upset in their diffusion gradients (Froehlich & Anastasslades, 1975), together with the release of cell-derived factors and waste products into the medium. A post-confluent culture contains three layers of cells, (i) those closest to the adherent surface, (ii) those surrounded by cells and (iii) those closest to the medium. This is distinct from the localised multi-layering that is observed with unevenly distributed cells. In the case of normal fibroblasts grown under standard conditions, the cells become organised in leaf-like parallel arrays with an orthogonal alternation between vertically adjacent leaves. Soon after a change of medium in a stationary fibroblast culture, there is a striking and often drastic resurgence of cell movement that causes temporary rents to appear in the cell sheet. This activity subsides within the course of a day, but is followed by division of 2-6% of the cell population (Bard & Elsdale, 1971), therefore not conforming to density inhibition by diminished nutrient supply. Inhibition of the top layer must in some way depend on the thickness of the middle layer if the growth regulating mechanism is to discriminate between degrees of over-confluence (or supra-confluence), hence the hypothesis of the "averaging of cell states". In the case of human diploid fibroblasts, it seems that the current ideas on growth regulation through density dependence are insufficient to account for their behaviour, and at least six more postulates have been proposed to control density-inhibition, combined in what is now termed the "message" hypothesis.

Since the observations of Hayflick and Moorhead (1961), it has been recognised that the continuous sub-culture of normal cells will lead to a steady decline in cell number and an eventual growth arrest. This in part depends upon the origin of the cells, the age of the donor (those obtained from embryonic tissue having a greater growth capacity than those from adult tissue) and the site of biopsy. *In vitro* senescence, also referred to as terminal passage, mean population doublings maximum (MPD_{max}) or phase III, is correlated with the progressive loss of telomere sequences from the chromosome ends and can be observed as an increase in both the cytoplasmic and nuclear size, but more importantly a decrease in the nucleocytoplasmic ratio. The process of cell ageing has been shown to involve progressive alterations in a number of cell characteristics, some of which can be seen in **Table 1.10**. The life-span of normal human diploid fibroblasts is generally quoted to be in the range of 50-70 population doublings. It must be noted this is solely for embryonic derived tissue, and that of adult has in the region of 30-40 doublings. In most other species, and especially with rodents, cultures cease to grow after some 5-7 passages in which the diploid cells will cease undergoing mitosis. Of course, the ranges quoted only serve as general guidelines, as some cell lines may show considerable deviation from them.

Culture media

Most studies utilise culture media made up of mixtures of salts, glucose, amino acids and vitamins, together with animal serum.

Commercial preparations

The content of culture medium is by far the most important single factor governing the cultivation of animal cells. Originally, the composition of media (or milieu) was based entirely on biological fluids such as

plasma, lymph, serum and principally embryonic tissue extracts. These chemically undefined media however, suffered the drawbacks of batch variation and contamination susceptibility.

Table 1.10 Typical profile of changes seen with normal diploid fibroblasts as a consequence of *in vitro* ageing (after Stanulis-Praeger, 1987)

Increased cell cycle time
Decreased number of cells in the cell cycle (i.e. decreased growth fraction)
Decreased saturation density
Increased incidence of polyploidy
Decreased adhesion to the substrate
Alterations in cell surface glycoproteins and proteoglycans
Altered cytoskeletal organisation
Reduced DNA synthesis
Decreased rate of protein synthesis, degradation and turnover
Decreased amino acid transport
Diminished response to growth factors

Today there are two main approaches to the composition of basal culture media. The first is loosely based on the analysis of plasma and leads to complex formulations such as Medium 199 (M199) that has 61 synthetic ingredients (Morgan *et al.*, 1950). The other is to reduce the number of components to the minimum number shown to be essential for cell growth. This second strategy lead to the design of Eagle's basal medium (BME) for the optimal growth of mouse L and HeLa cells (Eagle, 1955). It should be noted that even though BME has several different versions, they are not all commercially available. The formulation was later modified and improved as *minimum essential medium* with Earle's salts (EMEM or more commonly MEM) (Eagle, 1959), and like that of BME, has found widespread application for the growth of a variety of different cell lines.

Serum

While a basal medium provides a number of chemically defined low-molecular-weight components, it still requires in most cases, augmentation with serum to be fully effective. This limitation is no more apparent than with human diploid fibroblasts, where the addition of serum remains to a larger extent a necessity. Animal serum is a well-established media supplement, being a multifactorial reagent that satisfies both the nutritional and environmental requirements of cells (**Table 1.11**). Serum serves to promote the attachment and spreading of adherent cells, as well as providing a wide range of essential components. These include trace elements and water-insoluble nutrients, growth stimulating factors such as hormones and proteases, and components which render biological protection such as antitoxins, antioxidants and antiproteases. Serum contains several hundred different proteins with a total protein content of around 70 g/L. The major components include albumin (35-55 g/L), immunoglobulin G (8-18 g/L), immunoglobulin A (0.8-4.5 g/L), immunoglobulin M (0.6-2.5 g/L), fibrinogen (3 g/L), transferrin (3 g/L), α_1-antiprotease (3 g/L), α_2-macroglobulin (2.5 g/L), haptoglobin (1.7-2.3 g/L) and fibronectin (0.3 g/L). The sera from a variety of animal species have been used as supplements for cell culture media, although foetal and new-born bovine and adult horse sera are most widely used. Foetal bovine or calf serum (FCS) is a particularly potent growth promoter for many types of cells and contains very low levels of immunoglobulins. The composition of FCS however, is known to have considerable batch-to-batch variation, which is reflected in its growth promoting properties.

Table 1.11 Major serum components essential for *in vitro* cell growth & survival (after Cartwright & Shah, 1994)

Component	Main function(s) known
Proteins	
Albumin	Carries lipids (cholesterol etc.), hormones (thyroxine and steroids), minerals (Ni^{2+} and Zn^{2+}) and fat-soluble vitamins; provides osmotic pressure, buffering capacity
Ceruloplasmin	Binds and carries Cu^{2+}, (cf. liver growth factor {GHL})
Transferrin	Binds and carries Fe^{2+}
α_1-Anti-protease (-trypsin)	Inhibit trypsin
α_2-Macroglobulin	
Collagens	Enhance cell-substratum attachment in anchorage-dependent cells
Fetuin — Matrix proteins	
Fibronectin	
Laminin	
Polypeptides & growth factors	
Epidermal growth factor (EGF)	Mitogen
Eye-derived growth factor	Mitogen
Fibroblast growth factors (aFGF and bFGF)	Bind strongly to heparin, (potency of aFGF augmented by heparin)
Insulin	Mitogen; anabolic, promotes uptake of glucose and amino acids
Insulin-like growth factors (IGF-I and -II)	Mitogens
Interleukins (IL-1 and -6)	IL-6 can replace feeder layers for hybridomas
Nerve growth factor (NGF)	Poor mitogen, but induces differentiation in neuroectoderm cells
Platelet-derived growth factor (PDGF)	Binds to α_2-macroglobulin in serum
Transforming growth factors (TGFα, β_1 and β_2)	TFGβ_1 synergistic with EGF or TGFα for growth in soft agar, but inhibits growth in monolayer cultures
Peptides	
Glutathione	Redox reactions
Other hormones	
Cortisol (hydrocortisone)	Promotes cell attachment, induces cell differentiation
Oestrogens and androgens	Mitogens
Thyroid hormones (T_3 and T_4)	Oxygen consumption, energy metabolism; promotes growth and differentiation
Lipids	
Cholesterol	Membrane biosynthesis
Linoleic acid	
Prostoglandins	
Metabolites	
Amino acids	Cell proliferation
α-Keto acids (pyruvate)	
Polyamines	
Vitamins	
Trace elements & minerals	
As, Co^{2+}, Cr, Cu^{2+}, F, Fe^{2+}, I, Mn^{2+}, $Mo_7O_{24}^{6-}$, Ni^{2+}, SeO_3^{2+}, Si, Sn, VO_3^-, Zn^{2+}	Enzyme activation among others

Concentrations of compounds likely to influence homocysteine metabolism

Although numerous media formulations are available, none contain amino acid and vitamin levels that fall within normal physiological limits (**Table 1.12**). Homocysteine flux and its related compounds have been measured in various complete media, sometimes with varying levels of methionine or various forms of folate. The value of such experiments remains doubtful since a number of other compounds which vary between media may also directly or indirectly influence homocysteine metabolism. For example, two of the most commonly used basal media for fibroblasts are MEM (in its various forms) and RPMI-1640. The differences between them however, are quite striking, as are those between the different forms of MEM. This is further complicated by the mixing of different media as in DMEM / M199 (1 : 1) for skin fibroblasts, and the use of low-protein or serum-free media, the latter usually employing basal combinations such as DMEM / F12 (1 : 1), RPMI-1640 / DMEM / F12 (2 : 1 : 1) or MCDB 110[2]. Although serum-free media have many advantages, an ideal multipurpose formulation has not yet been developed and is almost certainly an unachievable goal.

For this work it was necessary to produce a basal medium that contained at least known important substrates at levels that were within the normal physiological range. This allowed comparison between physiological or commercial preparations, the two media forms representing a basal or supplemented state respectively.

Table 1.12 Comparison of the concentrations of major homocysteine related metabolites within various widely used commercial basal media

	BME	EMEM	GMEM	DMEM	IMEM	RPMI-1640	α-MEM	M199	F12
Trace elements (mg/L)									
$CuSO_4$-$5H_2O$	-	-	-	-	-	-	-	-	0.0024
L-Amino acids & derivatives (mg/L)									
L-Cysteine-HCl	-	-	-	-	-	-	100.0	0.1	36.0
L-Cystine	12.0	24.0	24.0	48.0	70.0	50.0	24.0	20.0	-
Glutathione (GSH)	-	-	-	-	-	1.0	-	0.05	-
L-Glycine	-	-	-	30.0	30.0	10.0	50.0	50.0	7.5
L-Methionine	7.5	15.0	15.0	30.0	30.0	15.0	15.0	15.0	4.5
L-Serine	-	-	-	42.0	42.0	30.0	25.0	25.0	10.5
Lipids (mg/L)									
Choline-Cl	1.0	1.0	2.0	4.0	4.0	3.0	1.0	0.5	14.0
Vitamins / co-factors (mg/L)									
Folic acid	1.0	1.0	2.0	4.0	4.0	1.0	1.0	0.01	1.3
Pyridoxal-HCl	1.0	1.0	2.0	4.0	4.0	1.0	1.0	0.025	-
Pyridoxine-HCl	-	-	-	-	-	-	-	0.025	0.06
Riboflavin	0.1	0.1	0.2	0.4	0.4	0.2	0.1	0.01	0.037
Vitamin B_{12}	-	-	-	-	0.013	0.005	1.4	-	1.4

BME = *Eagle's Basal Medium*; **EMEM** = *Minimum Essential Medium with Earle's Salts*; **GMEM** = *Glasgow's Modified Eagle's Medium*; **DMEM** = *Dulbecco's Modified Eagle's Medium*; **IMDM** = *Iscove's Modified Dulbecco's Medium*; **RPMI-1640** = *Roswell Park Memorial Institute-1640*; **α-MEM** = *MEM Alpha Medium*; **M199** = *Medium 199*; **F12** = *Ham's F12 Nutrient Mixture*

[2] MCDB — initially based on F12, the first derivative 104 contained selenium that was latter replaced with various other factors, supports clonal cell growth in dialysed serum (McKeehan *et al.*, 1977)

Physiological medium design

No cultured mammalian cell has been shown to grow in the absence of the eight essential amino acids, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine required by man (Eagle & Lavintow, 1965). Five additional amino acids however, are also required by cultured cells. The requirements for arginine, glutamine and tyrosine are not unexpected since they are mainly synthesised by the liver in the intact organism. Glutamine also acts as both an energy source and carbon framework in the synthesis of nucleic acids. The same may be true for histidine, which is an essential amino acid in infants (Snyderman *et al.*, 1957). The requirement for cyst(e)ine however, is more difficult to explain, but may reflect the functional inconsistency of the transsulphuration pathway in certain cell types other than hepatocytes, coupled to an increased demand for glutathione.

Eight vitamins, choline, folic acid, inositol, nicotinic acid, pantothenic acid, pyridoxal, riboflavin and thiamine have been shown to be required by cells *in vitro*. These are commonly included in media although some contain additional vitamins. Folic acid, nicotinic acid, pantothenic acid, pyridoxal, riboflavin and thiamine function as cofactors in important steps in cell metabolism and their indispensability is hardly surprising (Eagle & Lavintow, 1965). The importance of the other water-soluble vitamins (vitamin B_{12}, biotin and vitamin C) are less clear. Vitamin B_{12} has been reported to be fundamental for certain cells and is included in the basal formulations of IMEM, RPMI-1640, α-MEM and F12. Data concerning the fat-soluble vitamins is more limited, only M199, NCTC-109, -135 etc. (Evans *et al.*, 1956) and Williams' E (for hepatocytes, Williams & Gunn, 1974) contain both vitamin A and E.

Quality control of media

Physicochemical properties - medium pH & osmolality

Preparation of a basal medium whether commercial or in-house should satisfy the following criteria. It should be a clear solution with the correct pH at RT, the correct osmolality[3] (280-320 mOsmol/kg H_2O) and contain amino acids and key elements (salts, glucose and vitamins) consistent with the specification. Further, it should meet protocol standards for sterility, contain endotoxin levels of less than 1 ng/ml and after serum supplementation, support the growth of cells through at least two sub-cultures.

Medium pH is probably the variable which is most difficult to control. Cells in culture must be maintained within small pH limits in order to grow and function properly. With most normal cells of human origin these limits are between pH 7.2-7.8. Some of the effects of an alkaline pH on cell behaviour are listed in **Table 1.13**. The buffering system first, and still extensively used, was that of bicarbonate in equilibrium with 5-10% carbon dioxide in the gas phase. The main disadvantages with this system are that a carbon dioxide enriched atmosphere is essential for adequate pH stability, and a pK_a of 6.1 at 37°C for bicarbonate results in sub-optimal buffering in the physiological range. Because of these difficulties, effort was devoted to the development of organic buffers independent of carbon dioxide, covering the pK_a range 6.15-8.60 (Good *et al.*, 1966; Eagle, 1971).

The synthetic buffer HEPES has a pK_a of 7.0 and maintains a pH of 7.2-7.6. Though its addition does not totally compensate for bicarbonate, it being an essential nutrient regardless of its buffering role, its use allows reduction of carbon dioxide in the incubator atmosphere to 2%. Disadvantages to its use include temperature dependent buffering e.g. pH 7.55 at 15°C and 7.25-7.31 at 37°C, expense and toxicity above 100 mmol/L, with a cessation of cell respiration probably through decarboxylation of oxaloacetate. Although, it has been reported that at even 25 mmol/L, long-term use can cause irregular growth characteristics (Pollard & Walker, 1997).

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[3] osmolality — by definition = (m)Osmol/kg H_2O (or {m}Osm); osmolarity = (m)Osmol/L H_2O, in dilute aqueous solution such as tissue culture media the difference between osmolality and osmolarity is small

Table 1.13 Alterations to cultured cells caused by alkaline medium

Property	Effect of alkaline pH	Reference
Cell morphology and locomotion	Larger and more spherical, smaller contact with the growth surface, larger part of the cell membrane is in contact with the medium, increased transport rates	Taylor, 1962
Glycolysis	Promotes lactate production	Zwartouw & Westwood, 1958
Cell Growth	Growth-promoting in confluent cultures	Eagle, 1973; Froehlich & Anastassiades, 1974; Ceccarini, 1975
Membrane transport	Increased influx of various nutrients (uridine), (pH dependent uptake of macromolecules via endocytosis)	Ceccarini & Eagle, 1971; Kroll & Schneider, 1974; Rubin & Koide, 1975
Mucopolysaccharide (MPS) metabolism	Inhibits intracellular MPS degradation, specific effect on lysosomal function	Lie, 1974; Schwartz *et al.*, 1976
Enzyme activity	β-gluconidase, aryl sulphatase, β-glucosidase increased; α- & β-galactosidase, α-glucosidase, β-glucuronidase, hexosaminidase decreased	Ryan *et al.*, 1972; Butterworth *et al.*, 1974; Wood, 1975

Osmolality is an important parameter in the quality control of tissue culture media and depends upon the total osmotic activity contributed by ions and non-ionised molecules in solution. In basal media this is usually provided by sodium chloride, but in complete media also by albumin. Its control is critical not only for maintaining tonicity, but also for regulating cell metabolism. Ion-transport and changes in ionic concentrations both extra- and intra-cellularly can additionally affect the flux of amino acids and sugars and in turn therefore, basic cellular synthetic systems. Changes can additionally affect Na^+, K^+-ATPase (one of the principal enzyme systems for active cation transport, Alexander & Lee, 1970), alkaline phosphatase (Nitowsky *et al.*, 1963) and RNA synthesis and polymerisation in cells (Stolkowski & Reinberg, 1959).

Intensive studies on cultured renal and neuronal cells have established that adaptive volume regulatory responses, to both acute and chronic cell shrinking or swelling, are mediated by both electrolytes and small organic molecules such as *myo*-inositol, betaine glycerophosphocholine and taurine, as well as various other amino acids. Uptake of betaine as an osmolyte could, in certain cell types, falsely affect the remethylation of homocysteine. In the model studied here, this is not likely to be the case, since cultured fibroblasts lack betaine-homocysteine S-methyltransferase (BHMT).

Different cell types respond optimally as regards their survival to different osmolalities, ranging from 230 mOsm with lymphocytes (Trowell, 1963) to 320 mOsm with granulocytes (Waymouth, 1970). Somatic cells are resistant to a slightly wider range of osmolality (±10% with fibroblasts, Paul, 1975) than erythrocytes, but are nonetheless sensitive to rather small osmotic changes.

In summary

The several factors including composition, pH, osmolality and serum level were all taken into consideration when designing the physiological medium (see page 95).

Table 1.14 Fasting plasma amino acids at different stages of development in comparison to their levels in MEM

Amino acid	Fw.	Premature (first 6 weeks)			0-1 Month			1-24 Months		
		Range (µmol/L)	Mean (µmol/L)	±SD	Range (µmol/L)	Mean (µmol/L)	±SD	Range (µmol/L)	Mean (µmol/L)	±SD
L-Arginine	174.2	34-96	65.0	(±43.8)	6-140	73.0	(±94.8)	12-133	72.5	(±85.6)
L-Cystine	240.3	15-70	42.5	(±38.9)	17-98	57.5	(±57.3)	16-84	50.0	(±48.1)
L-Glutamine	146.1	248-850	549.0	(±425.7)	376-709	542.5	(±235.5)	246-1182	714.0	(±661.9)
L-Histidine	155.2	72-134	103.0	(±43.8)	30-138	84.0	(±76.4)	41-101	71.0	(±42.4)
L-Isoleucine	131.2	23-85	54.0	(±43.8)	26-91	58.5	(±46.0)	31-86	58.5	(±38.9)
L-Leucine	131.2	151-220	185.5	(±48.8)	48-160	104.0	(±79.2)	47-155	101.0	(±76.4)
L-Lysine	146.2	128-255	191.5	(±89.8)	92-325	208.5	(±164.8)	52-196	124.0	(±101.8)
L-Methionine	149.2	37-91	64.0	(±38.2)	10-60	35.0	(±35.4)	9-42	25.5	(±23.3)
L-Phenylalanine	165.2	98-123	155.5	(±17.7)	38-137	87.5	(±70.0)	31-75	53.0	(±31.1)
L-Threonine	119.1	150-330	240.0	(±127.3)	90-329	209.5	(±169.0)	24-174	99.0	(±106.1)
L-Tryptophan	204.2	28-136	82.0	(±76.4)	0-60	30.0	(±42.4)	23-71	47.0	(±33.9)
L-Tyrosine	181.2	147-420	283.5	(±193.0)	55-147	101.0	(±65.1)	22-108	65.0	(±60.8)
L-Valine	117.1	99-220	159.5	(±85.6)	86-190	138.0	(±73.5)	64-294	179.0	(±162.6)

2-18 Years			Adult			Mean					
Range (µmol/L)	Mean (µmol/L)	±SD	Range (µmol/L)	Mean (µmol/L)	±SD	Range (µmol/L)	(µmol/L)	±SD	(mg/L)	±SD	Ratio MEM : plasma
10-140	75.0	(±91.9)	15-158	86.5	(±101.1)	6-158	74.4	(±7.8)	13.0	(±1.4)	9.7 x ↑
5-45	25.0	(±28.3)	5-82	43.5	(±54.4)	5-98	43.7	(±12.1)	10.5	(±2.9)	2.3 x ↑
254-823	538.5	(±402.3)	205-756	480.5	(±389.6)	205-1182	564.9	(±87.8)	82.5	(±12.8)	3.5 x ↑
41-125	83.0	(±59.4)	0-8	4.0	(±5.7)	0-138	69.6	(±38.8)	10.7	(±5.9)	3.9 x ↑
22-107	64.5	(±60.1)	30-108	69.0	(±55.2)	22-108	60.9	(±5.9)	8.0	(±0.8)	6.5 x ↑
46-216	131.0	(±120.2)	72-201	136.5	(±91.2)	46-220	131.6	(±34.0)	17.3	(±4.4)	3.0 x ↑
48-284	166.0	(±166.9)	0-39	19.5	(±27.6)	0-325	141.9	(±75.5)	20.8	(±11.0)	3.5 x ↑
7-47	27.0	(±28.3)	10-42	26.0	(±22.6)	7-91	35.5	(±16.4)	5.3	(±2.4)	2.8 x ↑
26-91	58.5	(±46.0)	35-85	60.0	(±35.4)	26-137	82.9	(±42.7)	13.7	(±7.1)	2.3 x ↑
35-226	130.5	(±135.1)	60-225	142.5	(±116.7)	24-330	164.3	(±58.4)	19.6	(±7.0)	2.5 x ↑
0-79	39.5	(±55.9)	10-140	75.0	(±91.9)	0-140	54.7	(±22.7)	11.2	(±4.6)	0.1 x ↓
24-115	69.5	(±64.3)	34-112	73.0	(±55.2)	22-420	118.4	(±93.4)	20.8	(±17.5)	1.7 x ↑
74-321	197.5	(±174.7)	119-336	227.5	(±153.4)	64-336	180.3	(±34.5)	21.1	(±4.0)	2.2 x ↑

Table 1.15 Vitamins & provitamins found in human plasma, their solubility, concentration & relevance to MEM

Vitamin or provitamin group	Commercial form available	Solubility in water (mg/ml) at 25°C	Plasma concn. mean ±SEM (mg/L)	Ratio MEM : plasma
Fat-soluble vitamins				
Vitamin A	Vitamin A-acetate	Insoluble	0.5 (±0.1)	Not present
Provitamin A	β-Carotene	"	0.5 (±0.7)	"
Vitamin D	Ergocalciferol, vit. D_2	"	0.04 (±0.01)	"
Vitamin E	α-Tocopherolphosphate, di-Na-salt	"	8.6 (±0.9)	"
Vitamin K	Menadione-Na-bi-sulphite, vit. K_3	500	0.0004 (±0.0004) (vit. K_1, ♀, n = 30)	"
Water-soluble vitamins				
Vitamin B_1	Thiamine-HCl	1000	0.02 (±0.02)	47.6 x ↑
Vitamin B_2	Riboflavin	0.07-0.33	0.2 (±0.06)	0.6 x ↓
Vitamin B_6	Pyridoxal-HCl	220	0.02 (±0.02)	41.7 x ↑
Vitamin B_{12}	Cyanocobalamin	12.5	0.002 (±0.003)	Not present
Biotin	D-Biotin, vit. H	0.3-0.4	0.0004 (±0.0001)	"
Vitamin C	Ascorbic acid	333	9.1 (±0.8)	"
Folic acid	Folic acid	0.002	0.01 (±0.002)	100.0 x ↑
vit. B_3 { Niacin		Soluble	4.0	Not present
	Nicotinamide	1000	0.8	1.3 x ↑
Pantothenic acid	D-Ca-pantothenate	356	0.3 (±0.2)	2.9 x ↑
Questionable "vitamins"				
Choline chloride	Choline chloride	Very soluble	nd	nd (1.0 x ↔)
Myo- (or *meso*)-Inositol	i-Inositol	140	nd	nd (1.0 x ↔)

vit. = vitamin; nd = not determined, no variation from MEM

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(References in addition to those below, cited in relevance to work in subsequent Chapters, are listed at the end of each Chapter.)

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Theoretical aspects of cell culture

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Project aims

This thesis describes work within the ongoing overall project "the effect of cofactors and vitamins on homocysteine metabolism in health and disease", performed at the Metabolic Unit, Universitäts-Kinderspital beider Basel (UKBB).

Homocysteine metabolism is regulated by a series of complex mechanisms. One important mechanism is its own export, along with levels of B vitamins especially folate, AdoMet, AdoHcy and amino acids, as well as the proper function of various enzymes. These mechanisms in human cells, particularly export studies, have so far been performed in media containing un-physiological substrate levels. Difficulties in understanding these mechanisms are compounded by:

(i) the use of media supplemented with serum, which may exacerbate its un-physiological composition depending on the batch and amount added, resulting in an only partially-defined medium for cell growth; and

(ii) the need to unravel the metabolic interrelationships by piecing together information from various model systems.

The aims of this thesis therefore, were to study homocysteine export in association with levels of regulatory compounds in a human fibroblast model using conditions more comparable to the physiological situation than those obtained in previous studies. In order to achieve this, the following experimental approach was undertaken:

(i) the formulation and production of a basal physiological medium that would sustain cell growth;

(ii) the development and application of methods for metabolite determination;

(iii) the modification of sample handling and preparation procedures;

(iv) improvements to the existing methods of analysis by HPLC;

(v) the measurement of total, free and bound sulphydryl compounds in media from fibroblast cultures;

(vi) the measurement of intracellular sulphydryl compounds, 5-MeTHF, AdoMet, AdoHcy and amino acids in control and CBS-deficient cells; and

(vii) the effect of methionine and folate on the sulphydryl compounds, 5-MeTHF and AdoMet in control fibroblasts.

In performing this, it will be possible to:

(i) give support and validation to our knowledge of export from previous studies as applicable to the human physiological situation;

(ii) add to the existing work by obtaining complete data on the regulatory compound levels in human fibroblasts under the same culture conditions; and

(iii) better understand homocysteine metabolism.

Cell culture methods

Sub-culture of fibroblasts

Fibroblast cultures were maintained in standard T_{75} tissue culture (t/c) flasks (Nunc, Falcon) containing 20 ml laboratory prepared MEMc. They were kept at 37°C in a humid atmosphere containing a 5% CO_2-air mixture. The maintenance of cells in MEMc prior to their use in experiments ensured that the starting medium for each experiment was identical. Medium was renewed every 4-5 days between sub-cultures to maintain nutrient supply (and remove waste products), in an attempt to sustain optimal "growth". Cells were not routinely maintained in P-medt to avoid any possible long-term nutrient deficits. Cells were sub-cultured at a split ratio of 1 : 2 according to a slightly modified standard protocol using trypsin-ethylenediaminetetraacetic acid (EDTA)-4Na (Life Technologies™) to release cells.

Harvesting of cells

Trypsinisation - for sub-culture & freezing of cells

The medium was removed by aspiration and the adherent monolayer washed with 10 ml Ca^{2+} and Mg^{2+} free Hanks' balanced salt solution (HBSS, Life Technologies™) to remove all traces of medium and more importantly serum. This was then removed and 2-3 ml (1x) 0.25% (w/v) trypsin-1 mmol/L EDTA solution was added for 1 minute. After removal of the trypsin-EDTA solution, flasks were returned to 37°C for 2 minutes. Flasks were then removed and their edges sharply tapped to dislodge the attached cells. 2-3 ml HBSS was then added and the suspended cells were transferred in two equal aliquots into new flasks containing 20 ml MEMc.

Dissociation solution (non-enzymatic cell treatment) - for cell extract metabolite assays

This is essentially an alternative to using trypsin-EDTA solution (**Figure 3.1**). A 5 ml volume (1x) dissociation solution (Sigma, cat.-no. C5914), prepared in Ca^{2+} and Mg^{2+} free PBS, was added to the cells. After incubation at 37°C for 5-10 minutes, cells were treated as for trypsin-EDTA solution and obtained as a pellet by centrifugation (Sorvall® RT 6000D) at 850 x g for 10 minutes and the supernatant removed by aspiration.

Cell viability & counting

Cell viability was ascertained prior to all cryo-preservation and experimental procedures using the trypan blue exclusion test. Cell suspensions for experiments were initially prepared in 2-2.5 ml HBSS/flask. Aliquots of 100-200 µl from these were mixed in a 1 ml eppendorf tube with an equal volume of 0.4% (w/v) trypan blue solution (Sigma) and allowed to stand for 10 minutes. With the cover slip in place, approximately

10 µl of the cell suspension-trypan blue mixture was introduced into both chambers of a haemocytometer (Sigma, Bright-Line®). Cell counts were performed in duplicate in each chamber using an inverted light microscope (Zeiss) at x 100 magnification.

The number of cells/ml was calculated as follows:

number of cells counted in 5 squares x 25 x 10^4 (chamber volume correction factor) x 2 (dilution factor)

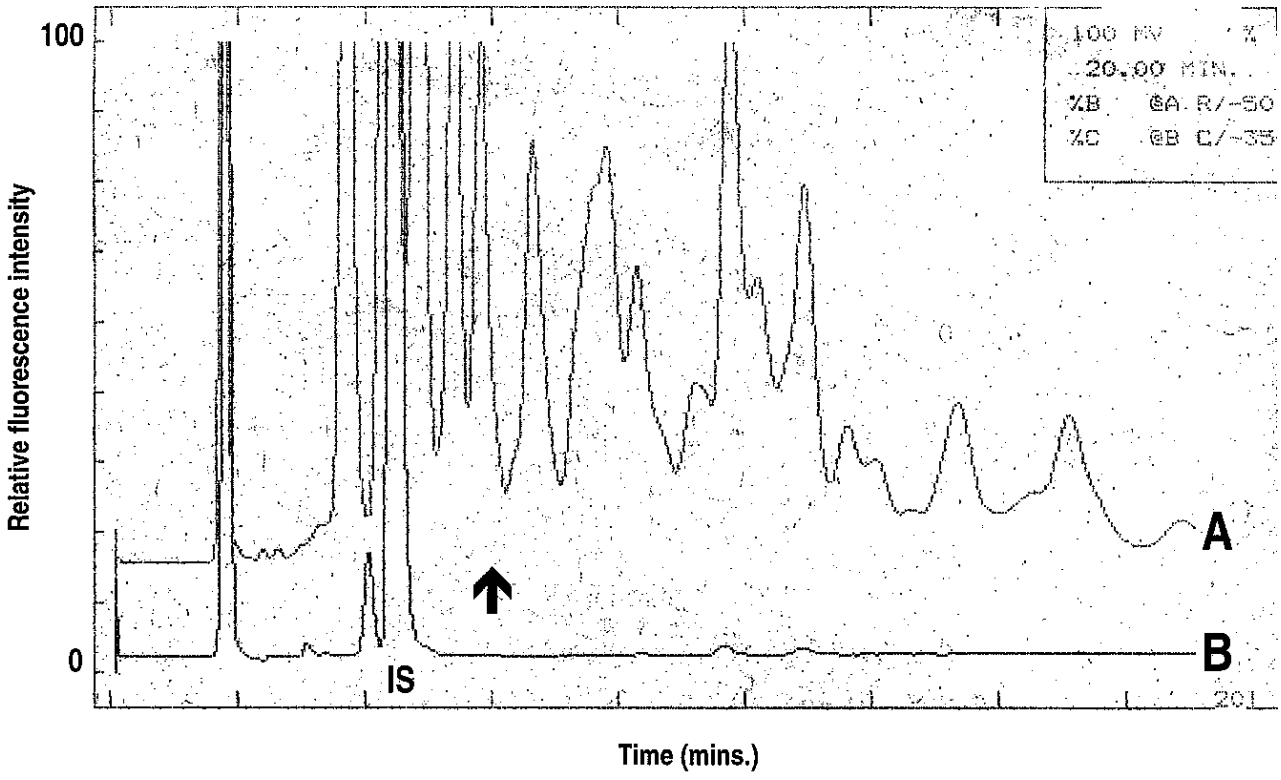
Cell viability was expressed in % as:

total unstained cells ÷ total stained and unstained cells x 100

N.B. Cell viability was always above the 80% minimum reported to be required for cell culture experiments

Figure 3.1 tHcy measurement - chromatogram of trypsin-EDTA solution vs. dissociation solution

interference caused by trypsin-EDTA solution contaminating cell extract tHcy measurement
A = trypsin-EDTA solution; **B** = dissociation solution; ↑ = retention time of homocysteine; **IS** = internal standard



Cryo-preservation & retrieval of cells

Freezing

Cells from post-confluent cultures from 3-5 flasks were treated with trypsin-EDTA solution and pooled, re-suspended in an equal volume of MEMc and obtained as a pellet by centrifugation at 850 x g for 10 minutes. After removal of the supernatant, cells were diluted to $5x10^5$-$1x10^6$ cells/ml in cryo-medium (RPMI-1640 containing 10% {v/v} glycerol) and aliquots of 1-2 ml were transferred to cryo-vials. Vials were packed into an insulated polystyrene box and placed at –70°C overnight before transferring to liquid nitrogen storage.

Thawing

After removal of cryo-vials from liquid nitrogen onto dry ice, they were quickly thawed in a water-bath at 37°C. Vials were cleaned with 70% ethanol, opened carefully keeping a tissue soaked in 70% ethanol around the cap to maintain sterility. Cell suspensions were transferred to individual 10 ml plastic screw-top tubes containing 7 ml MEMc and obtained as a pellet by centrifugation at 850 x g for 10 minutes. Supernatants were removed by aspiration, leaving approximately 1 ml of medium covering each cell pellet. Tubes were then sharply tapped to dislodge the cells that were transferred to T_{25} t/c flasks containing 5 ml MEMc. These were incubated until the cultures reached confluence and sub-cultured into T_{75} t/c flasks using 1 ml of HBSS and 1 ml trypsin-EDTA solution.

Protein determination (after Lowry *et al.*, 1951)

For the cell extract metabolite assays, confluent monolayers were dissociated non-enzymatically and depending on the metabolite preparation, pellets were lysed with 100 or 120 µl (for M+HT, page 73) lysing buffer which contained 2.5% (v/v) igepal CA-630 (nonidet P 40, Sigma cat.-no. C56741) in PBS, final pH 7.3. Protein was measured by the Lowry reaction using a 5 or 10 µl aliquot of the untreated lysate and measured analytes expressed as nano or picomol/mg protein.

A 5 or 10 µl volume of lysate was transferred to a 5 ml plastic tube and made up to 200 µl with distilled-water and stored at –20°C, or assayed directly. A 1 ml solution containing 0.1 ml 1% (w/v) copper sulphate (Cu_2SO_4), 0.1 ml 2% (w/v) potassium-sodium tartrate ($C_4H_4KNaO_6$-$4H_2O$) and 10 ml (2% {w/v} in 0.1 mol/L NaOH) sodium carbonate ($NaCO_3$)[1] was then added and the whole mixture allowed to stand for 10 minutes. After the addition of 0.1 ml folin-ciocalteau reagent (Merck, diluted 1 : 1 with distilled-water), the solution was mixed and left for 30 minutes. The mixture was then centrifuged at 100 x g for 5 minutes to remove any precipitate and the absorbance of the supernatant measured at 500 nm against a distilled-water blank. A standard curve (**Figure 3.2**) was prepared in duplicate each time using 0-50 µg bovine serum albumin (BSA).

In order to relate protein measured by the Lowry reaction to cell number (**Figure 3.3**), cells from 1-2 flasks were harvested with dissociation solution and pooled, cells were serially diluted from $2x10^6$-$3x10^4$ cells/ml, obtained as a pellet by centrifugation at 850 x g for 10 minutes, lysed and the protein measured. Conversion of protein to cell number was performed for all cell extract metabolites.

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[1] a modification involving the addition of 1% (w/v) lauryl sulphate (SDS, Sandermann & Strominger, 1972) allows assays to be performed in virtually any detergent and avoiding precipitate

Figure 3.2 Protein Lowry standard curve

BSA diluted in distilled-water to 12.5, 25.0, 37.5 & 50.0 µg protein/tube against absorbance @ 500 nm

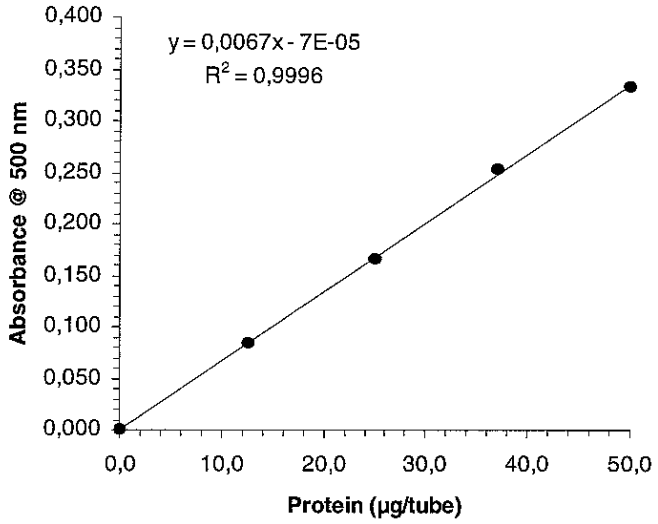
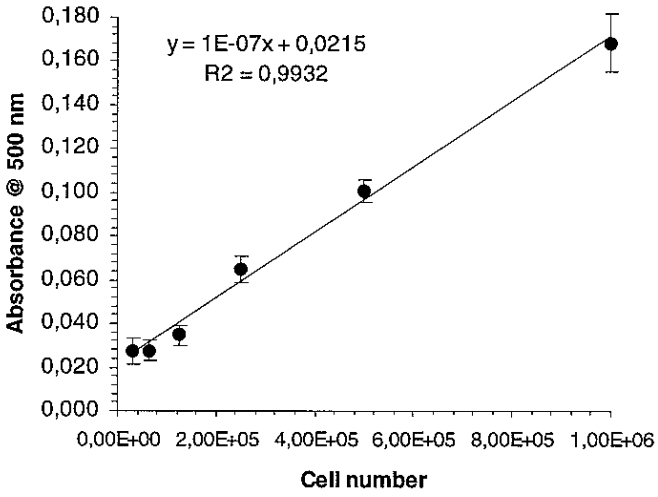


Figure 3.3 Conversion of protein Lowry to cell number

each point represents mean (±SD) from 8 different cell lines

e.g. absorbance of 0.100 = 14.94 µg protein = $7.9x10^5$ cells



Growth evaluation for comparison of extracellular metabolite levels

Introduction

Three colorimetric methods, ① giemsa, ② sulphorhodamine B (SRB) and ③ thiazolyl blue (MTT) were employed to determine cell proliferation in parallel to the 6-well plate or multi-time point experiments described in Chapter 6 (pages 117 & 118). Standard curves were produced by staining serially diluted cells ranging from $1x10^6$-$3x10^4$ cells/well in 4 ml MEMc.

The basis of these three methods were as follows:

① Giemsa (methylene blue + azure II eosin) is one of the so called Romanowsky permanent stains which has combined affinities for both basic and acidic substances within a cell. It is considered to be a good nuclear stain and an adequate cytoplasmic stain.

② SRB (Sigma) is a bright pink aminoxanthene dye with two sulphonic groups. Its histochemistry is similar to that of other related protein stains (e.g. Coomassie blue, bromophenol blue and naphthol yellow S). Under slightly acidic conditions, SRB binds to protein basic amino acid residues to provide a sensitive, linear index of cellular protein. Staining can be achieved without the need for digestion of samples and yields a product that can be stored indefinitely, either air-dried in plates or as sealed solutions, without deterioration.

③ MTT is a yellow/orange water-soluble tetrazolium salt (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) that forms an insoluble blue/purple formazan product by cleavage of the tetrazolium ring by active intracellular mitochondrial and other unspecific dehydrogenase enzymes (or external reducing agents). The formazan can be solubilised by the addition of acidic isopropanol (0.04-0.1 mol/L HCl in absolute isopropanol) or DMSO and measured spectrophotometrically yielding absorbance as a function of converted salt, or in this case, cell number.

Method for giemsa & SRB (after Skehan *et al.*, 1990)

After each sampling time point, the excess medium was removed by aspiration and the cells were washed three times with 3 ml HBSS. Cells were then fixed with 1 ml absolute methanol for 30 seconds, and after its removal by aspiration, allowed to air-dry completely before returning the 6-well plates to the incubator.

At completion of incubation, cells were stained with:

(i) giemsa, using 1 ml of a freshly prepared 10% (v/v) giemsa in distilled-water for 10 minutes and,
(ii) SRB, with 2 ml 0.4% (w/v) or 6.75 mmol/L SRB in distilled-water containing 1% (v/v) glacial acetic acid for 1 hour.

Unbound stain solution was removed by immersion of the whole plate five times in tap water. Excess water was removed and plates allowed to air-dry.

Cells were destained as follows:

(i) giemsa, by the addition of 1 ml 50% (v/v) trichloroacetic acid (TCA) for 3-5 minutes using a plate shaker (Heidolph Promax 1020). Absorbances were measured by spectrometer (Perkin Elmer Lambda 11/Bio

uv/vis) at 641 nm against a TCA blank, after a 1 in 4 dilution in TCA to maintain linearity, in paired, standard 10 mm semi-micro cuvettes (Hellma®) and,

(ii) SRB, by the addition of 1 ml 10 mmol/L tris(hydroxymethyl)aminomethane (TRIS) in distilled-water final pH 10.5. Absorbances were measured at 564 nm against a TRIS blank, after a 1 in 20 dilution in TRIS. Backgrounds (which were typically zero) were measured in parallel plates incubated with cell-free media.

Method for MTT (after Mosmann, 1984)

A 5 mg/ml stock solution of MTT (Sigma) was prepared in HBSS and filtered through a 0.2 µm syringe filter. This was stored at 4°C for daily use or stored at –20°C for extended (> 3 day) periods. The volume added to the wells was equal to one tenth the original medium volume (i.e. 400 µl/well for standard curves and 250 µl/well for experimental plates, made up to the original volume with HBSS) and incubated at 37°C for 3 hours. Following incubation, the medium-dye overlay was removed by aspiration and 1 ml DMSO was added to the well to solubilise the converted dye. Absorbances were measured at 570 nm and 660 (630-690) nm (backgrounds) against a DMSO blank, after a 1 in 4 dilution in DMSO.

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Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, Warren J, Bokesch H, Kenny S, Boyd M (1990): New colorimetric cytotoxicity assay for anticancer-drug screening. *J. Natl. Cancer Inst.* 82 (13): 1107-1112.

Methods of analysis of sulphydryl compounds, 5-MeTHF, AdoMet, AdoHcy & amino acids

Apparatus

Metabolites were measured using two independent methods, reverse-phase high-performance liquid chromatography (HPLC) for the sulphydryl compounds (homocysteine, cyst{e}ine, cysteinyl-glycine and glutathione), 5-MeTHF and adenosyl compounds (AdoMet and AdoHcy), and ion-exchange chromatography for the free amino acids.

Reverse-phase HPLC

Analyses were performed with two pumps (Jasco PU-980) which could be used isocratically or coupled together to provide a gradient system, a Uniflows 4-channel degasser (Degasys DG-2410), an auto-sampler with sample cooling (Jasco AS-950), a solvent mixer, various columns and pre-columns (Crom, Macherey-Nagel), a column oven (Croco.cil™) and two fluorescence detectors, the Fluor LC304 from Linear Instruments™ and the FD-1520 from Jasco. Integration was carried out using the Axxi-Chrom 747 Chromatography Datasystem software package (revision 747.0689D).

Ion-exchange chromatography

Analyses were performed by either a S 432 Sykam or Biochrom 20 Plus amino acid analyser. The S 432 Sykam system contained the following components, a reagent organiser (S 7110), a HPLC controller (S 2000), two HPLC independently controllable pumps (S 1000 and S 1300), an auto-sampler (Jasco 851-AS), a low pressure gradient mixer (S 2110), a column oven (S 4110), a high temperature post-column reactor (S 4210) and a uv-detector (Linear Instruments™ UVIS 204). The Biochrom 20 Plus system contained two pumps (each pump head having sapphire pistons and valves) with piston flush, an auto-sampler, a column oven, a reaction coil and a single flow cell photometer with optical beam splitter. Both systems were fully automatic, with refrigerated auto-samplers for sample cooling, column temperature and reaction coil regulation and data processing controlled by software from Borwin (version 1.20).

HPLC methods - chromatographic conditions

A number of methods were used for the various metabolites.

The conditions used to obtain separation are shown in **Table 4.1**. Each mobile phase was freshly prepared and filtered through a 0.45 µm filter. Reverse-phase chromatography was accomplished by means of an identically packed chromatographic and pre-column. Compound separation was isocratic except for the measurement of AdoMet and AdoHcy which required gradient elution. External standards were used in all methods except for homocysteine, where cysteamine (Sigma) was used as an internal standard (IS). Peak areas were used to quantify the different compounds.

Table 4.1 Reverse-phase HPLC analysis conditions for the sulphydryl compounds, 5-MeTHF, AdoMet & AdoHcy

	Sulphydryl compounds		5-MeTHF		AdoMet & AdoHcy	
Mobile phase (MP) †					A	B ‡
Buffer	13.61 g Potassium dihydrogen phosphate		2 ml 85% (v/v) OPA		13.61 g Sodium acetate trihydrate	
Modifier	-		-		2.00 g Heptanesulfonic acid *	
Acetonitrile (ACN, % {v/v})	4.4-4.5		8.0 or 10.0 (5.0-10.0)		5.0	20.0
Orthophosphoric acid (OPA, 85% {v/v})	10 ml		-		-	
PH	2.10-2.35 with 85% (v/v) OPA		2.33 with solid NaOH		4.25 with 100% (v/v) Glacial acetic acid	
Pump					A	B
Flow rate (ml/min.)	2.0 Continuous flow (CF)		1.5 CF		1.5 CF	
Pressure (kg/cm²)	≈ 230		≈ 180		≈ 260	
Column						
Main column packing & dimensions	Nucleosil 120 C18 250 mm x 4.6 mm x 5 µm		Nucleosil 120 C18 250 mm x 4.6 mm x 5 µm		Nucleosil C18 High-density 250 mm x 4 mm x 5 µm	
Pre-column packing & dimensions	Nucleosil 120 C18 20 mm x 4.6 mm x 5 µm		Nucleosil 120 C18 20 mm x 4.6 mm x 5 µm		Nucleosil C18 High-density 8 mm x 4 mm x 5 µm	
Temp. (°C) **	≥ 25.0		≥ 25.0		≥ 25.0	
Fluorescence detector ***						
Exitation (Ex, nm)	384		296		270	
Emission (Em, nm)	516		360		410	
Gain	1000		1000		1000	
Response time (s)	5		5		5	
Attenuation	S (recorder output 0)		S		S	
Bandwidth (nm)	18		18		18	
	Media & FCS	**Cell extracts**	**Media & FCS**	**Cell extracts**	**Media & FCS**	**Cell extracts**
Auto-sampler						
Run time (min.), auto-sampler/PC Axxi-Chrom	15/14	15/14	20-30/19-29	20-30/19-29	55/54	55/54
Injection vol. (µl)	30	80	30 & 100	40-80	30	40 & 80

† for 1 L; ‡ MP B = 85% (v/v) MP A + 15% (v/v) ACN; * add after filtration; ** column heater could only be used when the ambient RT was < 25°C (which unaffected separation); *** always set for maximum sensitivity; underlined values of pH, run time & injection volume were the ones most frequently used

Homocysteine & associated sulphydryl compounds (after Ubbink *et al.*, 1991)

Pinciple of assay

Essentially a two-step procedure whereby disulphides are reduced by tri-n-butylphosphibe (TNBP) and conjugated with the thiol-specific fluorescent reagent ammonium 7-fluorobenzo-2-oxa1,3-diazole-4-sulphonate (SBDF, Sigma).

Sample preparation & derivatisation

Total, free (non protein-bound) and protein-bound levels were determined in media and FCS, and just total levels for cell extracts.

For media & FCS

Thawed samples of 150 µl were assayed as follows:

(i) total, was determined directly,

(ii) free, was determined by addition of an equal volume of 10% (v/v) perchloric acid (PCA) in distilled-water, thorough mixing and centrifugation at 10000 x g for 10 minutes. 150 µl of this supernatant (dilution factor = 2) was used for total homocysteine measurement,

(iii) bound, the resulting pellet from the deproteinised samples was washed three times with 1 ml PBS pH 7.3, the remaining liquid removed completely and reconstituted in 150 µl distilled-water. Disruption of the pellet was better achieved by manual mixing or in case of high protein content sonication (Telesonic Ultrasonics TPC-25) after addition of cysteamine and TNBP.

Samples of 150 µl were mixed with 50 µl internal standard, 0.025 mmol/L cysteamine hydrochloride (prepared in 0.1 mol/L borax $\{K_2B_4O_4\text{-}4H_2O\}$ buffer pH 9.5 containing 2 mmol/L Na-EDTA, final concentration 6.25 µmol/L) and 20 µl freshly prepared reducing agent, 10% (v/v) TNBP in N,N-dimethylformamide in 1 ml eppendorf tubes then left to stand in an ice (4°C) water bath for 30 minutes (bound fractions were additionally mixed every 10 minutes during this step). Proteins were precipitated by the addition of 125 µl cold 0.6 mol/L PCA containing 1 mmol/L Na-EDTA, with thorough mixing and removed by centrifugation at 10000 x g for 2-3 minutes. Supernatants of 100 µl were mixed with an equal volume of derivatising agent, 1 mg/ml SBDF in 0.1 mol/L borax buffer pH 9.5 in the presence of 200 µl 0.2 mol/L borax buffer pH 10.5 containing 5 mmol/L Na-EDTA in 5 ml glass tubes (Dade Behring). Complete derivatisation of thiols was accomplished after thorough mixing and heating at 60°C for 60 minutes in a dri-block (Techne DB-3). After cooling on ice water, samples underwent immediate analysis, over-night storage at 4°C or were stored at -70°C.

For cultured cell extracts

Assay details were essentially identical to those for media and FCS except that reagent volumes were scaled down (**Table 4.2**) to accommodate for the smaller sample size, and to prevent unnecessary dilution of metabolites. The use of the same volume of reduced sample for derivatisation had the additional benefit of increasing the peak areas by about 30%.

HPLC

Injection volumes of derivatised samples for HPLC measurement were 30 µl for media and FCS and 80 µl for cell extracts. Samples are known to be stable for at least 3 days, standards for at least 6 months.

Table 4.2 Reagent volumes used in the sulphydryl compound assays

Assay step	Media & FCS (µl)	Cell extracts (µl)
Initial sample size	150	90
0.025 mmol/L IS	50	30
10% TNBP	20	12
0.6 mol/L PCA	125	75
Supernatant	100	100
0.1 mol/L borax buffer pH 10.5	200	200
SBDF	100 (1 mg/ml)	20 (5 mg/ml)

Figure 4.1A Chromatogram of sulphydryl compounds in media

A = cell line 5 growing in MEMt for 72 hours; **B** = cell line 5 growing in P-medt for 72 hours;
a = cyst(e)ine (retention time 3.16 mins.); **IS** = internal standard (retention time 4.02 mins.); **b** = cysteinyl-glycine (retention time 5.05 mins.); **c** = homocysteine (retention time 5.53 mins.); **d** = glutathione (retention time 9.60 mins.)

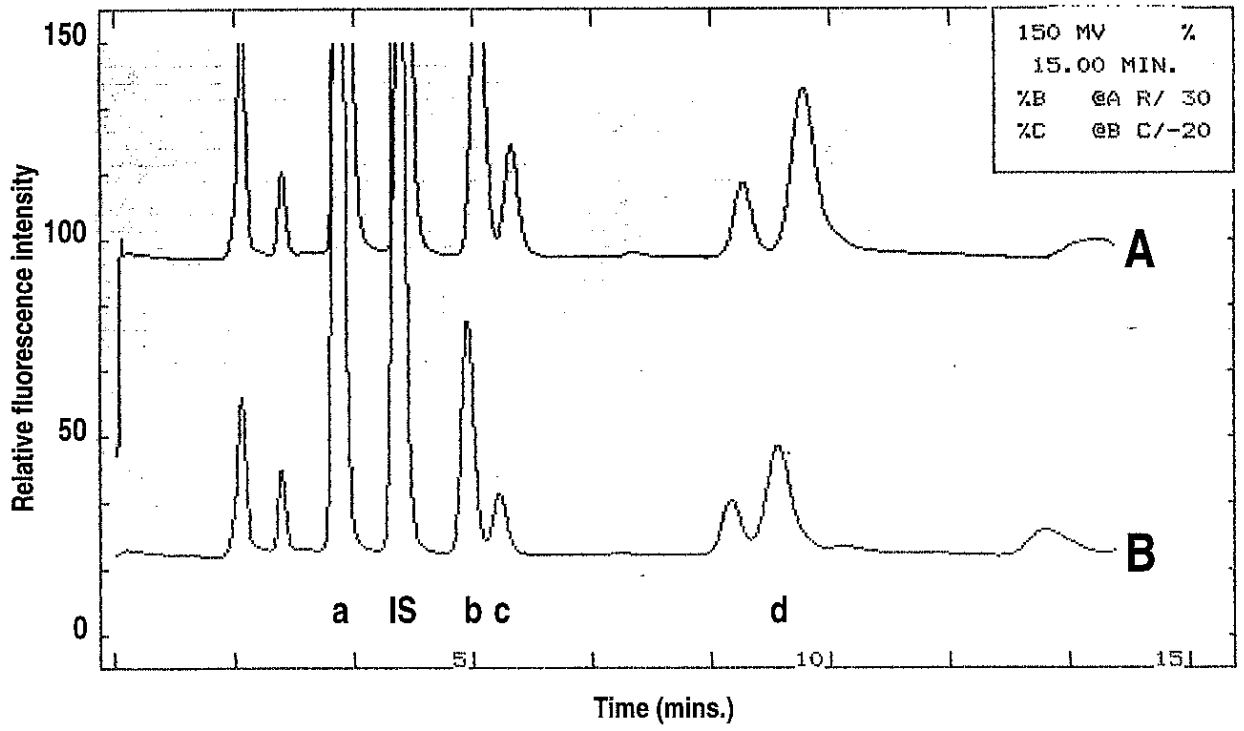
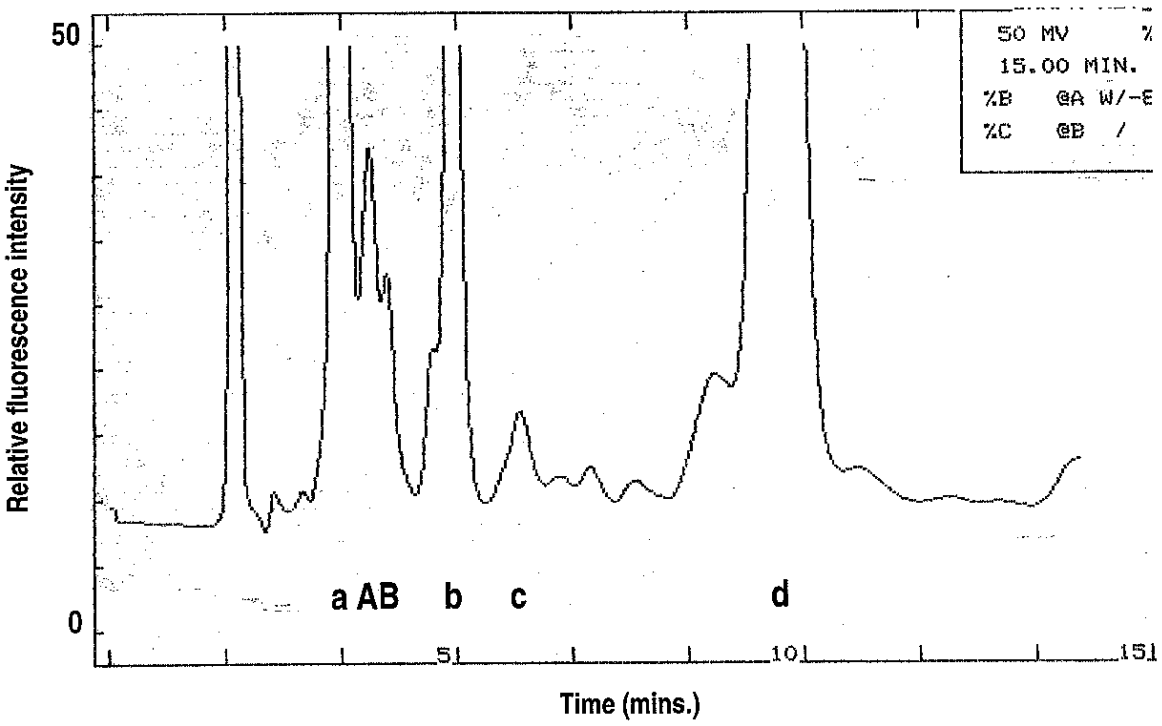


Figure 4.1B Chromatogram of sulphydryl compounds in cells

cell line 5 growing in MEMt for 7 days, no internal standard;
a = cysteine (retention time 3.29 mins.); **A** = unknown (retention time 3.72 mins.); **B** = unknown (retention time 3.97mins.); **b** = cysteinyl-glycine (retention time 4.93 mins.); **c** = homocysteine (retention time 5.90 mins.); **d** = glutathione (retention time 9.60 mins.)



Standards, calibration & linearity

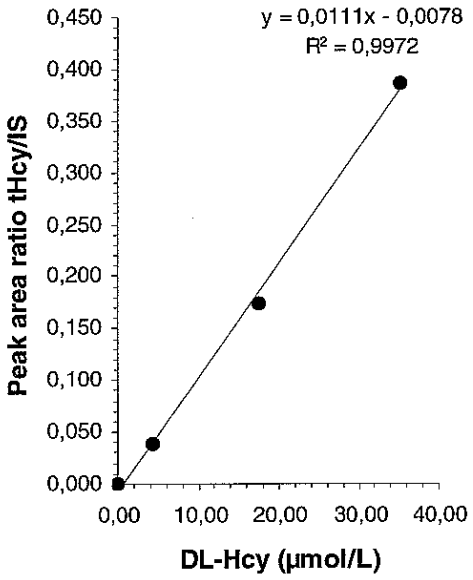
Calibration standards for homocysteine were prepared by the serial dilution of an accurately weighed amount of approximately 5 mg DL-homocysteine (Fluka, Sigma) in 10 ml IS. A four-point calibration curve (**Figure 4.2**) that had been previously shown to be linear over a concentration range of 2-70 µmol/L was included in all analyses. Initial analyses were performed with pooled human plasma, FCS, MEMt, P-medt, distilled-water and lysing buffer all spiked with homocysteine to check the effect of various matrices on the peak area and retention time, since it is known that plasma analysis requires standards added to plasma. After adjusting for the endogenous homocysteine concentrations in the plasma/serum and test media, the variation between each carrier was negligible. Subsequently, calibration curves were prepared in distilled-water or lysing buffer.

Calibration standards of L-homocystine (Sigma), L-cyst(e)ine (Calbiochem, Sigma), L-cysteinyl-L-glycine (Sigma) and glutathione (both GSH and GSSG, Sigma), were prepared by the serial dilution of a 10 µmol/L stock solution in distilled-water or lysing buffer. Four-point calibration curves or single/double calibration points, depending on the run size, were included in all analyses. Sulphydryl compound concentrations were determined by the line equation given by the standard curve. With homocysteine this was slightly modified to include the ratio of the homocysteine : cysteamine peak areas.

It was noted during the early analyses of intracellular compounds, that two unknown metabolites caused interference peaks in the position of the IS (mean retention time at 25°C for IS 3.97 {SD±0.02}

minutes, unknown A 3.73 minutes, unknown B 3.98 minutes). This was overcome by repeating the analysis but without IS and subtracting the difference (**Figure 4.1B**).

Figure 4.2 Standard curve for homocysteine



An example of the calibration curve for homocysteine — corrected for 85% purity (based on information provided by the supplier, Sigma), 30 µl injection volume (media & FCS samples), concentration range 0-35.11 µmol/L. Precision or the coefficient of variation (CV) in intra- & inter-day assays were 3.5 & 4.0%.

5-MeTHF (after Leeming *et al.*, 1990)

Principle of assay

5-MeTHF monoglutamate can be measured directly due to the intense natural fluorescence of its aromatic ring.

Sample preparation

For media & FCS

Samples were rapidly thawed in cold water and aliquots of 300µl were deproteinised by the addition of 100 µl freshly prepared 10% (v/v) PCA in distilled-water containing 10 mg/ml or 1% (w/v) ascorbic acid. After thorough mixing and centrifugation at 10000 x g for 2-3 minutes, supernatants were analysed by HPLC.

For cultured cell extracts - pre-analytical aspects

It is clear from the literature that the main intracellular folate(s) in mammalian cells exist in the form of polyglutamates. Although methods have been developed to look at these forms directly (Shane, 1982; Zorzopulous *et al.*, 1983; Selhub, 1989), detection in most cases depends on their extraction and enzymatic conversion to monoglutamates. Various methods (Rosenblatt *et al.*, 1979; Hoffman *et al.*, 1981; Selhub, 1989; Leeming *et al.*, 1990) have been developed for extraction and conversion, but no particular one is without its

limitations. Essentially, extraction methods involve either boiling cell extracts in the presence or absence of ascorbic acid, and conversion methods involve incubation with γ-glutamyl hydrolase.

(i) Extraction - It has been shown that many folate-dependent enzymes have a higher affinity for polyglutamate substrates (McGuire & Coward, 1984; Shane, 1989) and that boiling is a confirmed procedure to counteract inter-conversion (Hoffman *et al.*, 1981) and aid extraction. Kashani & Copper (1985) showed that disruption of fibroblasts by boiling appeared to cause less post-extraction changes of cell folates than freezing and thawing or sonication, but using methanol for protein precipitation instead of heat minimised the degradation of labile folates. Even so, folate recovery was > 90%, indicating that 5-MeTHF and THF, comprising > 50% and 6% of the intracellular folate respectively, were stable to the heating process.

Wilson & Horne (1983 & 1984) also showed that 5-MeTHF had excellent stability on heating (100°C for 10 mins.), but this was confined to HPLC-purified standard derivatives in the absence of ascorbic acid, which many have used to protect labile folates during cell disruption. Upon heating solutions of folate derivatives in the presence of 2% (w/v) sodium ascorbate, THF gave rise to 5,10-methylene-THF and 5-MeTHF, DHF gave 5-MeTHF and pteroylglutamate (PteGlu), 10-formyl-THF gave 5-formyl-THF and 10-formyl-PteGlu, and 5-formyl-THF gave a small amount of 10-formyl-PteGlu. These inter-conversions were only seen after heating, and were attributed to the presence of around 6 mmol/L formaldehyde in the reaction mixture. Solutions of ascorbic acid were therefore deemed not suitable for the extraction and subsequent assay of individual folate derivatives. Both Rosenblatt *et al.*, (1979), Hoffman *et al.*, (1981), Wilson & Horne (1983) and Selhub (1989) all used extraction procedures in the presence of ascorbate (8.5 mmol/L, 0.2% {w/v}, 2% {w/v} and 2% {w/v} respectively), and while this according to Wilson & Horne does not effect 5-MeTHF and PteGlu, assurance was not confirmed.

(ii) Enzymatic conversion - According to the IUBMB Enzyme Nomenclature Database, γ-glutamyl hydrolase (EC 3.4.19.9) is a *"lysosomal, thiol-dependent peptidase that is most active at acidic pH. Commonly studied with folylpoly-γ-glutamate as substrate, with which the initial cleavage may release glutamate or poly-γ-glutamate of two or more residues, according to the species of origin of the enzyme. Final products are folic acid and free glutamate. Highly specific for the γ-glutamyl bond, but not for the C-terminal amino acid. Action on γ-glutamyl bonds is independent of an N-terminal pteroyl moiety, but it is not known whether an N-terminal γ-glutamyl residue can be hydrolysed."*

In practice the use of the enzyme to elucidate intracellular folate levels can be complicated even when only one derivative e.g. 5-MeTHF is investigated. In particular, the choice of enzyme, its source, specific activity and the conditions that govern cleavage are important to the design of a reliable method. Reported assays have employed several different sources of hydrolase, including rat and normal human plasma (folate-depleted or -replete) (Rosenblatt *et al.*, 1979; Kashani & Cooper, 1985; Leeming *et al.*, 1990) to that extracted from hog kidney (Hoffman *et al.*, 1981), and with cleavage conditions ranging from pH 4.5-7.85 from room temperature to 37°C for 60-135 minutes.

Futher experiments involving the action of hydrolase, but not strictly pertaining to the conversion of folate polyglutamates, have shown that certain preparations have more than one pH optima, a narrow or broad temperature and pH profile, be inhibited or stabilised by various anions, be sensitive to redox agents and for complete hydrolysis may depend on enzymes acting together (Silink *et al.*, 1975; Rao & Noronta, 1977; Reisenaur *et al.*, 1977; Horne *et al.*, 1981).

Thus, depending on the hydrolase preparation chosen, its activity and the conditions that govern cleavage can be subject to many variables. The commercially available enzyme used here was purified from Pseudomonas species, and is supplied with defined specific activity and optimal conditions for conversion i.e. *Carboxypeptidase G: lyophilised powder containing approximately 10% protein (lowry). Unit definition: 1.0 unit will hydrolyse 1.0 μmole L-glutamic acid from (+) amethopterin/minute at pH 7.3 at 30°C. Activity: 30-150 units/mg protein.* For use, this was reconstituted in sterile PBS pH 7.3 and frozen in 40 μl aliquots at –20°C. As support for the use of a non-acidic pH, it has been shown that carboxypeptidase G(2)(CPG2) was very effective in the rescue of high-dose (HD)-MTX nephrotoxicity in cancer therapy after intravenous administration (Krackhardt *et al.*, 1999).

Due to the lack of standardised methods therefore, extraction using a detergent solution and conversion with a low level of enzyme (method without heat, M–HT) was initially tested. Subsequently, to better facilitate extraction and inactivate any endogenous enzymes that might utilise 5-MeTHF polyglutamates and/or formed monoglutamate during the enzymatic conversion, the method was modified to include heating (method with heat, M+HT). This method also contained 1,4-dithiothreitol (DTT) to protect 5-MeTHF monoglutamate from losses through oxidation, and employed a much higher level of enzyme. The two newly developed methods were as follows:

Method without heat (M–HT)

Cell pellets that were protected from light were lysed as described in Chapter 3 (page 62). 10 µl 0.0005 units enzyme was then added and the mixture incubated at 37°C for 40 minutes. 35 µl of 10 mg/ml ascorbic acid in 10% (v/v) PCA in distilled-water was then added to denature the enzyme and stop the reaction. Converted samples were analysed directly or stored at –70°C. For HPLC analysis, samples were thawed, centrifuged at 10000 x g for 2-3 minutes and supernatants analysed.

Method with heat (M+HT)

Again cell pellets were lysed as previously described, but this time heated to 90-95°C for 10 minutes in a water-bath. These were then cooled on ice, centrifuged at 10000 x g for 10 minutes, and a 90 µl supernatant treated with 10 µl 250 mmol/L (38.58 mg/ml) DTT made up in PBS, final pH approximately 7. 10 µl 0.05 units enzyme was then added and the mixture treated as described above.

Results

To validate each method, a series of experiments were undertaken to determine the required amount of enzyme and incubation time. The results of which are shown in **Figures 4.3-4.5** and **Table 4.3**.

(i) Enzyme concentration - Cells were grown in MEMt containing a ≥ 2 µmol/L folic acid level, obtained as a pellet and treated as previously described for M–HT and M+HT, but using a 10-fold dilution in enzyme. **Figure 4.3** shows the levels of 5-MeTHF detected by HPLC analysis after varying the enzyme concentration in the two methods. 5-MeTHF monoglutamate was detectable from both methods, but at 7 and 14 days only with M–HT. The methods showed similarity on using 0.005 units at 7 days, but unlike M–HT, M+HT showed the better correlation between enzyme concentration and 5-MeTHF levels.

(ii) Incubation time - Cells were again grown in MEMt, obtained as a pellet and treated as previously described for M–HT and M+HT, but using different incubation times. **Figure 4.4** shows the levels of 5-MeTHF detected by HPLC analysis after varying the incubation time in the two methods. 5-MeTHF monoglutamate was again detectable from both methods, and although the variation between the individual cell lines was large, was 10-fold higher with M–HT. Furthermore, with M–HT, 5-MeTHF levels decreased unexplainably between 20-40 minutes.

(iii) Substrate concentration - From the information supplied by Sigma, 0.0005-0.05 units would hydrolyse 0.1-10 nmol/L glutamic acid from 5-MeTHF polyglutamate/minute at pH 7.3 at 30°C, i.e. enough to hydrolyse 0.2-20-fold the level of 5-MeTHF found in human plasma, if it were polyglutamate. Nevertheless, as 5-MeTHF polyglutamate is not commercially available, and it remained a question as to whether sufficient enzyme had been included in M+HT, cells were grown in MEMt supplemented with approximately 23 µmol/L folic acid (≈ 1000x physiological levels) in an attempt to dramatically increase substrate levels. Lysates were obtained as previously described and subjected to serial dilution in PBS pH 7.3 before treatment with M+HT.

Figure 4.5 shows the levels of 5-MeTHF detected by HPLC analysis after varying the substrate concentration with M+HT. The relationship between substrate and 5-MeTHF levels were linear.

Figure 4.3 5-MeTHF extraction & conversion using M–HT & M+HT - varying enzyme concentration

each point represents mean (±SD, n = 4)

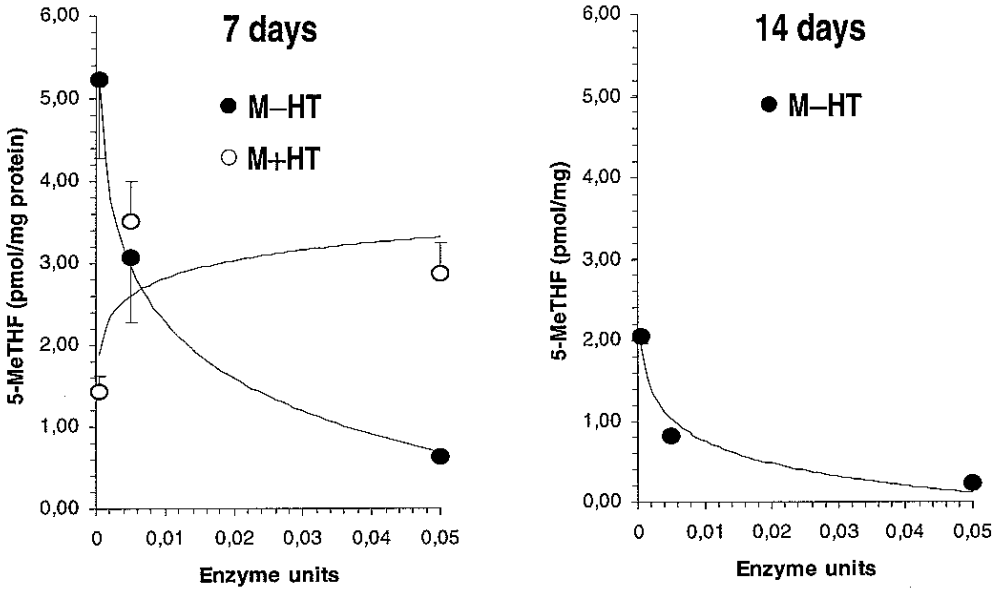


Figure 4.4 5-MeTHF extraction & conversion using M–HT & M+HT - varying incubation time

each point represents mean (±SD, n = 3) from each of 4 cell lines ②, ③, ⑤ & ⑥ (——), overall mean (——)

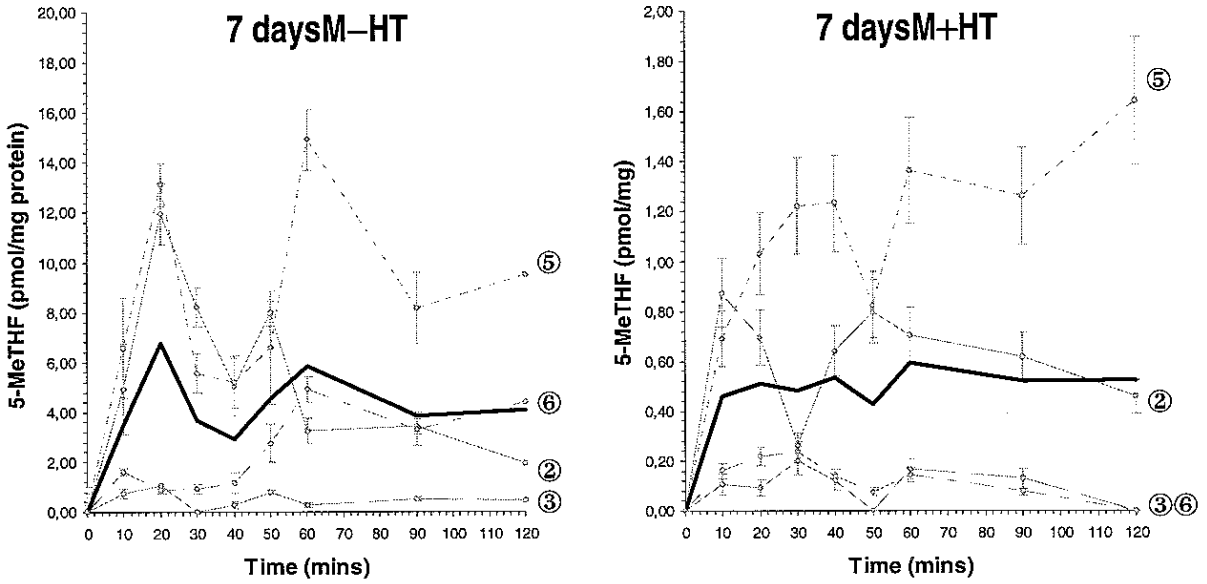
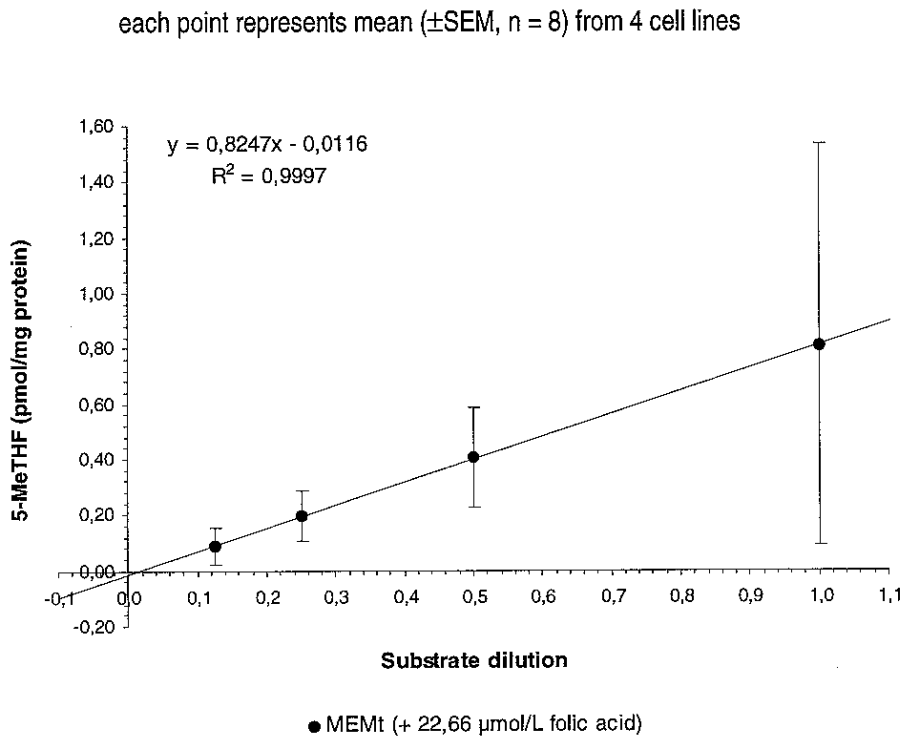


Figure 4.5 5-MeTHF extraction & conversion using M+HT - varying substrate concentration



Discussion

Many studies have indicated that much of the folate in cells is bound tightly to enzymes, indicating that there is not an excess of this cofactor and that its cellular availability is protected as well as being strictly regulated (Donnelly, 2001). Christensen & Ueland (1993) found that both skin and lung fibroblasts incorporate significant amounts of 5-MeTHF into proteins. Studies on rat liver cells have shown that as well as the association of 5-MeTHF with GNMT (Yeo *et al.,* 1999), folate can be distributed between the cytosol and mitochondria, and that folate isolated from the matrix was bound to protein. Treatment with acid was necessary to release the folate from the protein, but degrade products yielding mainly tetrahydrofolate (Cook & Blair, 1979). This binding of 5-MeTHF probably accounts for, in conjunction with the heating, the differences seen in the 5-MeTHF monoglutamate levels from the two methods. Thus, it is impossible to remove endogenous enzymes that utilise 5-MeTHF polyglutamate or formed monoglutamate by heating, and not simultaneously remove 5-MeTHF bound to these enzymes or protein. Nevertheless, on considering the results of varying the enzyme concentration, incubation time and substrate concentration, M+HT was chosen as the method for extraction and conversion for comparisons of levels in cells under different conditions, bearing in mind that recovery was not likely to be complete.

HPLC

Injection volumes of supernatants from deproteinised samples for HPLC measurement were 30 µl for media and FCS and 80 µl for cell extracts. Samples are known to be stable for at least 3 days, standards for at least 6 months.

Figure 4.6A Chromatogram of 5-MeTHF in media

A = MEMt (indicative of all cell culture media tested); **B** = MEMt spiked with 10 % (v/v) 5-MeTHF standard (57.75 nmol/L), retention time 6.89 mins.

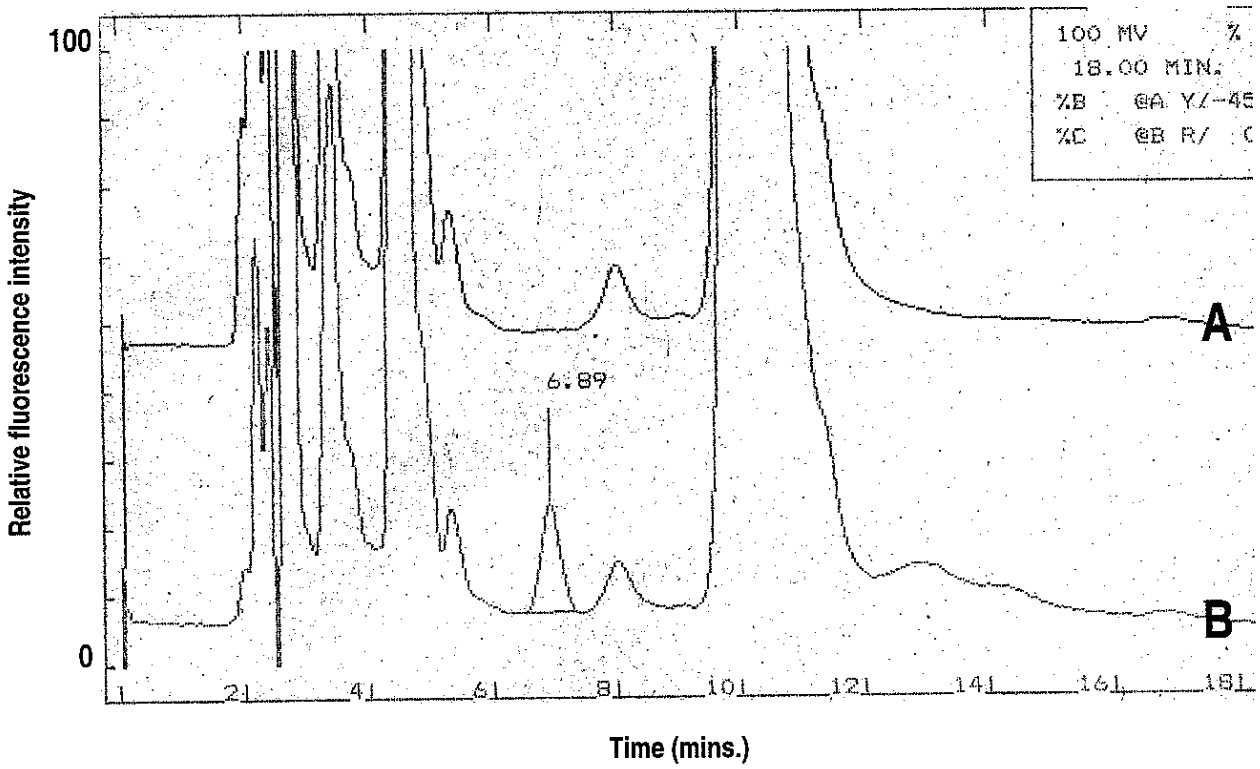
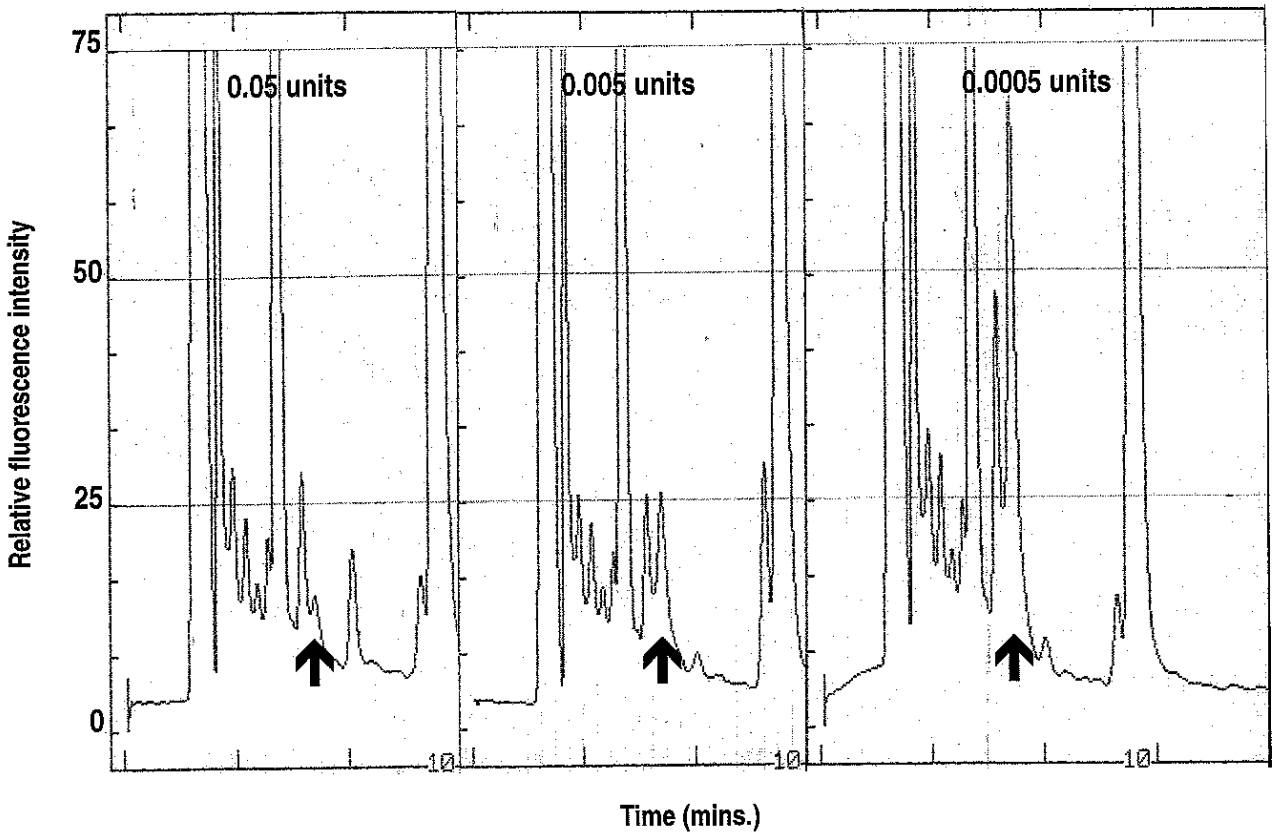


Figure 4.6B Chromatogram of 5-MeTHF in cells

detection of 5-MeTHF in cell line 2 growing in MEMt for 7 days with M–HT using 0.0005-0.05 units of enzyme;
↑ = 5-MeTHF, retention time = 5.65 mins., retention time of 5-MeTHF standard = 5.66 mins.

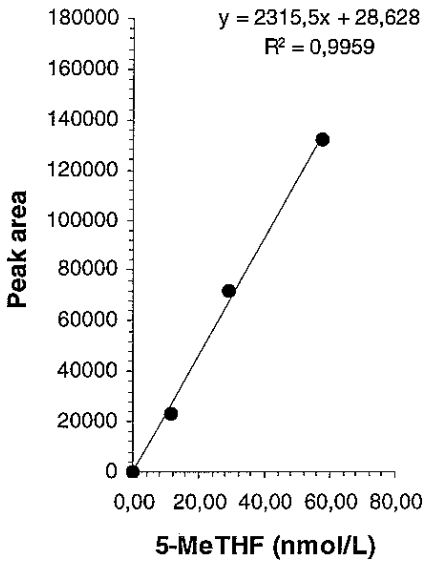


Standards, calibration & linearity

Calibration standards for 5-MeTHF were prepared by the serial dilution of an accurately weighed amount of approximately 1 mg 5-MeTHF disodium salt in 100 ml freshly prepared 0.5-1.0% (w/v) ascorbic acid in distilled-water. A four-point calibration curve was included in all analyses (**Figure 4.7**) that had been previously shown to be linear over a concentration range of 10-110 nmol/L.

In addition to the standards that were normally included in each run, the stability of 5-MeTHF was also monitored in series of analyses exceeding 3-4.5 hours (4 standards + 6 samples, depending on whether extracellular or intracellular samples were being anlaysed). This was achieved by including a standard (28.88 nmol/L) after the 9th and then after every 4 samples. The loss of 5-MeTHF, even in analyses that exceeded 40 samples, was found to be negligible.

Figure 4.7 Standard curve for 5-MeTHF



An example of the calibration curve for 5-MeTHF — corrected for 90 % purity (based on information provided by the supplier, Fluka), 80 µl injection volume (cell extracts), concentration range 0-57.75 nmol/L. Precision in intra- & inter-day assays were 3.8 & 5.7%.

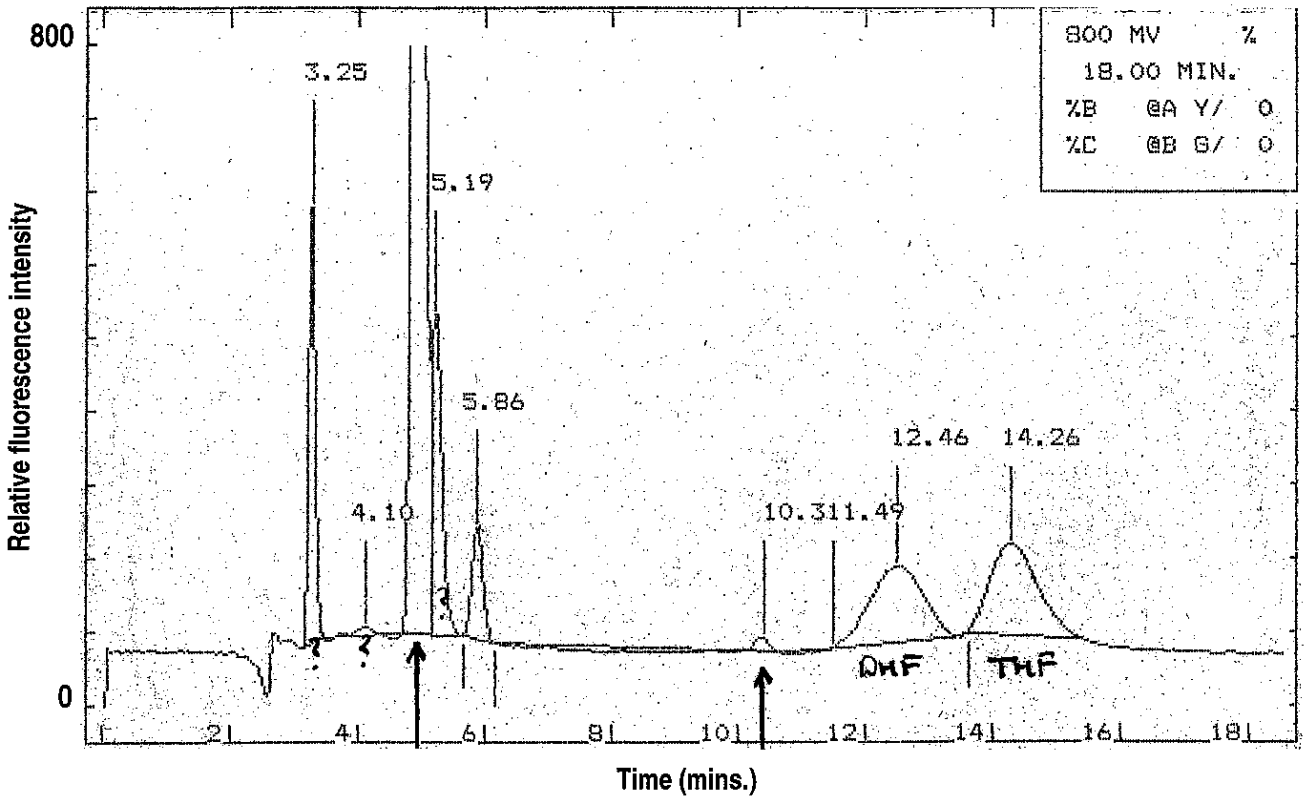
Possible interference by other folates

During the course of the analyses, both 5, 10-methylene-THF, DHF, THF, folic acid, folinic acid and formimino-L-glutamic acid (FIGLU) were also run to determine their possible detection with the conditions for 5-MeTHF and if so, their retention times in relation to 5-MeTHF (**Figure 4.8**).

Figure 4.8 Folate retention times in relation to 5-MeTHF

Retention times:

4.93 mins. = 5, 10-Methylene THF
5.86 mins. = 5-MeTHF
10.39 mins. = Folic acid or folinic acid
12.46 mins. = DHF
14.26 mins. = THF
FIGLU not detected



AdoMet & AdoHcy - simultaneous measurement

The analysis of these two compounds were achieved in a single run using a modification of the method of Loehrer *et al.,* (1996). This method employed gradient elution with a Nucleosil® C18, high-density column (Macherey-Nagel) and fluoresence detection.

Principle of assay

At the appropriate pH and temperature, adenosyl compounds react with monochloroacetaldehyde (CCA, Fluka) to form first the Schiff base then the etheno-derivative which strongly fluoresce.

Sample preparation & derivatisation of media, FCS & cultured cell extracts

Deproteinised media and FCS samples were rapidly thawed in cold water and centrifuged at 10000 x g for 2-3 minutes. Aliquots of supernatants of 200 µl were transferred to 5 ml glass tubes containing 50 µl 45 % (w/w) CCA in water and the pH corrected to between 3.5-4.0 with 3 mol/L sodium acetate (see pages 84-86). After thorough mixing and incubation at 40°C for 8 hours in a dri-block, samples were cooled in ice water and underwent immediate analysis or were stored at –70°C.

For deproteinised cell extracts, assay details were identical to those for media and FCS, except for the scaled down volumes to allow for the smaller sample size (**Table 4.5**).

HPLC

Injection volumes of supernatants from deproteinised samples for HPLC measurement were 30 µl for media and FCS and 40 or 80 µl for cell extracts. Samples are known to be stable for at least 3 days, standards for at least 6 months. **Table 4.3** shows the gradient from 0-60 minutes. With an initial flow rate of 1.5 ml/minute, both AdoMet and AdoHcy are eluted during the first 20 minutes. The column is then washed and re-equilibrated during the next 15 minutes. If another injection occurs at 60 minutes the run begins again, otherwise at 61 minutes the pump decreases the flow rate to 0.2 ml/minute.

Table 4.3 Gradient for AdoMet & AdoHcy elution

Time (mins.)	Pump 1, MP A (%)	Pump 2, MP B (%)	
0	100	0	AdoMet & AdoHcy elution
20	0	100	Column washing
21	0	100	
30	0	100	
35	100	0	Column re-equilibration
60	Flow rate 1.5 ml/min.		If next Injection
61	Flow rate 0.2 ml/min.		If no injection

pump 1 delivers MP A; pump 2 delivers MP B (**Table 4.1**)

Figure 4.9A Chromatograms of AdoMet & AdoHcy standards & in media

A ↑ AdoMet (retention time 7.45 mins.); ⇧ AdoHcy (retention time 4.81 mins.);
B ⇧ AdoHcy (retention time 4.87 mins.);
C upper trace = AdoMet & AdoHcy (retention time 6.59 mins.) in MEMt from cells growing for 24 hours; lower trace = mixed AdoMet & AdoHcy standards

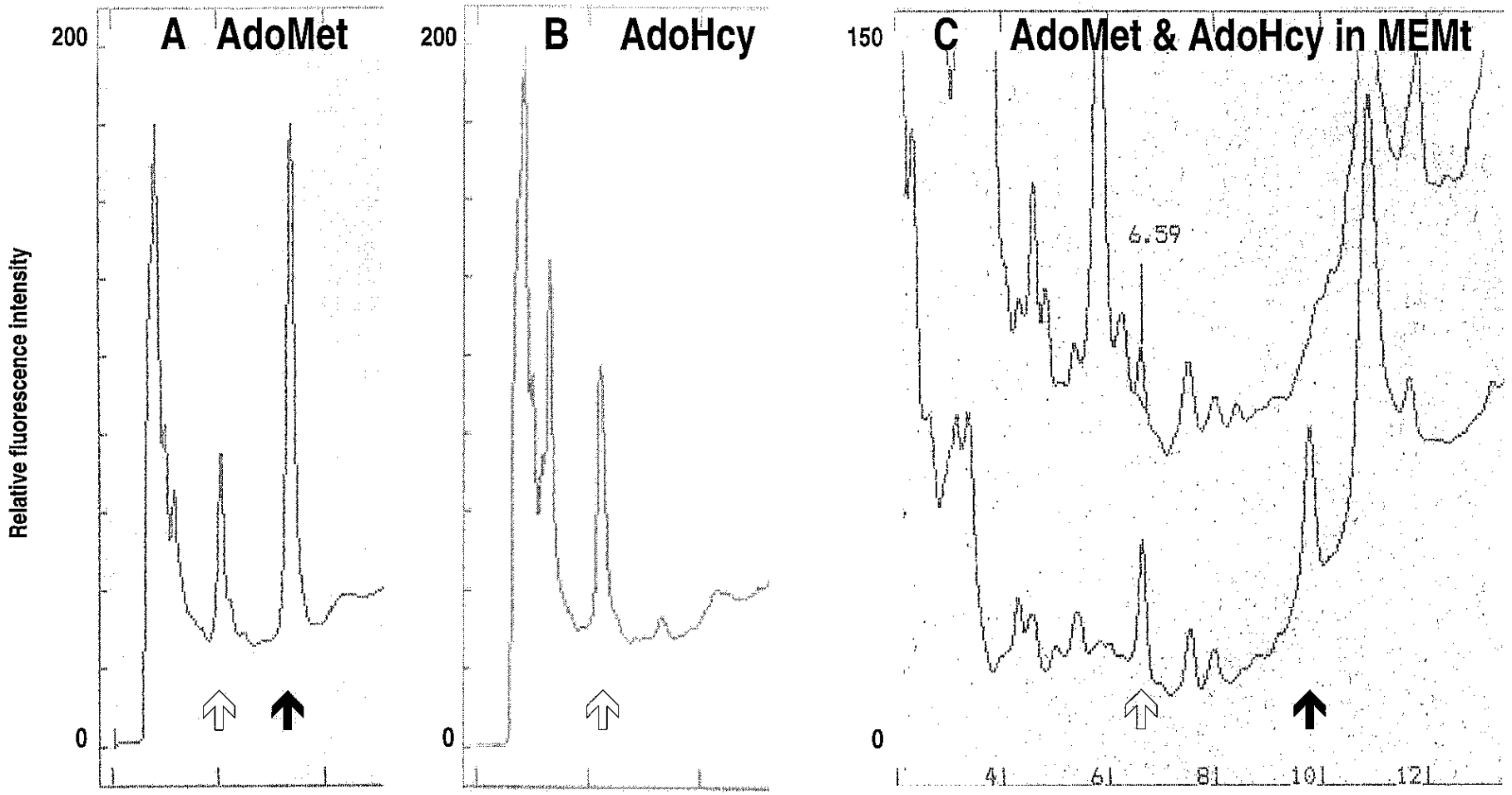
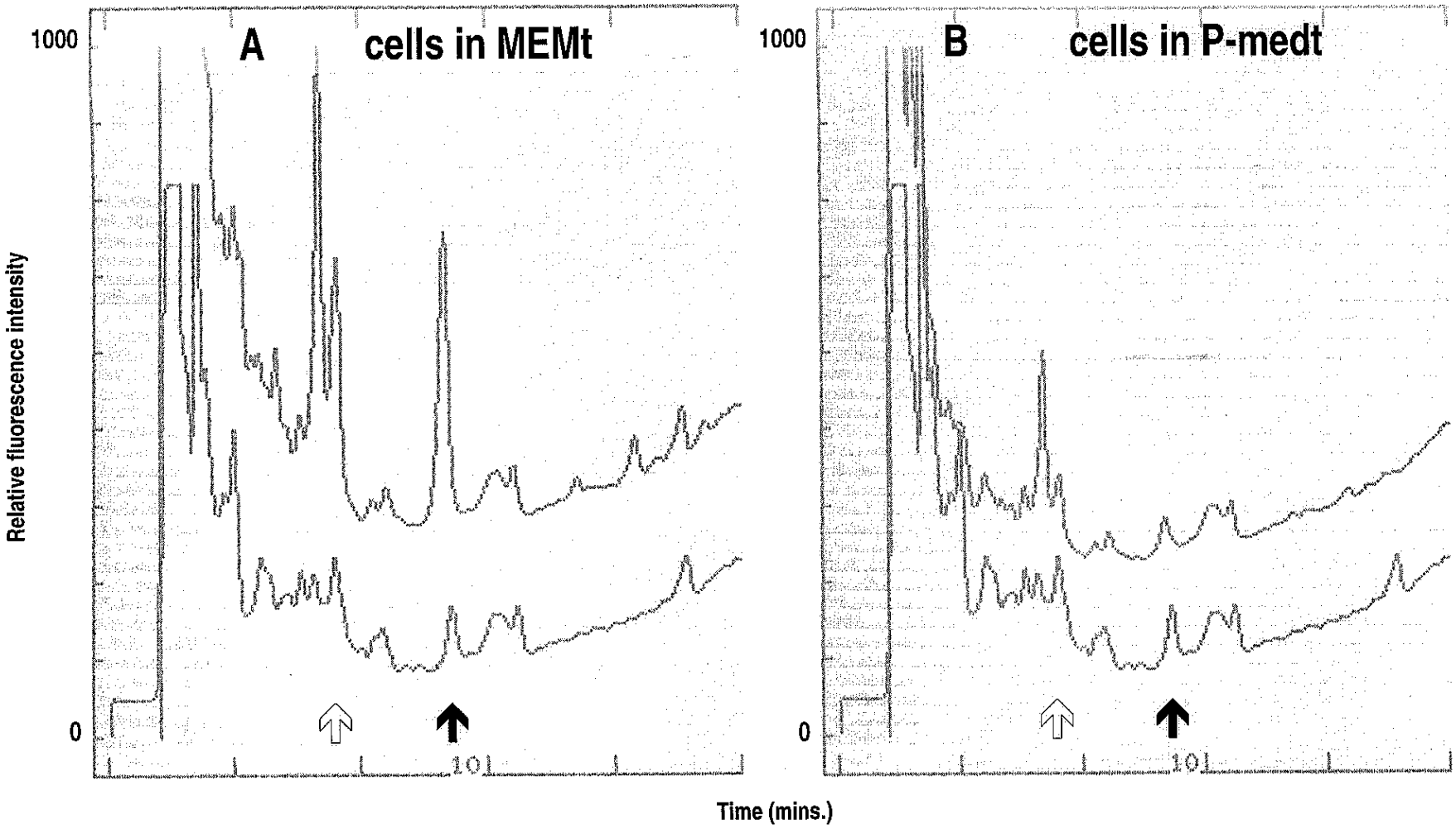


Figure 4.9B Chromatogram of AdoMet & AdoHcy in cells

A upper trace = cell line 6 growing in MEMt for 7 days; lower trace = mixed AdoMet & Adohcy standards;
⇑ AdoMet (retention times, 8.86 mins. for cells, 9.04 mins. for standard); ⇧ AdoHcy (retention times, 6.04 mins. for cells, 5.98 mins. for standard);

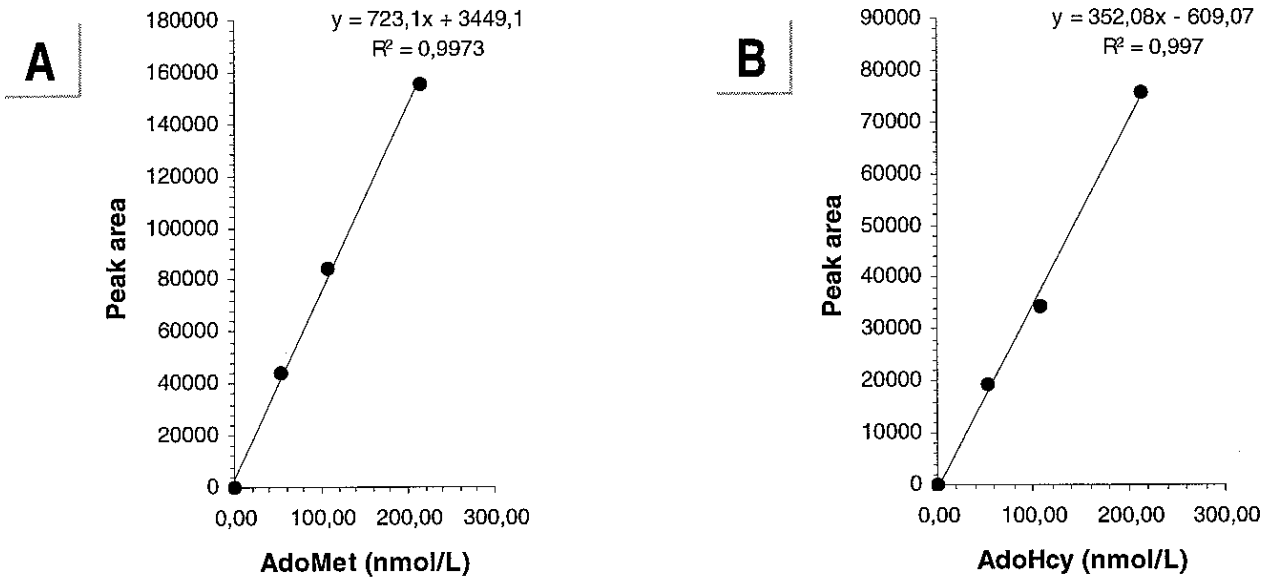
B upper trace = cell line 6 growing in P-medt for 7 days; lower trace = mixed AdoMet & Adohcy standards;
⇑ AdoMet (retention times, 8.88 mins. for cells, 9.04 mins. for standard); ⇧ AdoHcy (retention times, 6.03 mins. for cells, 5.98 mins. for standard);



Standards, calibration & linearity

Calibration standards were prepared by the serial dilution of an accurately weighed amount of approximately 1 mg AdoMet or AdoHcy in 5 ml 0.4 mol/L PCA in distilled-water. Further dilutions were made with distilled-water or lysing buffer, depending on the sample to be analysed. Even though separation of AdoMet and AdoHcy could be achieved in a single run, standards were always prepared and analysed separately, as AdoMet with a purity of only 72%, contains amounts of AdoHcy. A four-point calibration curve was included in all analyses (**Figure 4.10A** & **B**). The measurement of both adenosyl compounds were previously shown to be linear to 110 nmol/L for AdoMet and 160 nmol/L for AdoHcy.

Figure 4.10 Standard curves for AdoMet & AdoHcy



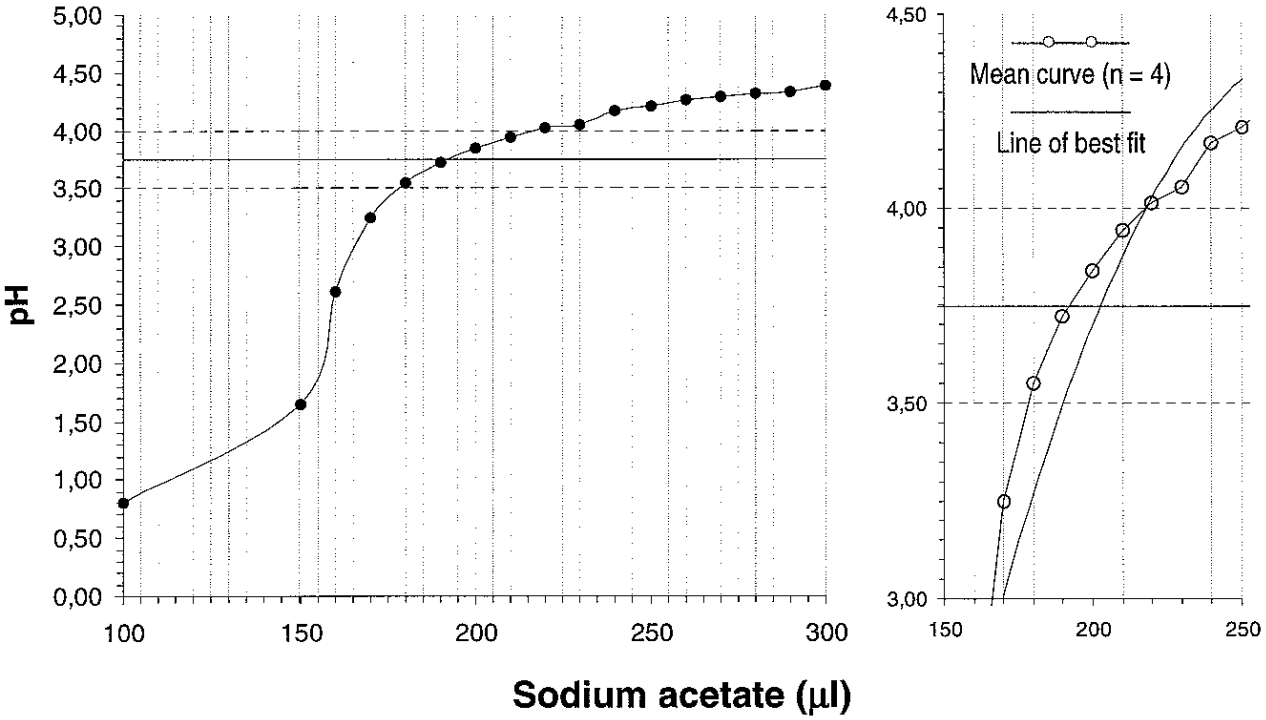
Examples of the calibration curves for the adenosyl compounds **A** AdoMet & **B** AdoHcy — corrected for purity (AdoMet 72 & 88%, AdoHcy 100%, based on information provided by the supplier, Sigma), dilution (50 : 50 with distilled-water, dilution = 2), 40 µl injection volume (cell extracts), separate runs, dilution factor from original sample = 1.625. Precision in intra- & inter-day assays were 4.0 & 4.5% for AdoMet & 2.8 & 5.0% for AdoHcy.

Modification of pH correction step

The existing method of sample preparation involved the individual pH adjustment of samples by the addition of sodium acetate and the use of pH paper (calibrated in 1 unit increments) as a means of control. To better facilitate this procedure, a number of representative deproteinised samples were prepared in 5- or 10-fold higher volumes than required, and after mixing with 250 µl CCA, the pH was measured by pH meter (Metrohm E 632) during the addition of sodium acetate. Similarly to the preparation of standards and samples for analyses, all determinations took place at RT. **Figure 4.11** shows the mean curve (n = 4) generated from the addition of 3 mol/L sodium acetate in 10 µl increments (from 150-300 µl) to a 1 ml aliquot containing 51.5% (v/v) lysing buffer pH 7.3, 28.5% (v/v) 10% (v/v) PCA in distilled-water and 20% (v/v) CCA that had been taken from a representative "deproteinised cell extracts" containing 900 µl lysing buffer pH 7.3 and 563 µl 10% (v/v) PCA in distilled-water. Similar curves were obtained for media and FCS. These were all 1 ml

aliquots that had been taken from representative "deproteinised media and FCS samples" made up in either 1 ml MEMt (pH 7.65), P-medt (pH 7.97) or FCS (pH 7.55) and 625 µl 10% (v/v) PCA in distilled-water. A full summary of the volumes determined along the line of best fit is shown in **Table 4.4.** For example, a pH of 3.75 (3.50-4.00) was obtained with media sample standard 0 (1 ml 50 : 50 mix of 0.4 mol/L PCA and distilled-water) by adding 219.2 µl (204.2-234.3 µl) sodium acetate (10-fold), and with cell sample standard 0 (1 ml 50 : 50 mix of 0.4 mol/L PCA and lysing buffer) by adding 108.4 µl (100.3-116.4 µl) sodium acetate. Based on this, a fixed volume of sodium acetate was chosen according to the nature of the sample, which meant that the procedure was less time consuming, thawed samples spent less time on the bench before derivatisation and no sample volume was lost.

Figure 4.11 pH adjustment of the lysing buffer preparation with sodium acetate



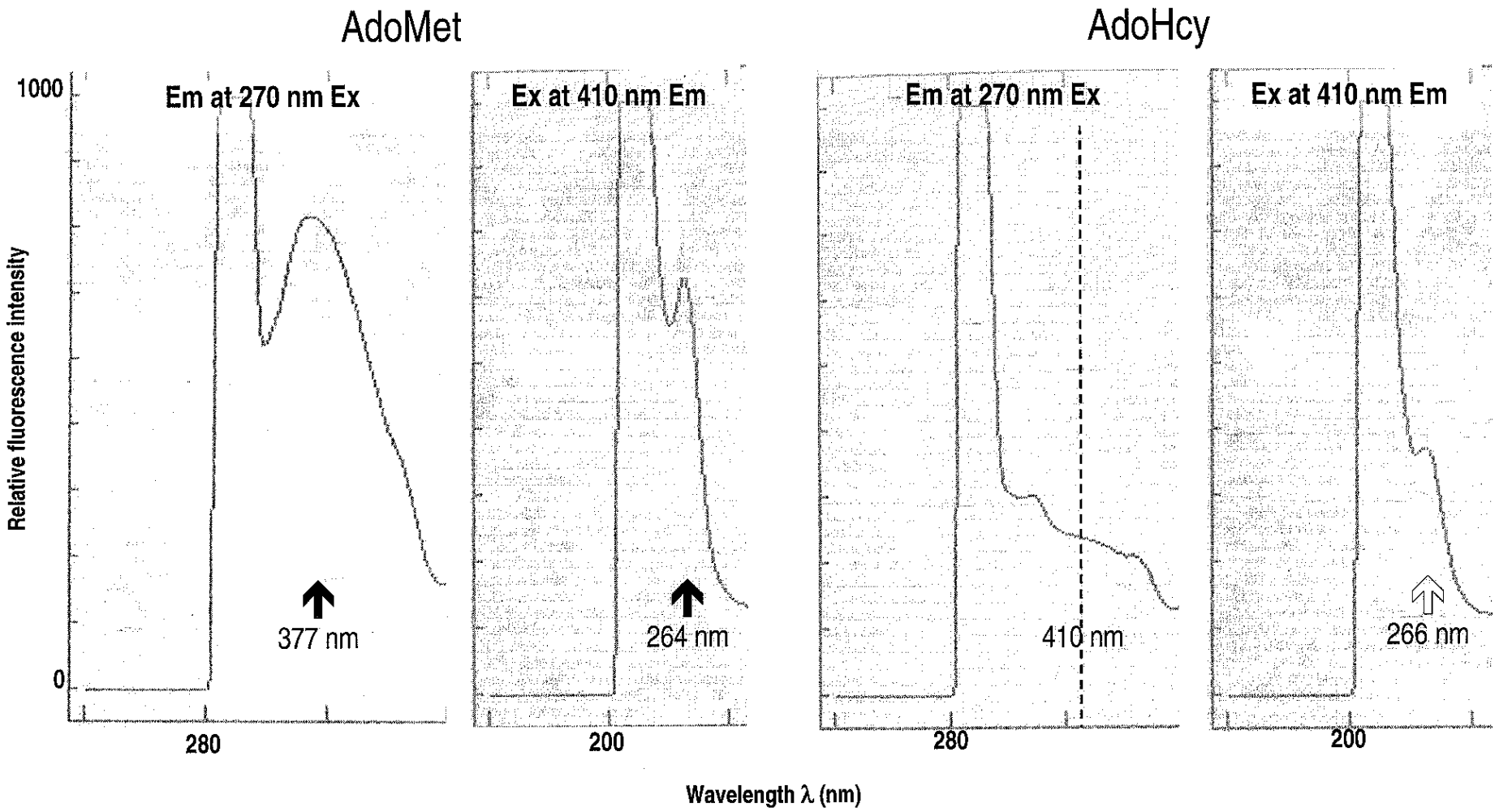
Scanning of emission & excitation spectra

During the measurement of the intracellular adenosyl compounds, it was noted that the levels of AdoHcy were very high in relation to both those previously reported and AdoMet (see Chapter 7, page 137). Although it was strongly suspected that another compound was co-eluting with AdoHcy, it proved difficult to confirm by simply altering the parameters controlling separation. Nevertheless, the rapid scan feature on the FP-1520 detector allowed on-the-flow spectral acquisition for both emission (Em) and excitation (Ex) spectra, taken at the relevant retention times, without interrupting the chromatographic elution. Stored spectra could then be retrieved and displayed on the PC (recorder, integrator) after designating an output range. Therefore, in attempts to verify the presence of AdoHcy in sample chromatograms, scanning of peaks was tested. As shown in **Figure 4.12** however, although AdoMet gave both Em and Ex maxima as expected, AdoHcy gave only an Ex maximum, this approach could therefore not be used.

Figure 4.12 Spectra for AdoMet & AdoHcy standards

AdoMet at 270 nm Ex, AdoMet gave maximum Em at 377 nm; and at 410 nm Em, AdoMet gave maximum Ex at 264 nm

AdoHcy at 270 nm Ex, AdoHcy gave no Em around 410 nm; but at 410 nm Em, AdoHcy gave maximum Ex at 266 nm



Liquid chromatography - mass spectroscopy (LC/MS) of AdoHcy

To further establish whether the peaks detected were AdoHcy or not, LC/MS was finally employed under the supervision of PD Dr. G. Imanidis in his laboratory at the Institute of Pharmaceutical Technology, Pharmazentrum, University of Basel.

Apparatus

Separation and analysis were performed with a HPLC series 1050 system (Hewlett Packard) and a reverse-phase ODS column and pre-column (Waters Spherisorb®, 125 mm x 4 mm x 5 µm), which were coupled to a mass spectrometer with uv-detector (Autosizer 1000 HsA and Mastersizer X, Malvern Instruments).

Principle of assay

After solution components have been separated by liquid chromatography, the molecule of interest is ionised by an ion source (e.g. electrospray ionisation {ESI}) inducing electron ejection, protonation or deprotonation. Ions are then differentiated according to their mass : charge ratio (m/z) by a mass analyser. The ion beam current is then measured by a detector, producing a mass spectrum that provides information on molecular weight and even structure.

Sample preparation

Cells from confluent stock cultures were harvested with dissociation solution, obtained as a pellet by centrifugation, lysed as described in Chapter 3 (page 62) and lysates treated as described for AdoMet and AdoHcy in Chapter 7 (page 126). For LC/MS analysis, both derivatised and underivatised lysates, positive controls of AdoHcy standards in 0.4 mol/L PCA and distilled-water, and negative controls of lysing buffer and 0.4 mol/L PCA were prepared. Modifications to the mobile phase included removal of the ion-pair reagent heptasulphonic acid and replacement of the 0.1 mol/L sodium acetate trihydrate with 0.01 mol/L ammonium acetate (Fluka). Flow rate and injection volume were 0.25 ml/minute and 40 µl respectively.

Results

With a wavelength of 258 nm and molecular weight limits of 384.7-385.7, AdoHcy was undetectable in derivatised and underivatised cell extracts, being only quantifiable at or above 26 nmol/L, 91 nmol/L lower than the mean level found in control cells in P-medt at 14 days by HPLC analysis. This supports the idea that the peak seen in chromatograms in the AdoHcy position is likely due to an interfering substance, so that it was unfortunately not possible to include AdoHcy data in this work.

Table 4.4 Summary of sodium acetate volumes calculated for a sample pH of 3.50-4.00

Sample (post-deproteinisation)	Mean sodium acetate volume (µl)		
	pH 3.50	pH 3.75	pH 4.00
Media & FCS (200 µl samples)			
100 µl 0.4 mol/L PCA (standard 0) + 100 µl distilled-water + 50 µl CCA + volume sodium acetate (µl)	20.42	21.92	23.43
100 µl adenosyl standard 1, 2, 3 etc. † + 100 µl distilled-water + 50 µl CCA + volume sodium acetate (µl)	"	"	"
200 µl MEMt + 50 µl CCA + volume sodium acetate (µl)	34.40	36.52	39.24
200 µl P-medt + 50 µl CCA + volume sodium acetate (µl)	37.20	38.73	40.82
200 µl FCS (100%) + 50 µl CCA + volume sodium acetate (µl)	34.13	36.40	39.33
Cells (100 µl samples)			
(100 µl 0.4 mol/L PCA {standard 0} + 100 µl lysing buffer) 100 µl mix + 25 µl CCA + volume sodium acetate (µl)	10.03	10.84	11.64
(100 µl adenosyl standard 1, 2, 3 etc. † + 100 µl lysing buffer) 100 µl mix + 25 µl CCA + volume sodium acetate (µl)	"	"	"
100 µl cell sample + 25 µl CCA + volume sodium acetate (µl)	18.84	20.11	21.70

† diluted 50 : 50 with distilled-water

Amino acid measurement by ion-exchange chromatography

Principle of assay

Since amino acids differ in their acid-base dissociation constants (or pK_a values), separation can be accomplished by using a cation-exchange resin (sulphonated divinylbenzene cross-linked polystyrene beads) column, temperature programming and a series of buffers of increasing pH and ionic strength.

The S 432 Sykam system used 3-lithium citrate buffers and separation was achieved by gradient elution of the acidic and neutral amino acids followed by isocratic elution of the basic amino acids, whereas with the Biochrom 20 Plus system which used 5-lithium citrate buffers, it was a completely stepwise isocratic elution. A full summary of the buffers and column conditions can be seen in **Table 4.5.** After separation, the column eluent was derivatised using ninhydrin (1,2,3-triketohydrindene hydrate) in a high-temperature reaction coil (with the S 432 Sykam system 125°C, the Biochrom 20 Plus system 135°C), which formed purple or yellow complexes with α-substituted amino acids. Quantities of amino acids were determined by comparing absorbances of the complexes measured at 570 and 440 nm (imino acids e.g. proline and hydroxyproline) with standard curves constructed for each amino acid to account for any differences in the degree of colour produced.

Table 4.5 S 432 Sykam & Biochrom 20 Plus buffer & column conditions

	S 432 Sykam		Biochrom 20 Plus	
Column	25 x 0.4 cm (LCA K07)		High pressure PEEK	
Cation-exchange resin	CK10F (Mitsubishi©)		Ultropac 8 (sodium or lithium)	
Column temperature (°C)	37-70		34-80	
Lithium citrate buffers	**Molarity (M)**	**pH**	**Molarity (M)**	**pH**
1	0.15	2.80	0.20	2.80
2	0.60	4.00	0.30	3.00
3	1.40	3.60	0.50	3.15
4	-	-	0.90	3.50
5	-	-	1.65	3.55

Sample preparation of media, FCS & cultured cell extracts

200 µl samples of media (basal and complete) and FCS were deproteinised with 50 µl 10% (w/v) sulphosalicylic acid (SSA, in distilled-water), allowed to stand at RT for 30 minutes, centrifuged at 10000 x g for 2-3 minutes and supernatants stored at –20°C. For cell extracts, cells from 3 flasks were pooled, obtained as a pellet by centrifugation at 850 x g for 10 minutes and lysed with 200 µl lysing buffer. After a 10 µl sample was removed for protein determination, 190 µl was deproteinised with 50 µl 10% (w/v) SSA (dilution factor = 2.53), centrifuged and supernatants stored as above.

Ion-exchange chromatography

Supernatant aliquots of 100 µl were mixed with an equal volume of sample dilution buffer containing 150 µmol/L norvaline as IS and analysed. Injection volumes were 100 µl for the Sykam system and 50 µl for the Biochrom system.

Figure 4.13 Chromatogram of amino acid standards

a-Aada = L-α-aminoadipic acid; a-ABS = L-α-amino-n-butyric acid; b-AIBA = β-aminoisobutyric acid; g-ABA = γ-aminobutyrate

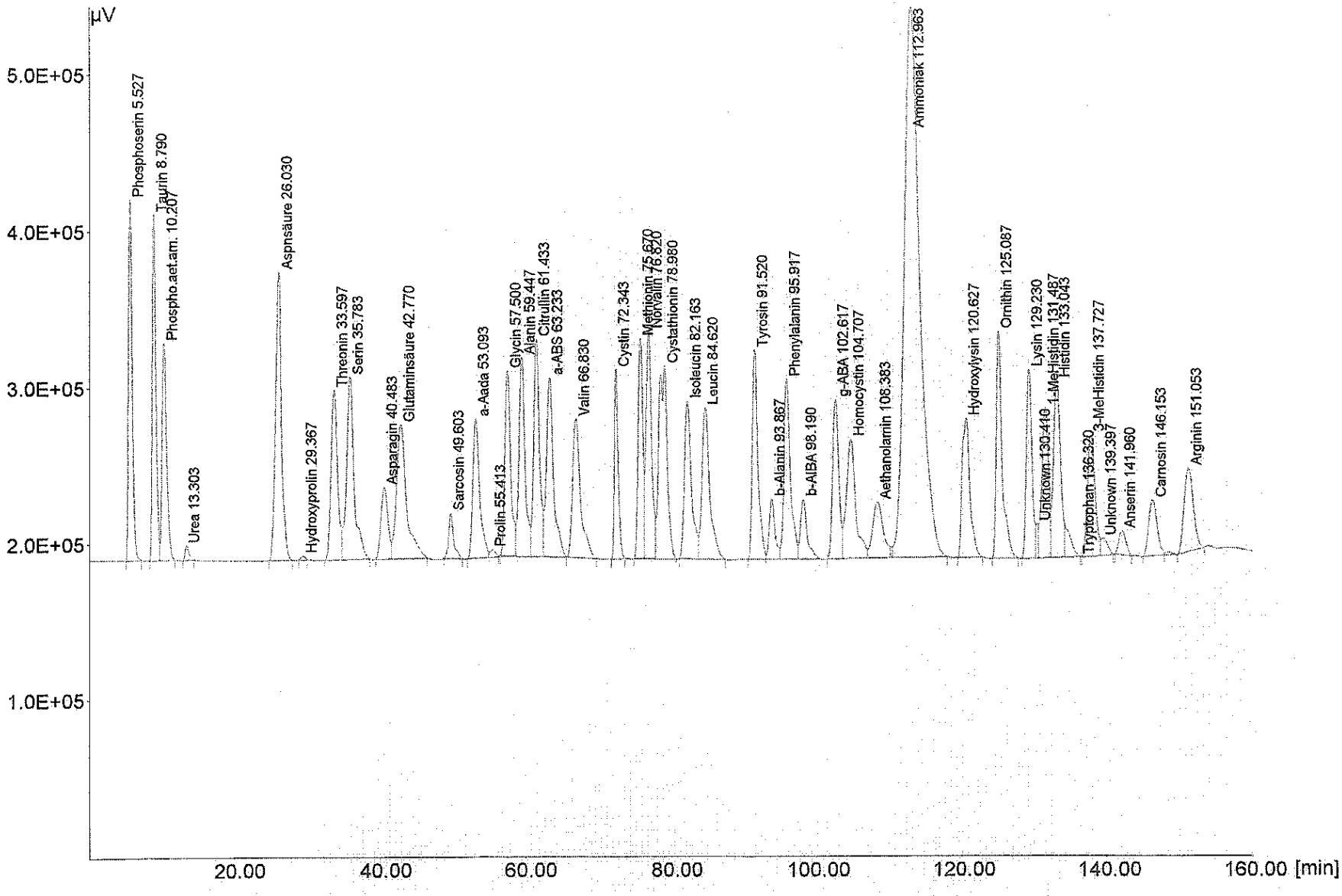


Figure 4.14A Chromatogram of free amino acids in cell line 5 growing in MEMt for 7 days

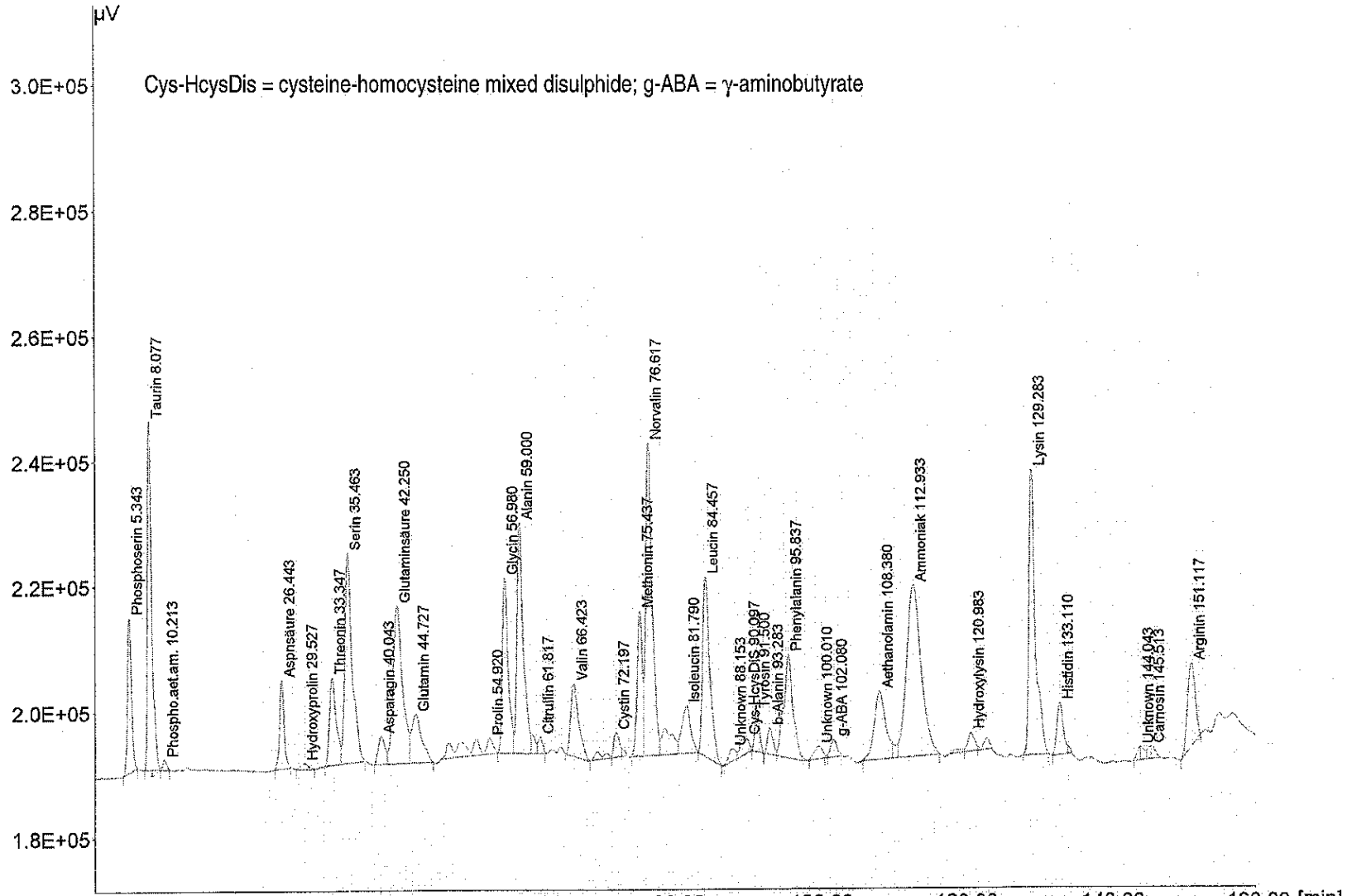
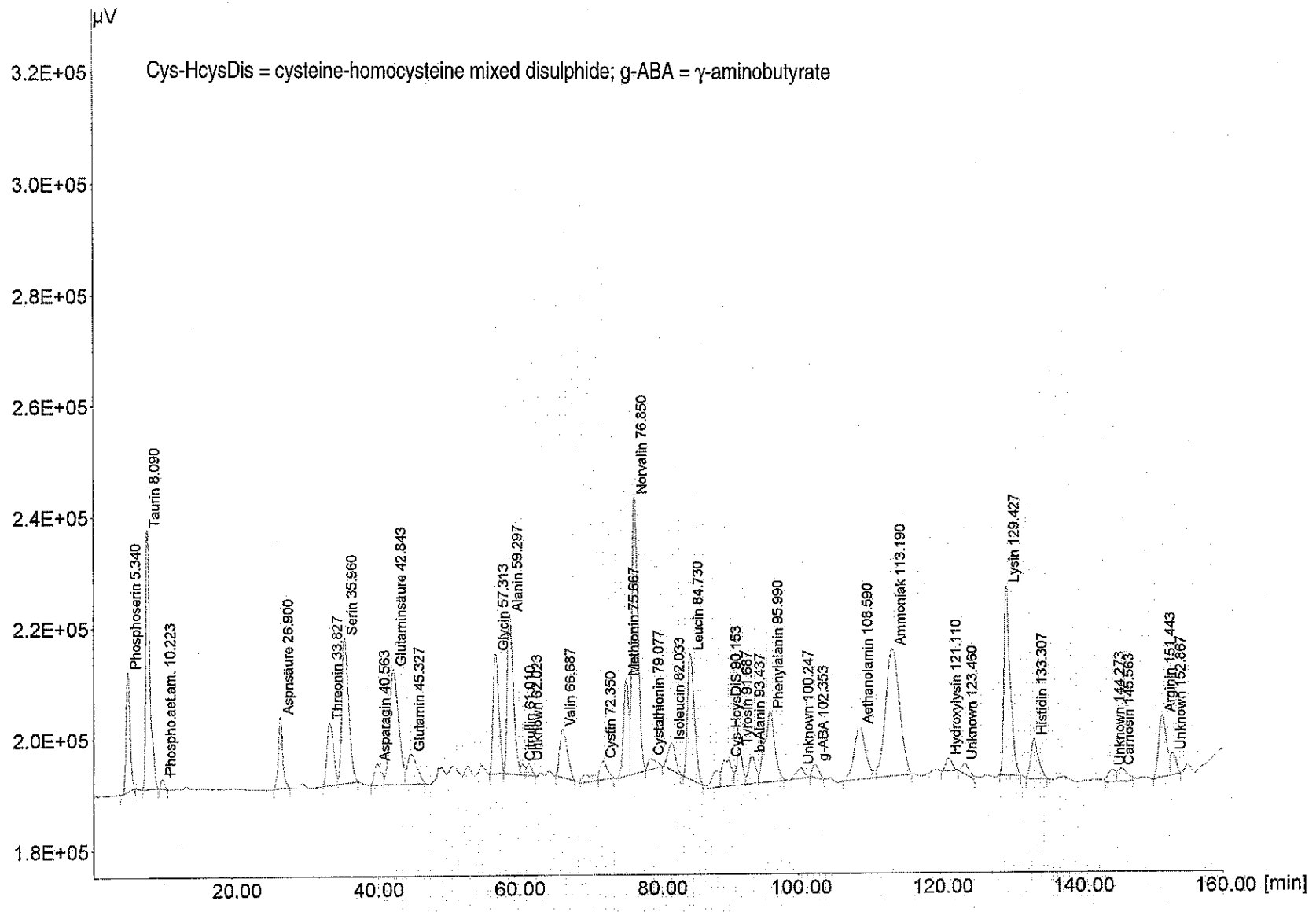


Figure 4.14B Chromatogram of free amino acids in cell line 5 growing in P-medt for 7 days



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Preparation & quality control of culture media

The basal physiological medium (P-med) for this work was designed on the components contained in two formulations of MEM and took into account the factors described in Chapter 1 (pages 42-44). Concentrations were based on mean reference values (from over 25 independent sources)[1] of the physiological levels of L-form amino acids and vitamins found in normal human plasma and summarised in **Tables 1.14** & **1.15**. This covered the age range from premature (first 6 weeks) to adult (> 18 years) and included the differences between sexes.

Basal media preparation

MEM was prepared in 500 ml batches from commercial MEM (Life Technologies™ cat.-nos. 041-01091 and 21090-022 or BioConcept Amimed® cat.-no. 1-31F01-I) containing Earle's salts but without L-glutamine, pH approximately 7.3 (7.2-7.4) at room temperature (RT) (for full composition refer to **Table 5.1**) + 25 mmol/L N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES), pH corrected to 7.3 at RT.

P-med was usually made up at working concentration in 2 L batches in double-distilled water (ddH_2O, < 10 μ{S}iemens/cm at RT)[2] using chemicals of the highest purity available, with 6 inorganic salts ($CaCl_2$ {anhyd.}, KCl, $MgSO_4$-$7H_2O$, NaCl, $NaHCO_3$ & NaH_2PO_4-H_2O), D-glucose, 12 amino acids (L-arginine-HCl, L-cystine, L-histidine HCl-H_2O, L-isoleucine, L-leucine, L-lysine-HCl, L-methionine, L-phenylalanine, L-threonine, L-tryptophan, L-tyrosine & L-valine), 1 peptide (L-alanyl-L-glutamine) and 8 vitamins (B_1, B_2, B_6, choline chloride, folic acid, *myo*-inositol, niacin & pantothenic acid) (**Table 5.1**) + 25 mmol/L HEPES, pH 7.3 at RT.

All the vitamins except choline chloride, i-inositol and nicotinamide were diluted in distilled-water from freshly made concentrates or well-mixed suspensions in the case of the folic acid and riboflavin before addition. Basal P-med was mixed by stirring overnight (after sealing the mixing vessel tightly with Parafilm M®) to insure complete dissolution of the components[3]. All media prior to storage was pH corrected to 0.02 units lower than the required pH with solid, then with 1 mol/L sodium hydroxide (NaOH) to avoid dilution of components. This was then filter sterilised through a 0.22 μm cellulose acetate, low protein binding membrane in a vacuum filtration system (Corning®). Basal media were stored at 4°C for no longer than three months. Samples from each fresh batch of basal P-med were taken and stored at –20°C for the purposes of quality control.

[1] see References (page 104)

[2] minimum acceptable level of purity is < 10 μS/cm, but was regularly measured at < 3 μS/cm at maximum

[3] also used as an alternative to preparing the cystine (being the only amino acid not readily soluble in distilled-water at RT) in a 25 mmol/L solution of NaOH

Table 5.1 Component comparison between P-med & MEM

	MEM		P-med
INORGANIC SALTS:	**mg/L**		**mg/L**
$CaCl_2$ (anhyd.)	200		200
KCl	400		400
$MgSO_4$-$7H_2O$	200		200
NaCl	6800		6800
$NaHCO_3$	2200		2200
NaH_2PO_4-H_2O †	140		140
OTHER COMPONENTS:	**mg/L**		**mg/L**
D-Glucose	1000		1000
Phenol red	10		-
AMINO ACIDS:	**mg/L**	**RATIO MEM : PLASMA**	**mg/L**
L-Arginine-HCl	126	9.7 x ↑	13.0
L-Cystine	24	2.3 x ↑	10.5
L-Glutamine	292	3.5 x ↑	-
L-Alanyl-L-glutamine	-	-	109.5
L-Histidine HCl-H_2O	42	3.9 x ↑	10.7
L-Isoleucine	52	6.5 x ↑	8.0
L-Leucine	52	3.0 x ↑	17.3
L-Lysine-HCl	72.8	3.5 x ↑	20.8
L-Methionine	15	2.8 x ↑	5.3
L-Phenylalanine	32	2.3 x ↑	13.7
L-Threonine	48	2.5 x ↑	19.6
L-Tryptophan	10	0.1 x ↓	11.2
L-Tyrosine	36	1.7 x ↑	20.8
L-Valine	46	2.2 x ↑	21.1

			PLASMA CONCENTRATION	
AMINO ACIDS:	**mg/L**	**RATIO MEM : PLASMA**	**mg/L**	**µmol/L**
L-Arginine-HCl	126	9.7 x ↑	11.3-15.1	64.9-86.7
L-Cystine	24	2.3 x ↑	6.0-13.8	25.0-57.4
L-Glutamine	292	3.5 x ↑	70.2-104.3	480.5-713.9
L-Alanyl-L-glutamine	-	-	-	-
L-Histidine HCl-H_2O	42	3.9 x ↑	0.6-16.0	3.9-103.1
L-Isoleucine	52	6.5 x ↑	7.1-9.1	54.1-69.4
L-Leucine	52	3.0 x ↑	13.3-24.3	101.4-185.2
L-Lysine-HCl	72.8	3.5 x ↑	2.9-30.5	19.8-208.6
L-Methionine	15	2.8 x ↑	3.8-9.6	25.5-64.3
L-Phenylalanine	32	2.3 x ↑	8.8-25.7	53.3-155.6
L-Threonine	48	2.5 x ↑	11.8-28.6	99.1-240.1
L-Tryptophan	10	0.1 x ↓	6.1-16.7	29.9-81.8
L-Tyrosine	36	1.7 x ↑	11.8-51.4	65.1-283.7
L-Valine	46	2.2 x ↑	16.2-26.6	138.3-227.2

				PLASMA CONCENTRATION	
VITAMINS:	**mg/L**	**RATIO MEM : PLASMA**	**mg/L**	**mg/L**	**µmol/L**
D-Ca-pantothenate	1	2.9 x ↑	0.4	0.2-0.7	1.0-3.4
Choline chloride	1	1.0 x ↔	1.0	nd	nd
Folic acid	1	100.0 x ↑	0.01	0.01	0.02
i-Inositol	2	1.0 x ↔	2.0	nd	nd
Nicotinamide	1	1.3 x ↑	0.8	0.8	6.6
Pyridoxal-HCl	1	41.7 x ↑	0.02	0.01-0.04	0.05-0.20
Riboflavin	0.1	0.6 x ↓	0.2	0.1-0.2	0.3-0.5
Thiamine-HCl	1	47.6 x ↑	0.02	0.01-0.04	0.6-1.9

† originally NaH_2PO_4-$2H_2O$; nd = not determined, no variation from MEM

Complete media preparation

Three different types of complete media were employed in the study, ① laboratory prepared MEM complete (MEMc) for the non-experimental sub-cultures, and two types of metabolite test (t) media ② MEMt (the supplemented state) and ③ P-medt (the basal state) for the experimental sub-cultures. These were all prepared as follows:

① MEMc = 500 ml (88.8% {v/v}) basal MEM + 50 ml (9.7% {v/v}) non heat-inactivated foetal calf serum (FCS-test {t}, FCS-A or FCS-B)[4] + 7 ml (1.3% {v/v}, 100x concentrated, 200 mmol/L) L-glutamine in a 0.85% (w/v) NaCl solution + 6 ml (1.1% {v/v}, 100x concentrated) antibiotic/antimycotic (AA) solution[5].

② MEMt = 88.0% (v/v) basal MEM + 10.0% (v/v) FCS-t + 1.0% (v/v) L-glutamine solution + 1.0% (v/v) AA solution.

③ P-medt = 88.0% (v/v) basal P-med + 10.0% (v/v) FCS-t + 1.0% (v/v) AA solution + 1.0% (v/v) 0.85% (w/v) NaCl solution[6].

MEMt and P-medt were always freshly prepared using the same stock concentrations of L-glutamine and AA solution for MEMc. Aliquots of the FCS-t (which were protected from light), FCS-A and FCS-B, L-glutamine and AA solution were stored at –20°C in volumes of 50 and 10 ml, 1 ml and 1 ml respectively. The non heat-inactivated foetal calf serum designated FCS-t was used only in the test media. There was no addition of L-glutamine to P-medt as the basal medium contained a physiological level of the peptide L-alanyl-L-glutamine (L-Ala-L-Gln, Sigma). This has a much greater stability than glutamine, which unlike most amino acids is labile in solution, and was required at the physiological concentration of 82.50 mg/L (the corrected concentration of glutamine due to the alanyl moiety was 109.48 mg/L). All complete media (MEMc, MEMt and P-medt) were pre-warmed to 37°C immediately before use.

Quality control of P-med(t) & MEM(t)

Physicochemical properties

Basal P-med was a clear, sterile solution with a pH of 7.3 at RT, an osmolality comparable to that of MEM and it contained amino acids at concentrations that were concordant with the chosen values based on average normal values. As a complete medium and by replenishing every 4-5 days in a similar manner to that with MEMc, it not only sustained cells over two passages but through long-term sub-culture (observational data). Cell growth measured as total protein increase by the SRB method was comparable to that of MEM, therefore excluding any significant variation in the levels of the other components such as salts, glucose and vitamins.

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[4] FCS-t, FCS-A and FCS-B refer to the various serum batches used

[5] AA solution contained 10,000 units Penicillin G (Na-salt), 10,000 µg Streptomycin sulphate and 25 µg Amphotericin B as Fungizone®/ml in a 0.85% (w/v) NaCl solution

[6] 0.85% (w/v) NaCl solution made up in distilled-water, sterilised through a 0.22 µm syringe filter, final pH 7.3

pH & osmolality

Before measurements were made, media constituents were thawed, combined and allowed to reach RT.

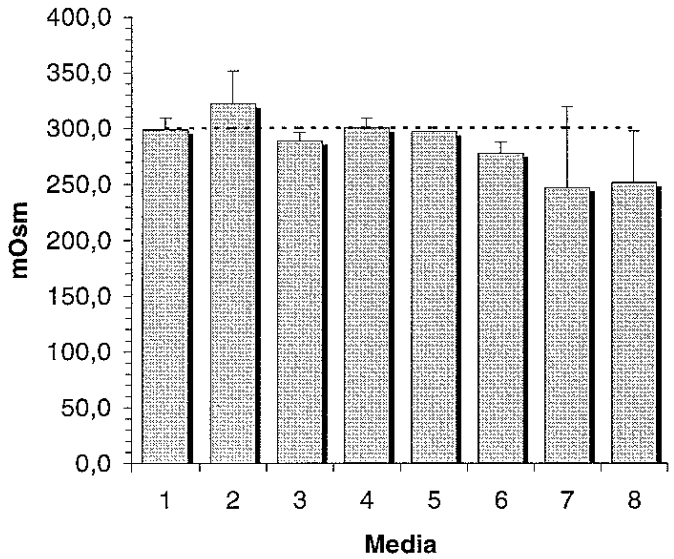
pH

It was decided to include in P-med as in MEM both bicarbonate in the form of $NaHCO_3$ at 26.19 mmol/L (with 5% CO_2) and HEPES at 25 mmol/L, thereby ensuring moderate to high buffering capacity. This was considered especially important with this model, to be sure substrate uptake was due to either metabolic requirement and/or extracellular concentration and not pH variation. The pH at RT after the addition of FCS of P-medt was 7.97 (SEM±0.58, n = 9) and MEMt 7.65 (SEM±0.02, n = 9). A mean difference of 0.32 units, but because of the nature of HEPES buffering, this was expected to fall between 0.2-0.3 units at 37°C and be within the required range for normal cell growth and function.

Osmolality

Chemically defined media should, if carefully prepared, be reproducible from batch to batch (**Figure 5.1**). The mean osmolality of P-med was 298.8 (SD±10.3, n = 6) mOsm and that of MEM was 322.3 (SD±29.7, n = 4) mOsm, a discrepancy of 23.5 mOsm or 7.6%. As both P-med and MEM contained identical inorganic salt, glucose and HEPES concentrations, their difference from one another was due to the physiological substrate levels, although with a mean increase of 11.3-fold in component concentration in MEM, the observed difference of 7.6% is less than expected. No osmoexpander (mannitol, sucrose etc.) was used in P-med to compensate for the lower osmolality, since the mean value was still within the 300 (±10%, 270-330) mOsm range stipulated for fibroblasts.

Figure 5.1 Osmolality of basal (P-med & MEM) & complete media (MEMc, MEMt & P-medt)



1 P-med mean = 298.8 (SD±10.3) n = 6
2 MEM mean = 322.3 (SD±29.7) n = 4

MEM mean reference values (Waymouth, 1970)
3 289 (SD±7) n = 25
4 300 (SD±9) n = 25
5 297 (range 278-314) n = 50

6 MEMc mean = 277.5 (SD±10.1) n = 4
7 MEMt mean = 247.0 (SD±72.3) n = 3
8 P-medt mean = 251.3 (SD±46.1) n = 3

As with plasma, dissolved oxygen and carbon dioxide in FCS can affect the osmolality (Meschia & Barron, 1956). Plasma or serum prepared from preserved blood may have raised osmolalities due to catabolism of high molecular weight components (proteins and polysaccharides) into smaller osmotically active

molecules, and to leakage of these ions from the cells before separation of the plasma (Popescu *et al.*, 1963). Addition of FCS-t (plus AA + glutamine for MEMt or AA + 0.85% {w/v} NaCl for P-medt) to the basal media however, caused a mean decrease in osmolality of 15.9% with P-med to P-medt (251.3 SD±46.1 mOsm, n = 3) and 23.4% with MEM to MEMt (247.0 SD±72.3 mOsm, n = 3). Even so, like that with pH, a difference in osmolality of < 2% between P-medt and MEMt would mean that substrate uptake was due to requirement and/or media concentration, not variation in osmolality.

Amino acids

As described in Chapter 4 (pages 89 & 90), amino acids except for cystine were analysed by ion-exchange chromatography (**Figure 5.2**). It was necessary to use the HPLC system described for homocysteine to measure cystine since this amino acid co-eluted with L-Ala-L-Gln in the ion-exchange system i.e. a retention time of 68.907 (SD±0.566, n = 4) minutes for cystine, 69.027 minutes for L-Ala-L-Gln. Cystine was consequently measured as its reduced thiol form and re-calculated using a four-point calibration curve. The L-Ala-L-Gln concentration was not determined. **Figure 5.3** shows the values obtained in P-med compared with the chosen target values (shown in **Table 1.14**). On comparing the obtained and target values, two main points were apparent, threonine, valine, isoleucine, leucine and arginine have standard deviations outside those of their corresponding target values, with isoleucine having the greatest discrepancy and cysteine, lysine, histidine and arginine were shown to be lower than their target values, the others higher. If the dissimilarity between the obtained and target value is expressed as a percentage of the target value, methionine and lysine had 0-5% disparity, cystine, tryptophan and arginine 5-10%, valine, leucine, tyrosine and phenylalanine 15-20%, and threonine, isoleucine and histidine > 20%, the modal difference being therefore 15-20%.

To better define the FCS composition, all batches were subjected to amino acid analysis (**Figure 5.4** & **Table 5.5**). This included both those for the routine culturing (FCS-A and -B) and the metabolite test media (FCS-t). With a 10% (v/v) dilution of FCS-t supplementing P-med, all amino acids still fell within the ranges of their physiological reference values.

Figure 5.2 Chromatogram of free amino acids levels in basal P-med

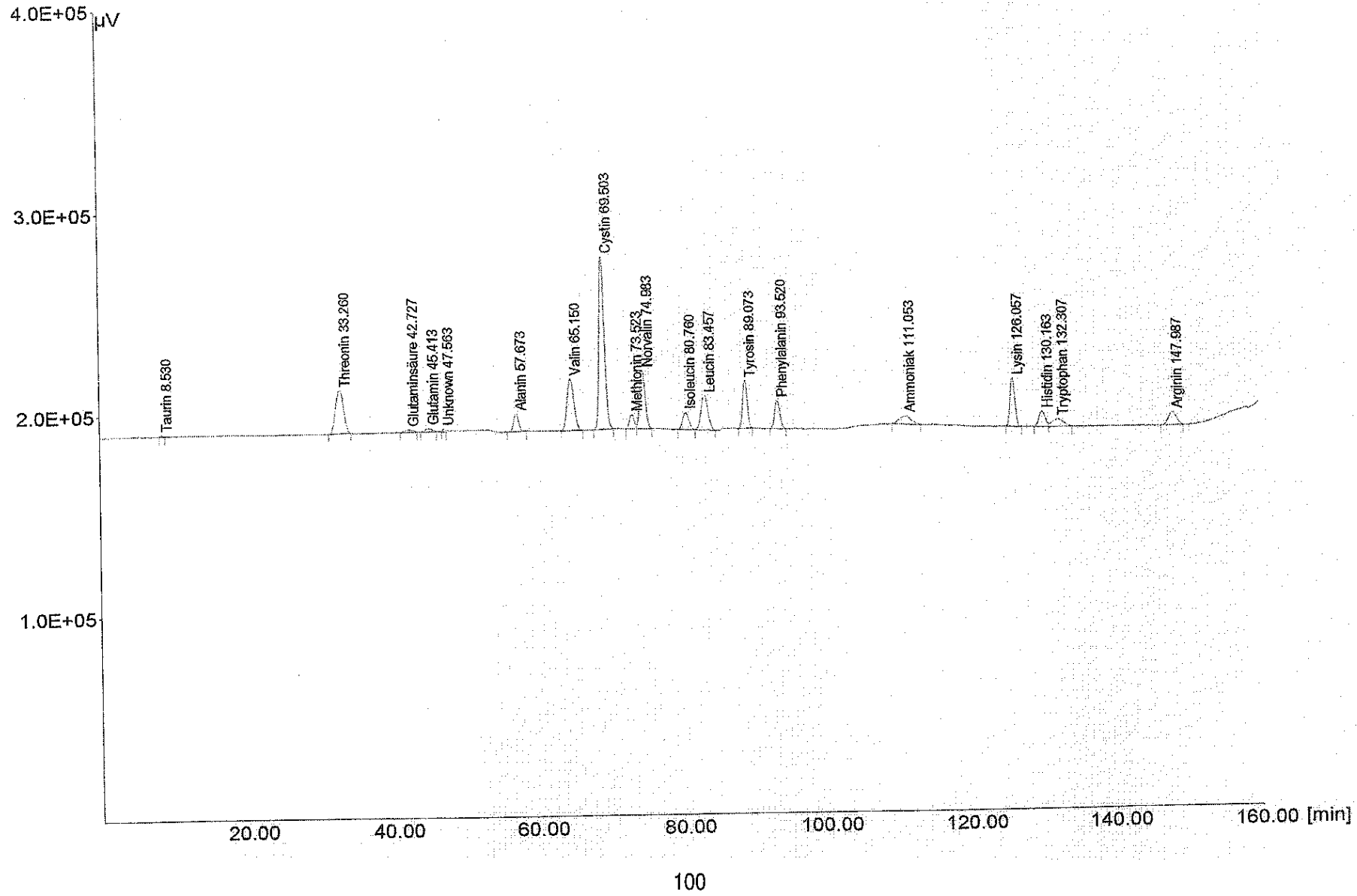
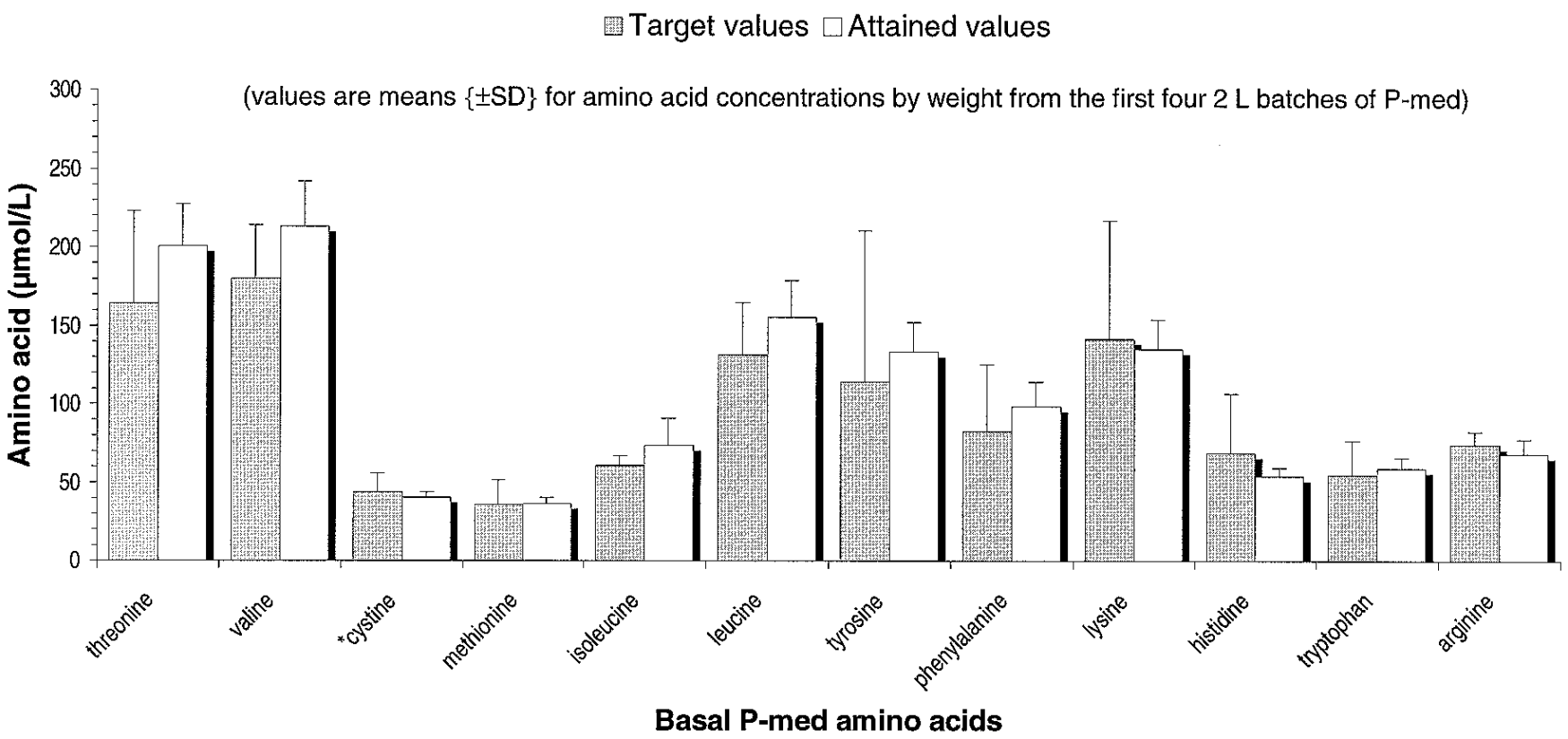


Figure 5.3 Ion-exchange chromatographic analysis of the free amino acid levels in basal P-med



* measured by the HPLC system described for homocysteine (see pages 66-71)

Figure 5.4 Chromatogram of free amino acids levels in FCS-t

a-AADA = L-α-aminoadipic acid; CHC = cysteine-homocysteine mixed disulphide

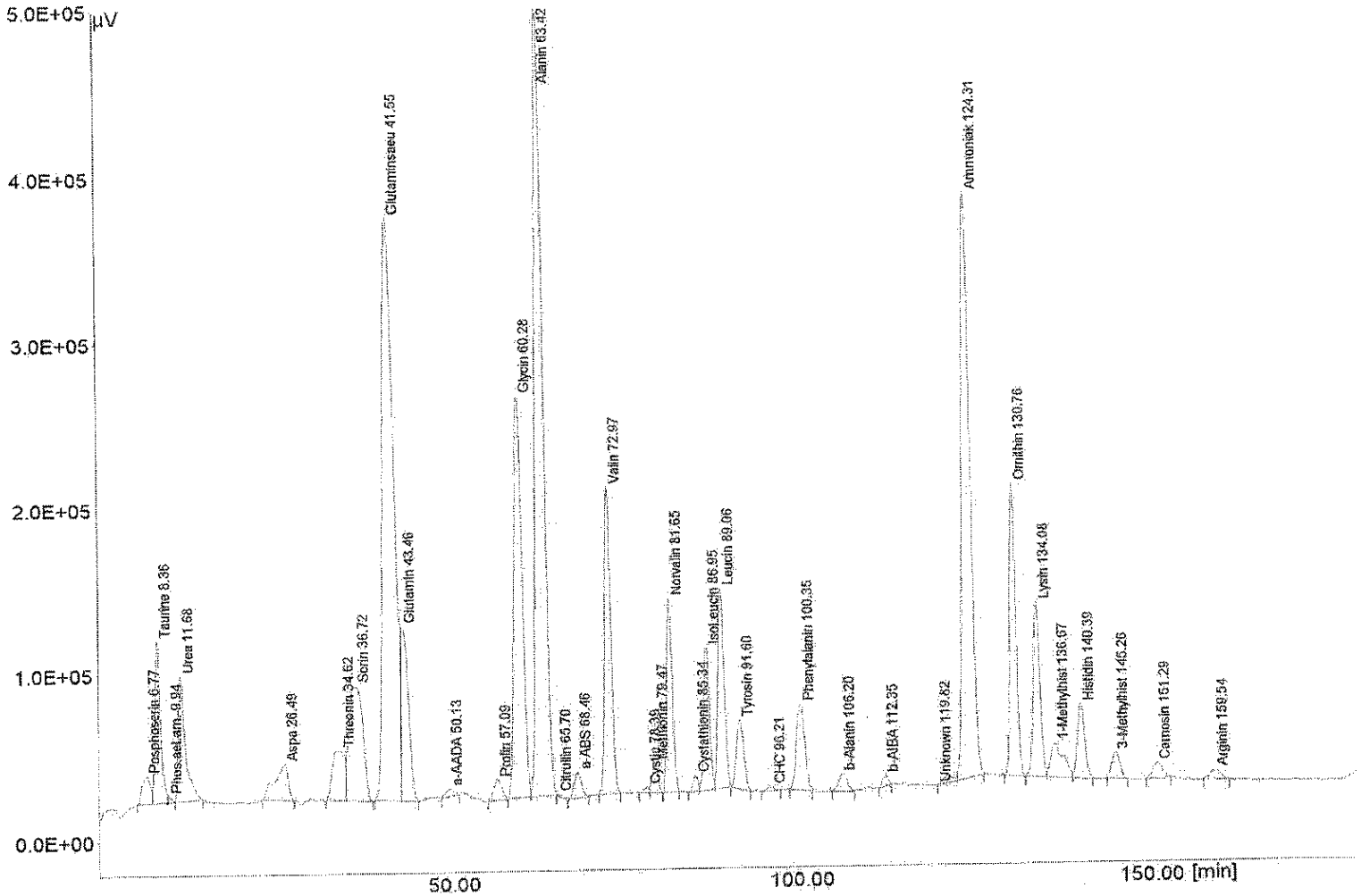


Table 5.5 Analysis of free amino acids in the three batches of FCS

Amino acid (or derivative)	FCS-t (µmol/L)	FCS-A (µmol/L)	FCS-B (µmol/L)
L-Taurine	173	120	122
L-Aspartic acid	95	62	63
L-Threonine	123	131	131
L-Serine	298	283	273
L-Glutamic acid	1287	906	860
L-Glutamine	232	470	523
L-Glycine	589	580	599
L-Alanine	1284	1020	992
L-Citrulline	nr	68	70
L-α-Amino-n-butyric acid (a-ABS)	35	31	39
L-Valine	376	346	329
L-Cystine	4	nd	nd
L-Methionine	29	30	29
L-Cystathionine	13	9	9
L-Isoleucine	155	128	130
L-Leucine	213	220	223
L-Tyrosine	83	96	89
β-Alanine (b-Alanin)	67	25	26
L-Phenylalanine	116	134	130
β-Aminoisobutyric acid (b-AIBA)	27	nd	nd
L-Ornithine	238	196	198
L-Lysine	184	185	194
$N^{\tau(1)}$-Methyl-L-histidine (1-Methylhist)	62	25	21
L-Histidine	82	79	67
$N^{\pi(3)}$-Methyl-L-histidine (3-Methylhist)	43	36	41
L-Carnosine	93	29	29
L-Arginine	19	36	32

nr = not resolved; nd = not detected

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Sulphydryl compound export from control fibroblasts

Introduction - preliminary studies

In order to measure sulphydryl compound export over time in the 6-well plate experiments, a number of preliminary studies were necessary to determine any differences in, ① background sulphydryl levels in media, ② stability of the measured sulphydryl compounds and ③ cell growth. A further consideration was as follows. In addition to the cystine in basal MEM and P-med, FCS-t contained homocysteine, cyst(e)ine, cysteinyl-glycine and glutathione. Although these compounds can exist as free (reduced) thiols, disulphides, mixed-disulphides and/or protein-bound mixed disulphides, the predominant forms are probably mixed-disulphides and/or protein-bound mixed disulphides. The dilemma faced with the presented data is that the method for HPLC determination i.e. derivatisation created all free (reduced) thiols or "total thiol" of each compound preventing distinction of these various forms. Nevertheless, to examine this a little further than just measuring total, it was decided to additionally measure free (non-protein bound) and protein-bound sulphydryl compound levels, where the total is the sum of the free and bound.

① Background sulphydryl compound levels in media

Sulphydryl compounds in the freshly prepared complete media P-medt and MEMt at time 0, would cause any measured changes in the sulphydryl compound levels from the experiments to be falsely raised. It was necessary therefore, for each individual experiment as described in Chapter 4 (pages 66-71), to measure and subtract the background levels of total, free and bound sulphydryl compounds caused by the basal media and FCS-t, the results of which are summarised in **Table 6.1.**

As no data exists on sulphydryl compound levels in FCS like those in human plasma, it is difficult to draw any conclusions from the presented data. On comparing FCS-t to human plasma, the percentages of free and bound homocysteine were more similar to one another than reported for human plasma (see page 20), but total homocysteine was 3-4-fold higher. It must be stressed that the reported values for bound homocysteine in human plasma were calculated and not measured. With the other compounds, total cyst(e)ine was 1-2-fold lower and total glutathione 4-5-fold higher, than in human plasma.

On comparing media with FCS-t (i.e. the 10% {v/v} figures), levels of total and bound homocysteine were in accordance with FCS-t, but free homocysteine was 2-fold higher. Total and free cysteinyl-glycine were in agreement with FCS-t, but bound cysteinyl-glycine was 3-fold higher. Total, free and bound glutathione corresponded with FCS-t. Total, free and bound cysteine could not be evaluated due to the cyst(e)ine levels in the basal media.

Table 6.1 Background levels of free & bound suphydryl compounds in media & FCS

	Sulphydryl compounds (µmol/L)											
	Homocysteine			Cyst(e)ine			Cysteinyl-glycine			Glutathione		
	total	free	bound	total	free	bound	total	free	bound	total	free	bound
MEMt	4.97±1.36	3.64±0.54 73.24%	1.83±0.62 36.82%	71.48±7.32	80.55±15.06 112.69%	4.13±1.21 5.78%	0.93±0.17	0.58±0.04 62.37%	0.28±0.10 30.11%	2.19±0.29	0.71±0.18 32.42%	0.80±0.11 36.53%
P-medt	4.55±0.59	3.55±0.36 78.02%	1.68±0.43 36.92%	33.06±3.01	35.26±5.61 106.65%	3.22±0.86 9.74%	0.89±0.11	0.60±0.09 67.42%	0.31±0.04 34.83%	2.12±0.26	0.78±0.15 36.79%	0.90±0.18 42.45%
FCS-t	36.70±5.48	17.00±5.41 46.32%	19.69±0.66 53.65%	26.69±3.64	16.47±5.23 55.47%	11.45±3.80 38.57%	8.14±0.22	6.93±2.04 85.16%	1.28±2.19 15.67%	20.19±1.17	10.84±3.39 53.69%	7.00±1.77 34.67%

values are mean (±SD) for media (n = 5), FCS (n = 7) and percentage of free & bound of the total

② Stability of measured sulphydryl compounds

Many studies have investigated changes in the levels of sulphydryl compounds in cell culture media, but none have concurrently examined compound stability, neither as endogenous components of the media nor post-export. The few studies performed have examined compound oxidation in cell-free media containing no or low (5%) levels of FCS, or in phosphate buffered solutions. Experiments were conducted mainly at pH 7.4, at room temperature or 37°C, and used a colorimetric method (Ellman's reagent, 5,5'-dithiobis-2-nitrobenzoic acid) to detect "loss" of -SH compounds.

Long & Halliwell (2001) observed that homocysteine, cysteine, cysteinyl-glycine and glutathione in the 0.5-10 mmol/L range were unstable in RPMI-1640, DMEM and MEM. The thiol group was lost at a significant rate, at least 20% or more in certain cases over 2 hours, with the relationships given as cysteine > homocysteine > cysteinyl-glycine > glutathione. Loss was accompanied by the accumulation of hydrogen peroxide to levels in the 20-30 μmol/L range, although less peroxide was produced on a molar basis than the amount of thiol lost. It had been previously shown that this complex concentration dependence between thiol loss and the generation of peroxide results from the additional reaction of thiols with peroxide (Aruoma *et al.*, 1989). The relative reactivities of thiols with either oxygen or peroxide being inversely related to the pK_a of the thiol group (Winterbourn & Metodiewa, 1999). A way to further elucidate these relationships is to perform similar experiments on loss, but with separate compounds in buffered solutions. This would also prevent the possibility of any FCS derived metal ions (copper and iron) or metal ion complexes and various enzymes (catalase, superoxide dismutase or xanthine oxidase etc.) oxidising or interacting with the sulphydryl compounds. Therefore, as compound oxidation increases with pH in the 5-9 range and the pH of basal MEM and P-med differed after FCS addition, it was necessary to determine the extent of any compound loss, and if possible correct the data presented in the subsequent chapters.

Sulphydryl compound stability in media

Methods

Sulphydryl compound stability was assessed by following the changes in media basal levels. Aliquots of MEMt and P-medt were incubated in 6-well plates (low-evaporation lid, well area 9.6 cm^2, costar®) at 37°C in a 5% CO_2-air mixture for 336 hours (14 days). Samples were removed daily, derivatised and analysed as described in Chapter 4 (pages 68-71). Media pH at 37°C was measured on day 0, 7 and 14 using pH indicator strips with a range of 6.5-10.0 in 0.2-0.5 increments.

Results

Figure 6.1 shows the percentage change in media levels of homocysteine, cyst(e)ine, cysteinyl-glycine and glutathione in MEMt and P-medt over 336 hours (14 days) at 37°C. Data was corrected for purity, background and expressed as % increase or decrease of the starting concentration. The pH of MEMt was 7.38 (SD±0.26, n = 35) and for P-medt was 7.57 (SD±0.24, n = 35). **Table 6.2** shows a summary of the changes in sulphydryl compound levels at 5, 7 & 14 days.

Figure 6.1 Percentage change in media sulphydryl compounds in MEMt and P-medt over 336 hours (14 days) at 37°C

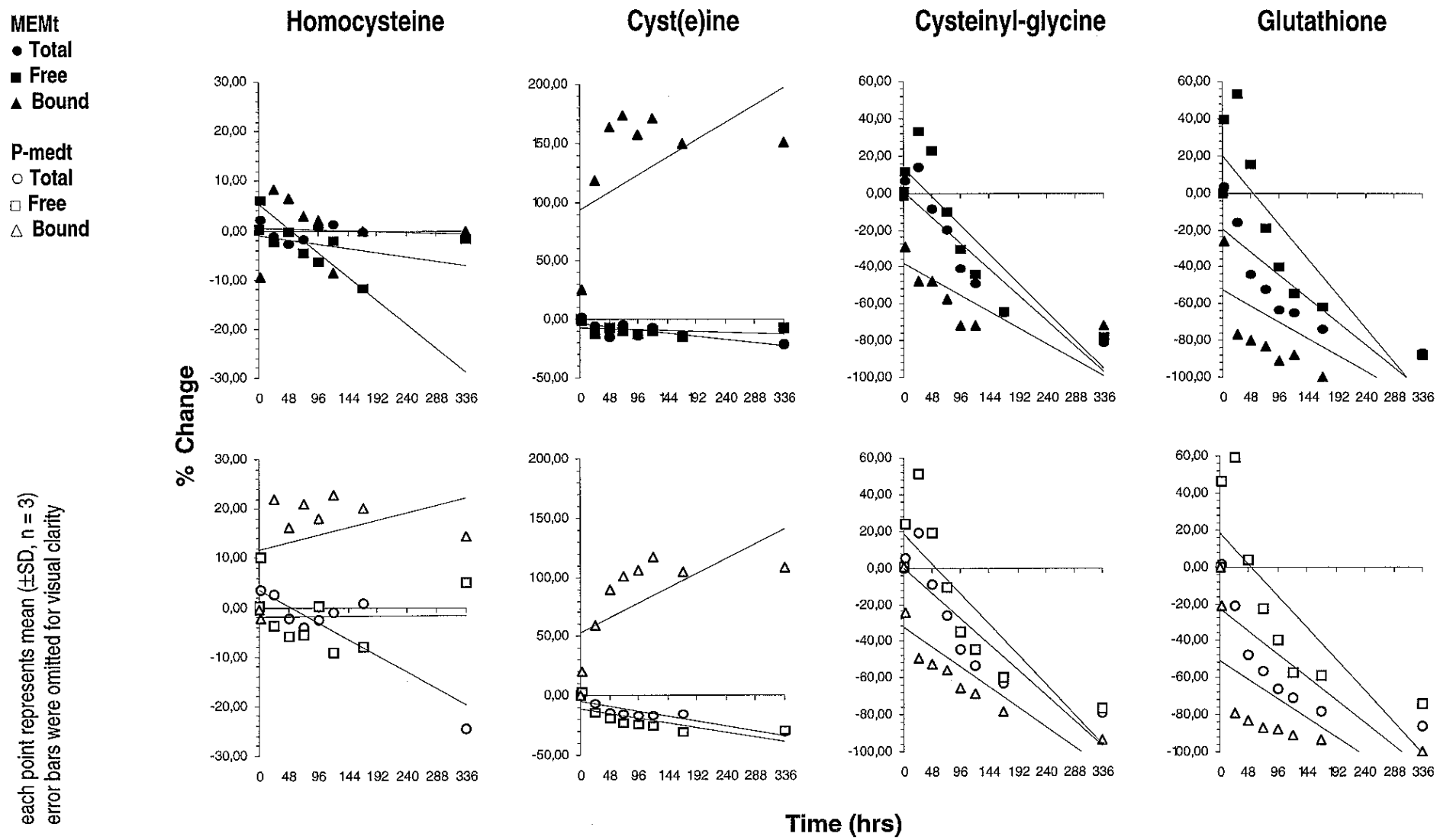


Table 6.2 Summary of changes in sulphydryl compound levels at 5, 7 & 14 days

	Fraction	% Change					
		5 days		7 days		14 days	
		MEMt	P-medt	MEMt	P-medt	MEMt	P-medt
Homocysteine	total	+ 1.1	– 1.1	– 0.5	+ 0.9	– 38.0	– 24.7
	free	– 2.3	– 9.1	– 11.8	– 7.9	– 1.9	+ 4.9
	bound	– 8.5	+ 22.7	0.0	+ 19.9	0.0	+ 14.4
Cyst(e)ine	total	– 7.5	– 16.8	– 13.9	– 16.0	– 21.3	– 31.3
	free	– 9.4	– 25.6	– 15.2	– 31.8	– 7.7	– 29.3
	bound	+ 170.9	+ 116.7	+ 150.0	+ 104.5	+ 150.4	+ 108.5
Cysteinyl-glycine	total	– 49.2	– 53.1	– 65.8	– 62.9	– 81.2	– 79.5
	free	– 44.4	– 44.4	– 64.5	– 59.8	– 78.4	– 76.8
	bound	– 71.7	– 68.5	– 101.4	– 77.4	– 71.7	– 93.7
Glutathione	total	– 65.2	– 71.1	– 74.3	– 78.1	– 87.2	– 86.1
	free	– 54.9	– 57.7	– 62.2	– 59.0	– 87.8	– 74.4
	bound	– 87.7	– 90.8	– 100.0	– 93.4	– 87.7	– 100.0

In **MEMt**, total homocysteine (tHcy, initially 4.20 µmol/L) remained unchanged until 168 hours, but decreased by 38% at 336 hours. This did not reflect concurrent changes in free (fHcy, initially 2.79 µmol/L) and bound (bHcy, initially 1.08 µmol/L) fractions, which remained unchanged at 336 hours. Total cyst(e)ine (tCys, initially 74.99 µmol/L) decreased by 8% at 120 hours, 14% at 168 hours and 21% at 336 hours, with free cyst(e)ine (fCys, initially 60.40 µmol/L) unchanged and bound cyst(e)ine (bCys, initially 2.30 µmol/L) increasing by 171% at 120 hours, by 150% between 168 and 336 hours. Total, free and bound cysteinyl-glycine (initially tCys-gly 0.77 µmol/L, fCys-gly 0.55 µmol/l and bCys-gly 0.18 µmol/L) and total, free and bound glutathione (initially tGSH 1.87 µmol/L, fGSH 0.82 µmol/L and bGSH 0.65 µmol/L) all decreased over 336 hours. tCys-gly decreased by 49%, fCys-gly by 44% and bCys-gly by 71% at 120 hours, with total, free and bound almost zero at 336 hours. tGSH decreased by 65%, fGSH by 55% and bGSH by 88% at 120 hours, with total, free and bound almost zero at 336 hours.

In **P-medt**, tHcy (initially 4.68 µmol/L) decreased by 25%, fHcy (initially 2.98 µmol/L) was unchanged and bHcy (initially 1.03 µmol/L) increased by 14% at 336 hours. tCys (initially 34.85 µmol/L) and fCys (initially 27.90 µmol/L) decreased by 17% and 26% at 120 hours and by 31% and 29% at 336 hours, bCys (initially 2.46 µmol/L) increased by 117% at 120 hours and by 109% at 336 hours. tCys-gly (initially 0.87 µmol/L) decreased by 53%, fCys-gly (initially 0.55 µmol/L) by 44% and bCys-gly (initially 0.27 µmol/L) by 69% at 120 hours, with total, free and bound almost zero at 336 hours. tGSH (initially 2.01 µmol/L) decreased by 71%, fGSH (initially 0.78 µmol/L) by 58% and bGSH (initially 0.76 µmol/L) by 91% at 120 hours, with total, free and bound almost zero at 336 hours.

In summary

The starting concentrations of sulphydryl compounds (except cyst{e}ine) in P-medt and MEMt were very similar. Most changes occurred over the first 72 hours, but with no clear involvement of one form or another. In some cases these changes seemed very large, but as they were related to the starting concentrations, they

altered the total pool very little e.g. in MEMt at 120 hours bCys increased from 2.30 μmol/L by 171% to 6.23 μmol/L. Levels of the free homocysteine and cyst(e)ine fractions were high in comparison to their total and bound fractions. Homocysteine appeared to bind to protein to a greater extent in P-medt than in MEMt probably due to the lower basal cystine level, but quickly reached a plateau. Cyst(e)ine binding to protein reached its maximum between 72-120 hours, then changed little, and was lower in P-medt than MEMt due to the lower basal cystine level in Pmed. Decreases in bound cysteinyl-glycine and glutathione were associated with increases in their free fractions. Total cysteinyl-glycine but not glutathione increased in both media over the first 24 hours.

Sulphydryl compound stability in buffered solutions

Methods

In addition, sulphydryl compound stability was assessed by following changes in buffered solutions at media pH. Freshly prepared stock solutions of DL-homocysteine, L-homocystine, L-cyst(e)ine, cysteinyl-glycine or glutathione (GSH and GSSG) made up in distilled-water were diluted to 10 μmol/L, a concentration of similar magnitude to that of the compounds in media, in (i) distilled-water, (ii) distilled-water adjusted to pH 7.38 with 25 mmol/L HEPES or (iii) distilled-water adjusted to pH 7.57 with 25 mmol/L HEPES. These solutions were incubated in 96-well plates (serocluster, flat bottom, costar®) at 37°C in a 5% CO_2-air mixture for 336 hours (14 days). Samples were removed daily, derivatised and analysed as described in Chapter 4 (pages 68-71).

Results

Figures 6.2A & B show homocyst(e)ine, cyst(e)ine, cysteinyl-glycine and glutathione stability at media pH and 37°C. Data was expressed as both mean increase or decrease in μmol/L and % decrease from the starting concentration.

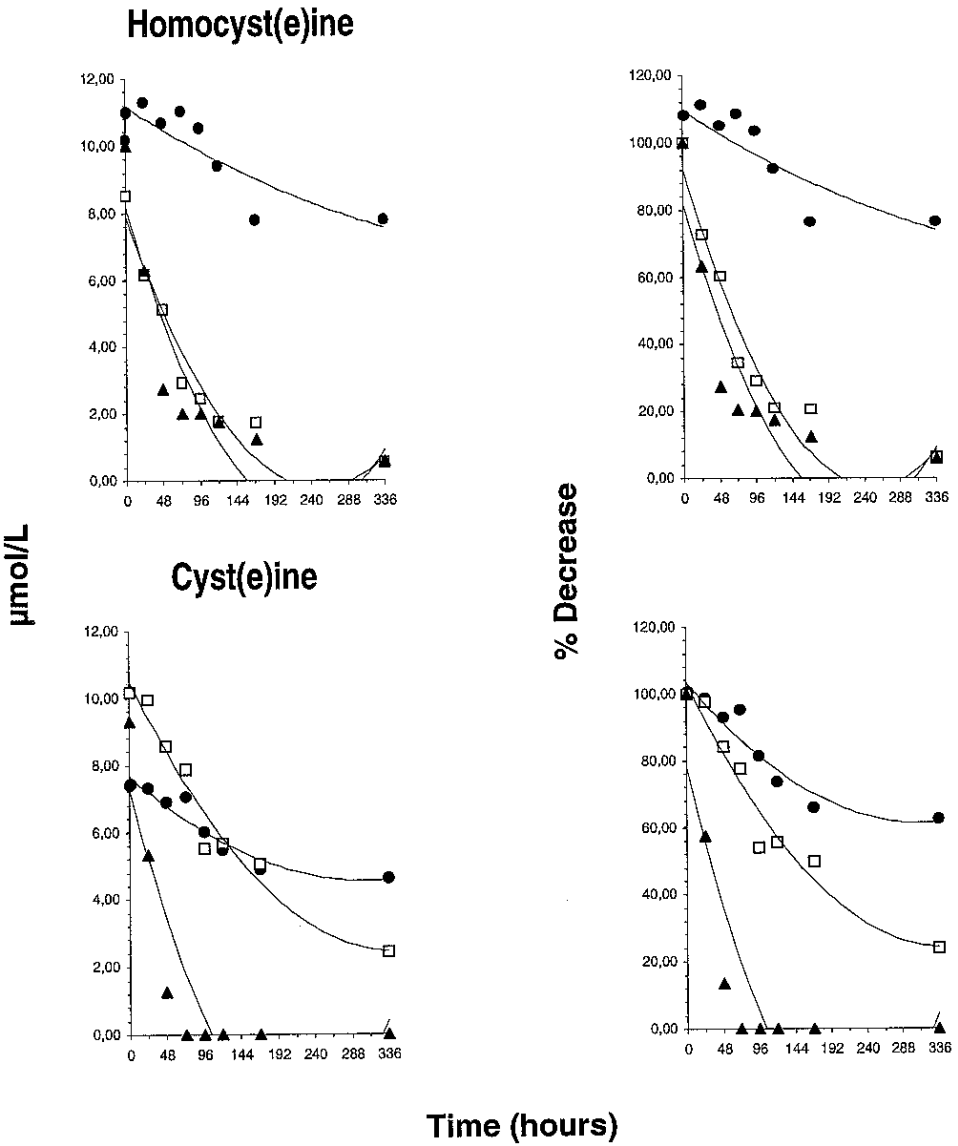
As seen in **Figures 6.2A** & **B**, homocyst(e)ine, cyst(e)ine and cysteinyl-glycine were more stable in distilled-water (lower pH, see **Figure 6.2A** legend) than at pH 7.38 or 7.57, with the relative stabilities as distilled-water > pH 7.38 > pH 7.57 for homocyst(e)ine and cyst(e)ine, and distilled-water > pH 7.38 = pH 7.57 for cysteinyl-glycine. Glutathione on the other hand, was more stable at pH 7.57, than in distilled-water or at pH 7.38. Only cyst(e)ine showed a difference in stability between the solutions tested. Homocyst(e)ine and cyst(e)ine were converted to compounds not measured in this system, whereas cysteinyl-glycine was converted to cyst(e)ine at pH 7.38. Glutathione was converted to cysteinyl-glycine at pH 7.38 and 7.57, and cysteinyl-glycine and cyst(e)ine in distilled-water.

On comparing the buffered solutions with media i.e. **Figures 6.2A** & **B** with **Figure 6.1**, homocysteine and cyst(e)ine were more stable in media, possibly due to the FCS-t content and protein binding (although any FCS-t derived metal ions would have possibly potentiated auto-oxidation), but glutathione and cysteinyl-glycine showed similar stability.

Discussion

Results shown in **Figures 6.1**, **6.2A** & **B** clearly demonstrate loss of sulphydryl compounds from the media and buffered solutions. This was especially apparent with cysteinyl-glycine and glutathione, and there appeared to be no simple relationship between the levels of mixed-disulphides and protein-bound mixed disulphides. These findings justify the measurement of free and protein-bound fractions, and not simply their calculation by subtracting the free fraction from the total.

Figure 6.2A Homocyst(e)ine & cyst(e)ine stability at media pH & 37°C



• distilled-water

□ pH 7.38

▲ pH 7.57

notes on **Figures 6.2A & B**

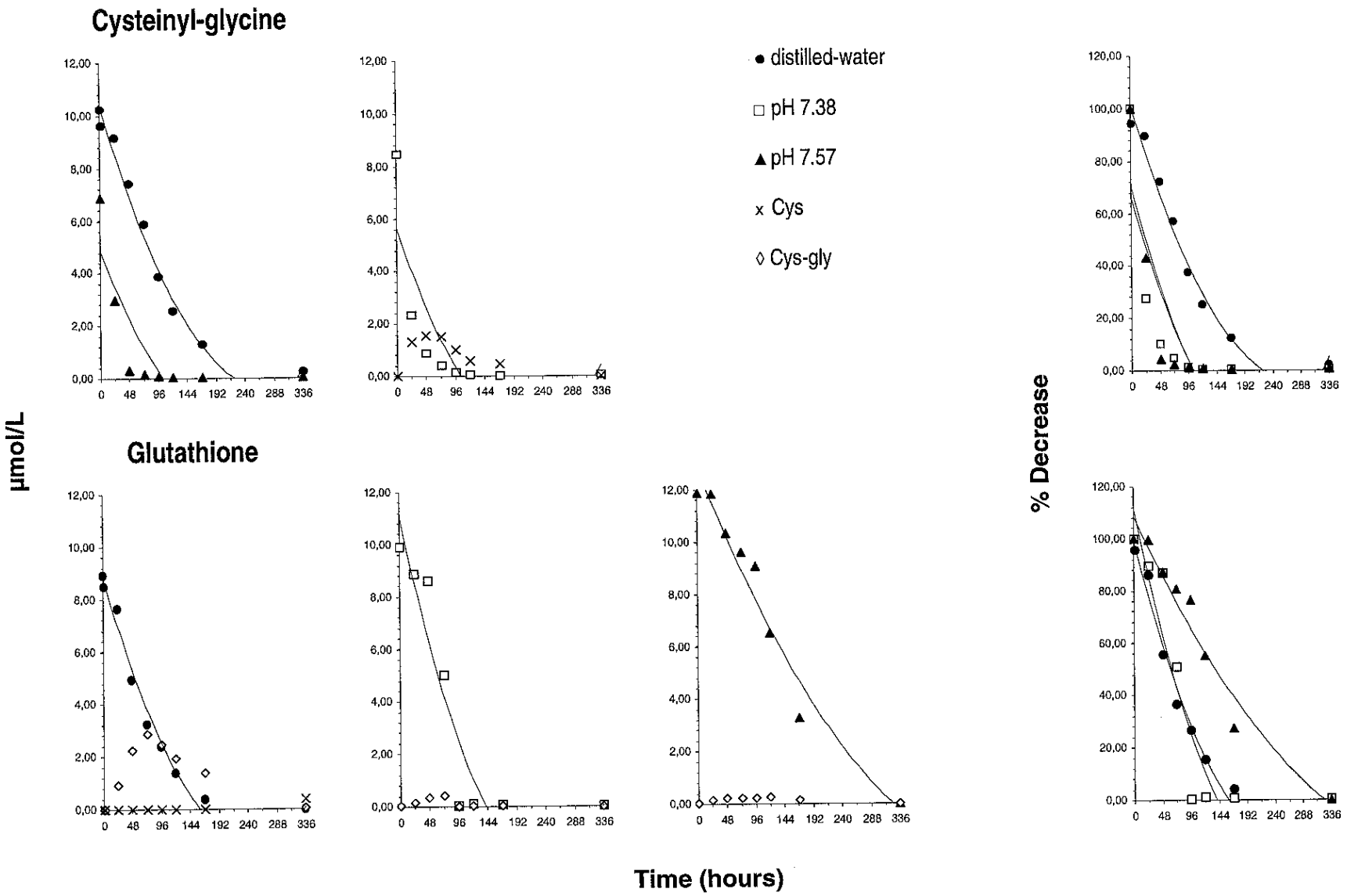
only thiols shown

cyst(e)ine made up in distilled- water containing 25 mmol/L NaOH, then a 1 in 1000 dilution in either distilled-water or buffered solution

mean pH (n = 3) in distilled-water at room temperature:
homocyst(e)ine = 6.20 (SD±0.01)
cyst(e)ine = 6.47 (SD±0.08)
cysteinyl-glycine = 5.78 (SD±0.17)
glutathione = 5.45 (SD±0.14)

error bars (±SD) were omitted for visual clarity

Figure 6.2B Cysteinyl-glycine & glutathione stability at media pH & 37°C



Possible reasons for the observed losses are oxidation and physical stability. The initial reaction between sulphdryl compounds as the thiolate anion and hydrogen peroxide produces a transient intermediate sulphenic acid, and only in conditions of excess thiol will the disulphide be formed. In the absence of excess thiol and under physiologically relevant conditions, further oxidation can irreversibly produce the sulphinic (RSO_2H) and sulphonic (RSO_3H) acid forms. Due to the derivatisation reaction, homocysteine and cyst(e)ine that form homocysteic (which is thought to inhibit cystine uptake like that of homocystine) and cysteic acid would not be detected. Additionally, breakdown of glutathione could cause an increase in cysteinyl-glycine, and that in turn, an increase in cyst(e)ine. For example, in MEMt at 24 hours, bound glutathione had decreased by 77% with a concomitant increase in free glutathione by 52%, and in P-medt at 24 hours, bound glutathione had decreased by 79% with a concomitant increase in free glutathione by 59%. But with cysteinyl-glycine, the total also increased, in MEMt by 14% and in P-medt by 20%, and probably accounts for the discrepancy in bound and free glutathione in MEMt of 25% and in P-medt of 20%. The discrepancy between loss of glutathione in this study and the finding of Long & Haliwell (2001) that glutathione was more stable, can be attributed to the poor specificity of Ellman's reagent compared to HPLC separation.

In theory the sulphydryl compound data presented later and in Chapters 7 (pages 128-133) and 8 (pages 148-155) could be corrected, but as the system is dynamic this is not possible. It must therefore be taken into account when interpreting the data that the measured concentrations during culture reflect not only metabolite production but also losses, and in fact the majority of results are actually higher than those reported.

③ Cell growth studies

A clear relationship between medium substrate concentration, cell growth and metabolism is expected. Thus studies on metabolism need to take into account any possible effects due to differences in cell growth characteristics between physiological medium and MEM. This growth was compared in the two media as below. Furthermore, by expression of metabolite levels in $\mu mol/L/10^6$ cells as described in Chapter 3 (pages 62-63) will minimise the influence of differences in cell growth between different experiments.

As described in Chaper 3 (pages 64-65), rates of cell growth were measured in fibroblasts in 6-well plate cultures by three colorimetric methods. Although the standard curves (**Figure 6.3**) were linear, further comparison of the absorbance based cell numbers with those obtained from actual haemocytometer counts revealed a better correlation with SRB than with giemsa or MTT, and therefore, only cell numbers obtained from the SRB method were used in the conversion of metabolite levels to $\mu mol/L/10^6$ cells. The results of which can be seen in **Figure 6.4**

Staining of protein basic amino acid residues with SRB - log phase cells **(Figure 6.4A)**

Cell numbers determined from the line of best fit over 120 hours increased from $6.1x10^4$-$2.6x10^5$ in MEMt and from $8.3x10^4$-$2.3x10^5$ in P-medt, a gain of 326.2% in MEMt and 177.1% in P-medt. Doubling times ($t^{1/2}$) from $1x10^5$-$2x10^5$ cells were 60.9 hours for MEMt and 79.1 hours for P-medt, a difference in growth rate of 23.0%.

- post-confluent cells **(Figure 6.4B)**

Cell numbers over 120 hours increased from $2.9x10^5$-$4.2x10^5$ in MEMt and from $3.8x10^5$-$4.3x10^5$ in P-medt, a gain of 44.8% in MEMt and 13.2% in P-medt. This was far in excess of the 2-6% growth observed with post-confluent fibroblasts following a medium change by Bard & Elsdale (1971).

Figure 6.3 Examples of standard curves

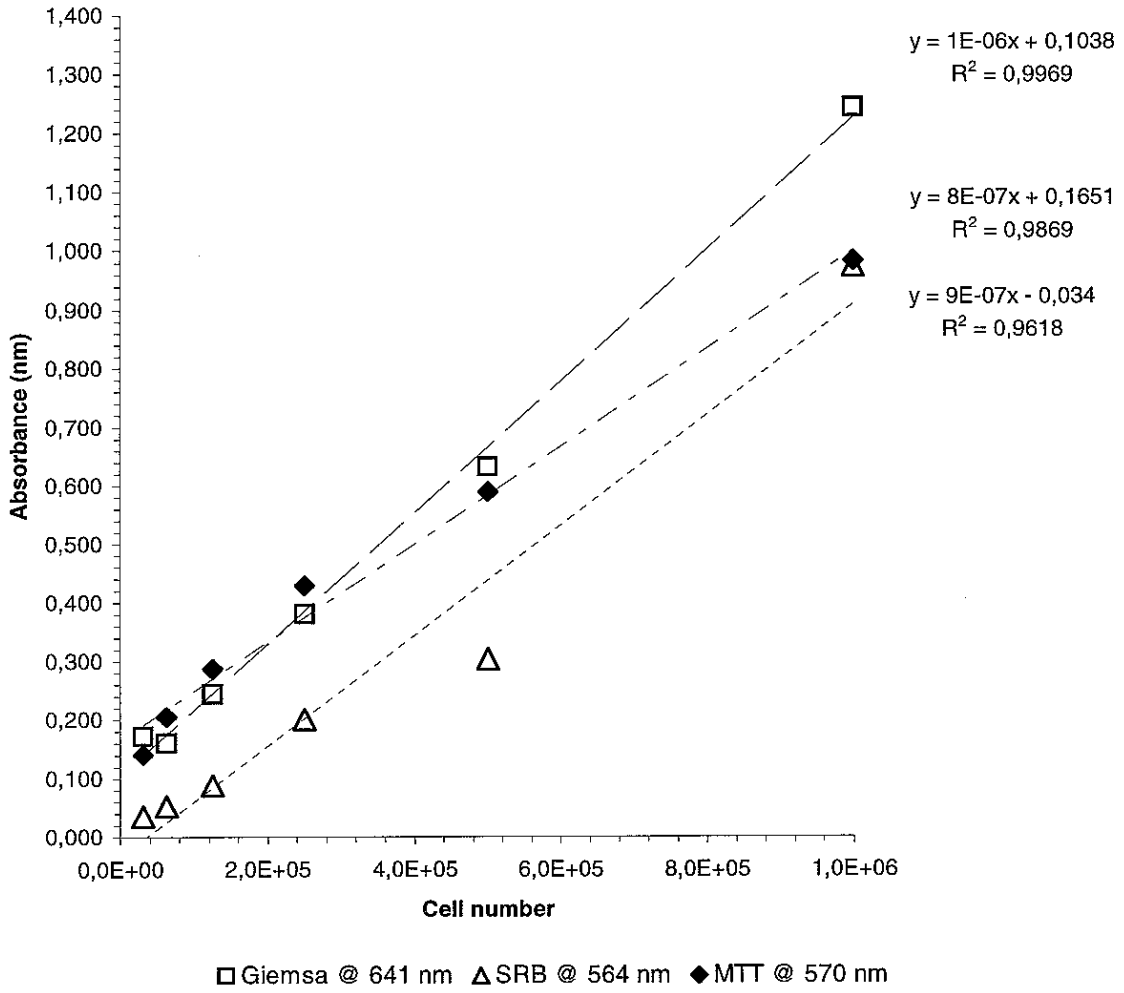
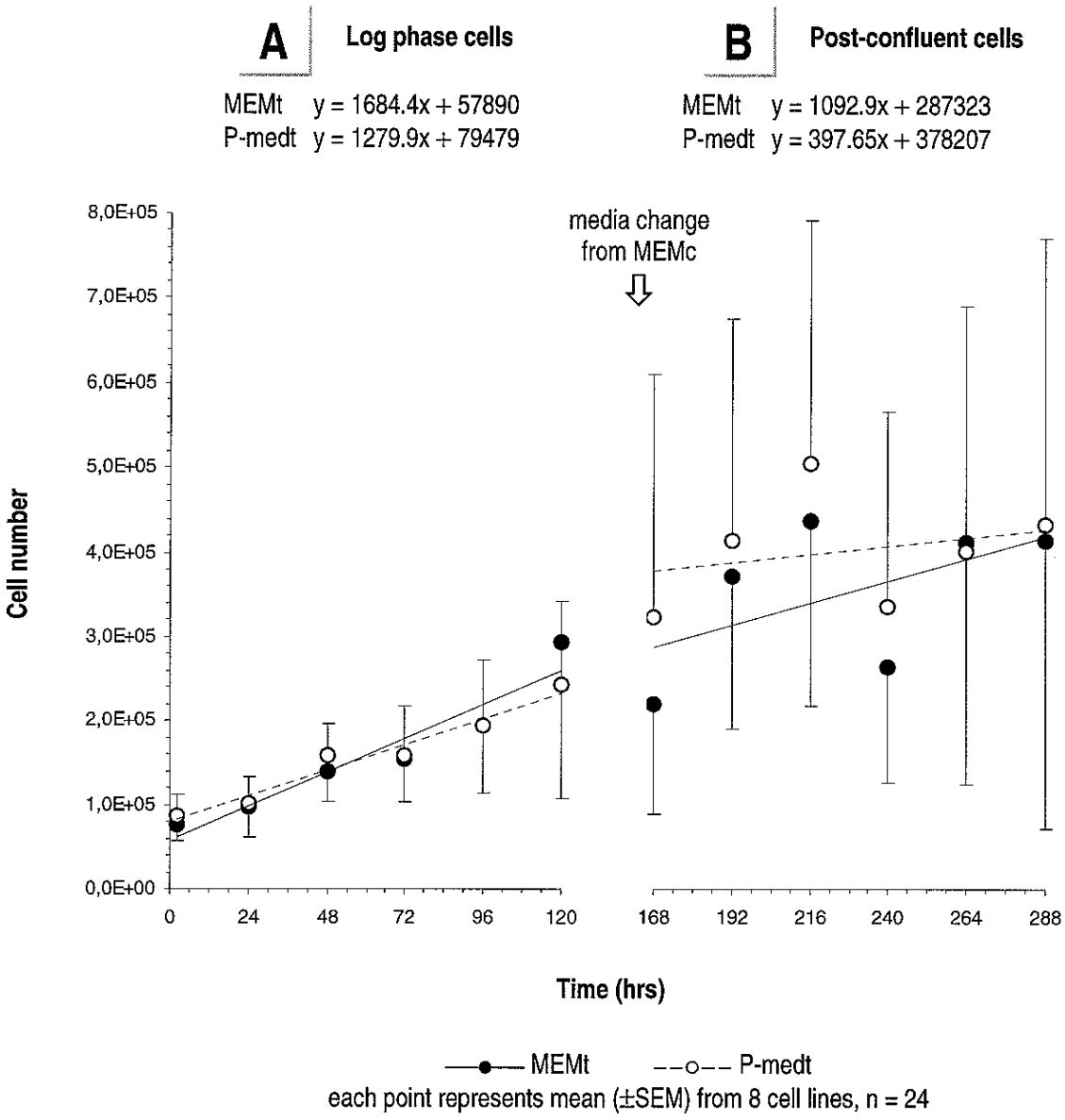


Figure 6.4 SRB growth curves



Thus based on **Figure 6.4** it can be concluded that there were little quantitative differences in cell growth and cell number between P-medt and MEMt, indicating that differences in cell growth can be excluded as an influential factor in the present studies on the changes in metabolite levels.

Measurement of sulphydryl compounds in media from fibroblast cultures

Materials

Selected cell lines

Sixteen human skin fibroblast cell lines that initially exhibited a good level of growth were chosen for experiments (**Table 6.3**). These had been sub-cultured from primary explant cultures established from either skin biopsies or cell cultures that had been obtained by UKBB for diagnostic studies during 1996-2001. Twelve control cell lines (nos. 1-12, passage nos. 2-10, mean = 7) were from individuals (8♂, 4♀, age 0-23 years) proven by fibroblast assays not to have homocystinuria. Six cell lines were from individuals with a non-metabolic disease (e.g. tumour or suspected tumour of the liver, kidney or stomach, suspected Pallister-Kilian syndrome or skeletal dysplasia), five cell lines were from individuals with a suspected metabolic disorder other than homocystinuria and one cell line was from an individual with a suspected remethylation defect (MMA with cblC), but in whom such defects were excluded by fibroblast assays. Four mutant cell lines (nos. 13-16, passage nos. 7-9, mean = 8) were from individuals (2♂, 2♀, age 9-34 years) with homocystinuria, proven by enzyme assay to be due to CBS deficiency (**Table 6.4**).

Table 6.3 List of selected human skin fibroblast cell lines

	Cell line	Mean passage no.	Gender	Date of birth	Date of death	Skin biopsy	Fibroblast culture
Controls	1	9	♂	01.07.99	-	✓	
	2	2	♂	06.04.98	-	✓	
	3	8	♂	11.04.98	-	✓	
	4	7	♀	25.05.00	25.06.00	✓	
	5	5	♂	14.02.98	-		✓
	6	5	♂	13.07.98	-	✓	
	7	9	♂	06.02.80	-	✓	
	8	6/7	♂	08.07.00	-	✓	
	9	9	♂	29.10.99	-		✓
	10	10	♀	29.08.95	-	✓	
	11	10	♀	23.06.98	-	✓	
	12	9	♀	18.07.79	-		✓
Mutants	13	7	♂	12.06.89	-		✓
	14	9	♂	14.03.85	-		✓
	15	9	♀	07.03.93	-		✓
	16	7/8	♀	27.07.68	-		✓

Table 6.4 Enzyme activity levels in the CBS-deficient cell lines

Cell line	CBS activity (nmol/h/mg protein)			
	Assayed – PLP		Assayed + PLP	
	– AdoMet	+ AdoMet	– AdoMet	+ AdoMet
13	nd	-	nd	-
control	4.7	-	8.1	-
14	nd	nd	nd	nd
control	2.6	4.9	5.4	8.1
15	nd	nd	0.03	0.03
control	2.2	5.3	5.1	15.2
16	0.04	0.03	0.09	0.13
control	2.6	6.2	5.4	10.0
control range (n = 20)	mean = 12.0 (5.1-27.7)	-	mean = 15.2 (6.8-37.6)	-

results obtained from metabolic lab; mean result from 2 separate batches of cells (Fowler *et al.*, 1978); nd = not detected

Methods

6-well plate experiments - media metabolites

Attempts were made to obtain the highest surface area (SA) : volume (V) i.e. cell density : medium ratio to obtain maximum metabolite concentrations. Seeding was also carefully calculated to ensure confluence at completion of experiments. Attempts were made to obtain sufficient cells in 6-well plates to increase levels of extracellular and intracellular metabolite measurements by incorporating poly-D-lysine hydrobromide[1] (Sigma) coated glass beads in the wells. Each well held 27 beads which increased the surface area from 9.6-39.5 cm^2 or 4.1-fold and the SA : V ratio, even after compensating for the necessary increase in volume to 4.0 ml, by 2.6-fold. This approach however, was complicated and too time consuming for practical purposes and had to be abandoned.

General aspects

Before experiments were initiated, cells from confluent (7-14 day) stock cultures were harvested using trypsin-EDTA solution and pooled as described in Chapter 3 (page 60). Cells were diluted in either physiological medium (P-medt) or original medium (MEMt) as control.

[1] lyophilised powder, γ-irradiated, Mw. $7x10^4$-$15x10^4$ (> $7x10^4$ promotes cell adhesion to solid substrates)

Log phase cells

Cells were diluted in MEMt or P-medt to $5x10^4$ cells/ml and seeded into 6-well plates at $1x10^5$ cells/well and made up to a total volume of 2.5 ml. Samples (0.5 ml for homocysteine, cysteine, glutathione and cysteinyl-glycine, 0.5 ml for 5-MeTHF and 1 ml for AdoMet and AdoHcy) were removed from the media at time points ranging from baseline (the seeding media), 2 hours (when the cells had fully adhered) and then at 24 hour intervals until 120 hours (confluence). They were processed for metabolite determination taking the precautions described by Loehrer *et al.*, (1996) and outlined in **Table 6.5**.

Table 6.5 Precautions required for sample processing (after Loehrer *et al.*, 1996)

Media metabolite	Sample size	Precautions required	Stored
Homocysteine †	0.5 ml		–70°C
5-MeTHF	0.5 ml	+ 5 mg ascorbic acid, mixed thoroughly, (light protection in aluminium foil)	–70°C
AdoMet & AdoHcy	1 ml	+ 625 µl 10% (v/v) perchloric acid (PCA, $HClO_4$) in distilled-water, mixed thoroughly	–70°C

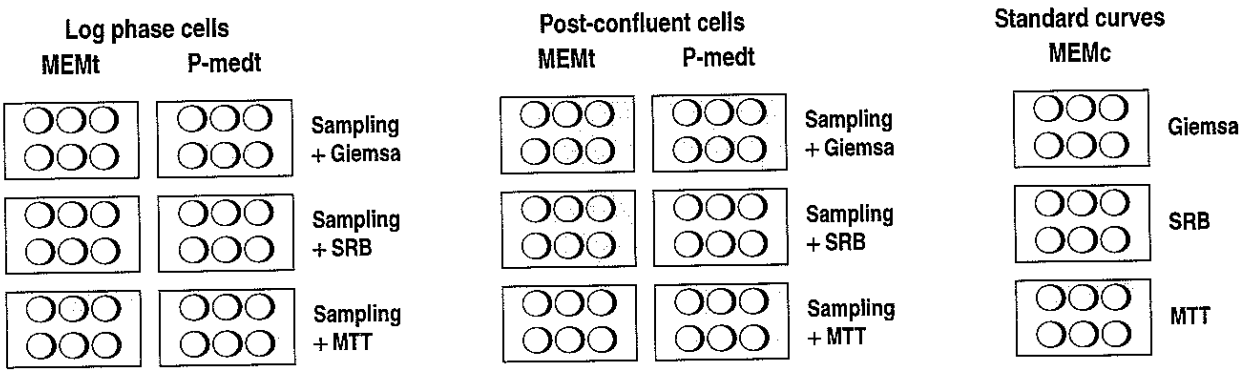
† as well as for cyst(e)ine, cysteinyl-glycine and glutathione

Post-confluent cells

Cells were suspended in MEMc and seeded as before. They remained in culture for 7 days, whereby they had formed confluent monolayers. After removal of the medium by aspiration, cells were washed three times with 3 ml HBSS. MEMt or P-medt was then added in volumes of 2.5 ml before returning the plates to the incubator. Sampling (baseline to 120 hours) and processing took place as previously described.

Staining of cells was performed as described in Chapter 3 (pages 64 & 65), a summary of the 6-well plate experiments can be seen in **Figure 6.5**.

Figure 6.5 Summary of 6-well plate experiments



Results

Sulphydryl compound concentrations (μmol/L) measured as total, free and bound are shown in media from log phase cell cultures in **Table 6.6** and in media from cultures of post-confluent cells in **Table 6.7. Figures 6.6** & **6.7** show the changes in sulphydryl compound concentrations compared to baseline, in log phase and post-confluent cells grown in either MEMt or P-medt.

Statistical analyses in this and subsequent chapters were performed between metabolite levels in media and cells with the paired student's t-test, but noted only when $p < 0.05$ which was considered statistically significant.

Log phase cells

Homocysteine — total homocysteine (tHcy) levels increased in both media over 96 hours indicating net export. This reflected concurrent increases in the free (fHcy) and bound (bHcy) fractions, although in comparison to MEMt, P-medt showed lower fHcy and higher bHcy levels. The rate of export was maximal between 2-24 hours, but reached a plateau between 72-120 hours.

Cyst(e)ine — total cyst(e)ine (tCys) levels decreased in both media over 72 hours indicating net uptake as the decrease was greater than the loss described in the preliminary studies. As bound cyst(e)ine (bCys) changed very little, the decrease in tCys was entirely due to the uptake of free cyst(e)ine (fCys). The rate of uptake was maximal between 2-24 hours, but changed very little from then on.

Cysteinyl-glycine — although total cysteinyl-glycine (tCys-gly) levels increased in both media over 72 hours, this did not necessarily indicate net export, as cysteinyl-glycine is derived from the breakdown of glutathione. Nevertheless, like that of tCys, the increase in tCys-gly was entirely due to the free cysteinyl-glycine (fCys-gly).

Glutathione — total glutathione (tGSH) levels changed very little in both media over 120 hours indicating no net export or uptake. This reflects an increase of the free (fGSH) fraction together with a proportional decrease in the bound (bGSH) fraction.

Post-confluent cells

Homocysteine, cyst(e)ine & cysteinyl-glycine — all showed changes similar to those observed with log phase cells.

Glutathione — tGSH levels increased over 48 hours in MEMt and 24 in P-medt indicating net export. As bGSH changed very little, the increase in tGSH was entirely due to the fGSH. The rate of export was maximal between 2-24 hours, but decreased from then on.

Table 6.6 Sulphydryl compound concentrations (μmol/L) with log phase cells in culture media

Time (hours)	Homocysteine					
	MEMt			P-medt		
	total	free	bound	total	free	bound
0	5.23 ±1.44	3.83 ±0.56	1.92 ±0.65	4.79 ±0.62	3.74 ±0.38	1.77 ±0.45
2	5.78 ±1.63	3.79 ±0.96	1.83 ±0.27	4.94 ±1.23	3.53 ±0.43	1.64 ±0.27
24	9.45 ±3.73	6.44 ±1.02	2.48 ±0.56	8.47 ±3.32	5.26 ±0.50	2.52 ±0.62
48	13.82 ±6.35	9.23 ±2.13	3.40 ±1.00	12.35 ±4.45	6.97 ± 1.11	4.19 ±0.98
72	18.58 ±7.43	11.91 ±2.79	4.68 ±1.51	17.83 ±7.10	10.08 ±4.59	5.88 ±1.41
96	23.82 ±10.09	14.07 ±5.38	5.56 ±1.67	22.04 ±8.62	11.49 ±2.89	7.42 ±2.10
120	29.75 ±13.08	18.44 ±7.30	6.77 ±2.20	26.72 ±11.02	13.30 ±4.18	9.05 ±2.70
Time (hours)	Cyst(e)ine					
	MEMt			P-medt		
	total	free	bound	total	free	bound
0	71.48 ±7.32	80.55 ±15.06	4.13 ±1.21	33.06 ±3.01	35.26 ±5.61	3.22 ±0.86
2	70.45 ±11.09	77.95 ±8.52	4.71 ±0.66	31.23 ±4.02	33.09 ±2.55	2.87 ±0.36
24	68.29 ±11.69	73.57 ±9.76	6.28 ±1.25	29.32 ±3.35	28.97 ±3.49	4.10 ±0.90
48	67.20 ±11.22	73.07 ±7.73	6.45 ±1.22	27.12 ±3.22	27.04 ±2.79	4.57 ±0.95
72	65.26 ±10.15	71.34 ±9.24	6.69 ±1.42	26.52 ±2.69	27.20 ±2.98	4.56 ±1.05
96	64.12 ±9.73	67.32 ±6.90	6.18 ±1.01	25.78 ±2.03	24.76 ±2.61	4.43 ±1.00
120	63.16 ±7.38	66.84 ±6.50	6.59 ±1.44	24.65 ±2.20	23.80 ±2.29	4.47 ±1.05
Time (hours)	Cysteinyl-glycine					
	MEMt			P-medt		
	total	free	bound	total	free	bound
0	0.93 ±0.17	0.58 ±0.03	0.28 ±0.10	0.89 ±0.11	0.60 ±0.09	0.31 ±0.04
2	0.94 ±0.13	0.73 ±0.17	0.24 ±0.07	0.83 ±0.10	0.59 ±0.15	0.25 ±0.07
24	1.24 ±0.20	1.12 ±0.20	0.14 ±0.03	1.14 ±0.17	1.00 ±0.20	0.14 ±0.04
48	1.51 ±0.34	1.56 ±0.55	0.13 ±0.04	1.31 ±0.24	1.13 ±0.23	0.17 ±0.04
72	1.67 ±0.44	1.61 ±0.49	0.15 ±0.05	1.43 ±0.31	1.39 ±0.44	0.19 ±0.06
96	1.74 ±0.48	1.69 ±0.58	0.14 ±0.04	1.45 ±0.28	1.21 ±0.21	0.20 ±0.06
120	1.94 ±0.63	1.84 ±0.68	0.15 ±0.07	1.44 ±0.21	1.29 ±0.29	0.20 ±0.07
Time (hours)	Glutathione					
	MEMt			P-medt		
	total	free	bound	total	free	bound
0	2.19 ±0.29	0.71 ±0.18	0.80 ±0.11	2.12 ±0.26	0.78 ±0.15	0.90 ±0.18
2	2.24 ±0.33	1.11 ±0.13	0.79 ±0.14	1.95 ±0.23	0.88 ±0.18	0.73 ±0.12
24	2.22 ±0.47	1.73 ±0.31	0.33 ±0.08	2.05 ±0.42	1.47 ±0.22	0.35 ±0.09
48	2.30 ±0.66	1.90 ±0.52	0.25 ±0.07	1.99 ±0.51	1.56 ±0.36	0.28 ±0.06
72	2.29 ±0.66	1.98 ±0.65	0.24 ±0.07	1.94 ±0.48	1.61 ±0.34	0.25 ±0.05
96	2.29 ±0.74	1.88 ±0.46	0.19 ±0.05	1.85 ±0.46	1.44 ±0.33	0.24 ±0.03
120	2.54 ±1.31	2.00 ±0.53	0.18 ±0.05	1.79 ±0.43	1.50 ±0.41	0.24 ±0.03

each result represents mean (±SEM) from 8 cell lines, n = 24

Table 6.7 Sulphydryl compound concentrations (µmol/L) with post-confluent cells in culture media

Time (hours)	Homocysteine					
	MEMt			P-medt		
	total	free	bound	total	free	bound
0	5.23 ±1.44	3.83 ±0.56	1.92 ±0.65	4.79 ±0.62	3.74 ±0.38	1.77 ±0.45
2	4.66 ±0.47	4.06 ±0.65	1.95 ±0.58	4.88 ±0.75	3.86 ±1.14	2.16 ±0.37
24	11.47 ±3.90	7.37 ± 2.00	3.26 ±0.76	9.17 ±3.32	6.05 ±2.37	3.86 ±1.16
48	17.58 ±6.70	10.70 ±2.83	4.83 ±1.10	12.81 ±5.59	6.89 ±2.05	5.51 ±2.20
72	22.66 ±9.73	13.71 ±4.59	6.17 ±1.15	15.42 ±6.98	8.76 ±3.73	10.36 ±11.42*
96	27.59 ±9.19	18.60 ±5.94	7.96 ±1.37	17.76 ±8.69	9.38 ±4.14	9.35 ±6.97*
120	32.62 ±9.46	23.62 ±8.01	9.38 ±1.34	20.32 ±10.36	10.69 ±5.26	8.95 ±4.34
Time (hours)	Cyst(e)ine					
	MEMt			P-medt		
	total	free	bound	total	free	bound
0	71.48 ±7.32	80.55 ±15.06	4.13 ±1.21	33.06 ±3.01	35.26 ±5.61	3.22 ±0.86
2	65.22 ±9.30	72.01 ±13.46	4.06 ±0.84	31.41 ±4.31	28.43 ±8.38	3.46 ±0.78
24	60.56 ±6.43	61.61 ±12.05	4.68 ±1.04	25.34 ±2.07	22.12 ±6.99	3.84 ±0.67
48	54.93 ±3.92	59.29 ±13.14	4.77 ±1.01	23.52 ±1.85	19.56 ±6.24	3.83 ±0.83
72	53.76 ±3.76	55.37 ±11.39	5.00 ±1.30	22.83 ±2.72	19.28 ±6.76	4.22 ±1.19
96	48.92 ±3.71	53.76 ±11.38	4.57 ±1.02	21.27 ±2.78	18.71 ±7.36	3.93 ±0.97
120	47.66 ±4.97	52.42 ±11.52	4.07 ±1.00	20.85 ±2.93	18.03 ±6.93	3.86 ±1.16
Time (hours)	Cysteinyl-glycine					
	MEMt			P-medt		
	total	free	bound	total	free	bound
0	0.93 ±0.17	0.58 ±0.03	0.28 ±0.10	0.89 ±0.11	0.60 ±0.09	0.31 ±0.04
2	0.83 ±0.19	0.62 ±0.13	0.14 ±0.03	0.94 ±0.14	0.60 ±0.20	0.24 ±0.06
24	1.36 ±0.32	1.10 ±0.30	0.09 ±0.02	1.30 ±0.17	0.98 ±0.30	0.15 ±0.03
48	1.89 ±0.50	1.56 ±0.39	0.13 ±0.03	1.58 ±0.32	1.13 ±0.35	0.20 ±0.03
72	2.17 ±0.63	1.69 ±0.50	0.14 ±0.05	1.61 ±0.47	1.11 ±0.39	0.23 ±0.06
96	2.33 ±0.60	1.97 ±0.57	0.17 ±0.06	1.59 ±0.44	1.14 ±0.41	0.24 ±0.03
120	2.51 ±0.61	2.13 ±0.60	0.17 ±0.05	1.54 ±0.48	1.11 ±0.42	0.24 ±0.04
Time (hours)	Glutathione					
	MEMt			P-medt		
	total	free	bound	total	free	bound
0	2.19 ±0.29	0.71 ±0.18	0.80 ±0.11	2.12 ±0.26	0.78 ±0.15	0.90 ±0.18
2	2.15 ±0.44	1.07 ±0.22	0.62 ±0.13	2.31 ±0.28	1.02 ±0.34	0.78 ±0.13
24	3.19 ±0.74	2.09 ±0.67	0.23 ±0.07	2.81 ±0.43	1.79 ±0.55	0.32 ±0.07
48	4.04 ±1.14	2.62 ±0.91	0.19 ±0.06	3.15 ±0.94	1.98 ±0.64	0.29 ±0.03
72	4.26 ±1.52	2.59 ±1.12	0.20 ±0.08	2.88 ±1.12	1.77 ±0.65	0.29 ±0.06
96	4.48 ±1.55	2.99 ±1.35	0.17 ±0.05	2.74 ±1.25	1.74 ±0.60	0.28 ±0.04
120	4.96 ±1.81	3.29 ±1.50	0.18 ±0.05	2.67 ±1.35*	1.68 ±0.63	0.27 ±0.04

each result represents mean (±SEM) from 8 cell lines, n = 24; * large SEM, reflects wide variation in individual cultures

Figure 6.6 Sulphydryl compound changes (µmol/L/10^6 cells) with log phase cells

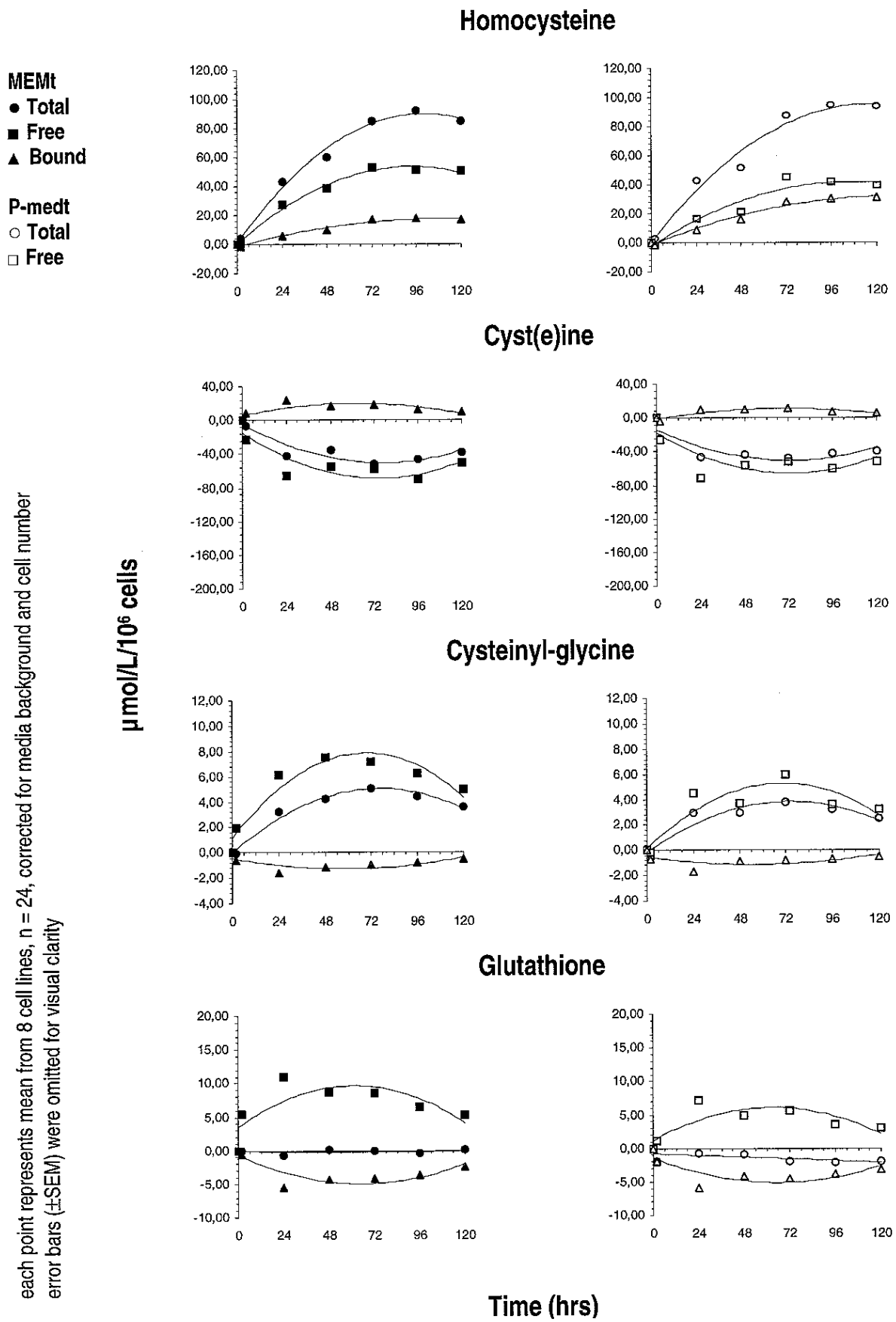
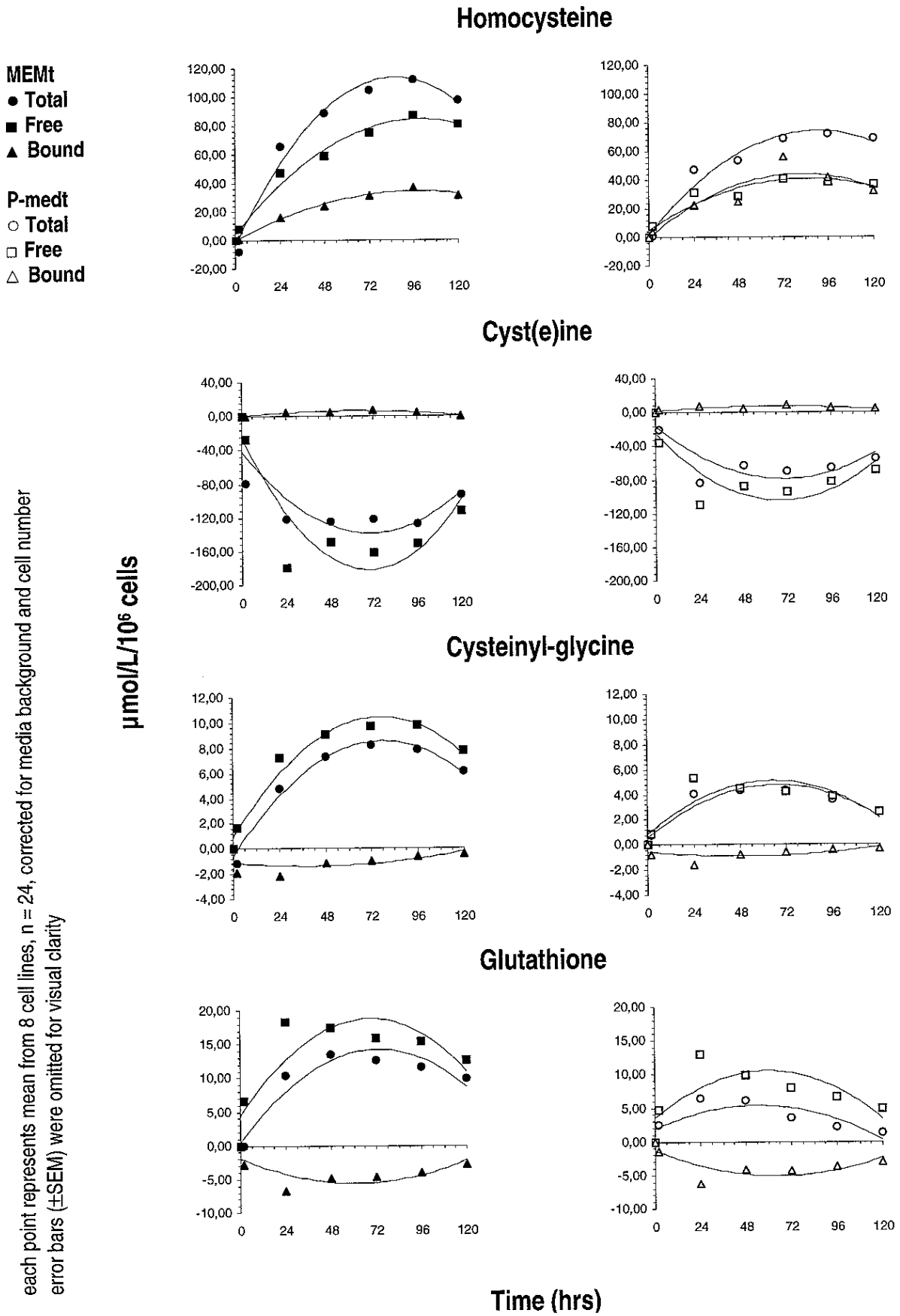


Figure 6.7 Sulphydryl compound changes (µmol/L/10^6 cells) with post-confluent cells



Discussion

At first glance, the plots of the changes in homocysteine, cyst(e)ine, cysteinyl-glycine and glutathione between the two media comprising **Figure 6.6** show no apparent differences. With **Figure 6.7** however, these differences are more dramatic. The most noticeable from **Figures 6.6** & **6.7** being that most metabolite changes occurred during the first 72 hours, and more specifically with the free fractions between 2-24 hours.

Log phase cells

As the substrates in basal P-med were based on physiological levels and not proportionally reduced in relation to MEM, the similarity in homocysteine export between the two media is quite surprising. In trying to explain this, both the levels of methionine, folic acid, vitamin B_6, cystine, uptake and FCS must be considered. In terms of cell growth, the data presented here is in direct agreement with previous reports stating export from growing cells is greatest during early to mid-exponential phase and then decreases as a function of cell density (Christensen *et al.,* 1991). If methionine uptake between the cells in P-medt and MEMt was similar, the level and rate of homocysteine export gave no indication of the difference in methionine between them, since basal MEM contained roughly a 3-fold higher level. Previous reports could not discount a direct enhancement by methionine (Christensen *et al.,* 1991), but experiments were performed in either MEM or RPMI-1640 containing additionally higher folic acid levels. Folic acid supplementation has been shown to reduce homocysteine export in a dose-dependent manner (van der Molen *et al.*, 1996 & 1997). Basal P-med contained a 100-fold lower folic acid level that could conceivably reduce homocysteine remethylation and consequently increase export, even with lower medium methoinine. If transsulphuration had been affected by the almost 42-fold lower level of vitamin B_6 in basal P-med, causing a reduction in CBS and CTH activity, it could possibly be reflected in increased cyst(e)ine uptake, which was not the case. Similarly, there was no indication of homocysteine or homocystine uptake in P-medt in response to the lower methionine level, as again, cyst(e)ine uptake was similar between the two media. The lower basal P-med cystine levels do explain the differences in the free and bound homocysteine between the media, as homocysteine formed more mixed disulphides with free protein cysteine residues. One question that remains open is the cause of the decrease in bound glutathione and the increase in free glutathione and cysteinyl-glycine, considering cysteinyl-glycine export is not supported by the literature. Both homocysteine binding and the freeing and breakdown of glutathione were also observed in cell-free media. The poor stability of glutathione and cysteinyl-glycine may have meant that levels were underestimated, and export of glutathione did occur. Although metal ions have been shown to increase homocysteine export in endothelial cells linked to the level of FCS from which they are derived (Hultberg *et al.,* 1998c), this is unlikely to differ between P-medt and MEMt since both contained 10% (v/v) FCS.

Post-confluent cells

This was essentially the same as with the log phase cells, but the main issue here was the level of post-confluence growth caused by the change in media from MEMc to P-medt or MEMt. The effects were greater with MEMt, where the cell density surpassed that of P-medt by approximately 30%. With fibroblasts this effect is quite common, with cell movement so violent that temporary rents in the cell sheet sometimes occur (Bard & Elsdale, 1971).

Conclusions

This study demonstrates clear export of homocysteine, but with no real difference between P-medt and MEMt, both supporting and validating our knowledge of export from previous studies, but now under more physiological conditions. Considering the free and bound homocysteine, the finding of similar degrees of binding of homocysteine, as well as the other free sulphydryl compounds to protein, in spite of different substrate levels, emphasises the role of protein binding in the export of homocysteine. Similarities in the export of homocysteine reflect the complexity of the system. It is likely that an expected increase in homocysteine export with low folate is counteracted by a lower homocysteine production and export with low methionine. Since cell growth was similar, the difference in folic acid levels between the two basal media is the most probable cause of the similarities in homocysteine export from the cells growing in P-medt and MEMt. The resurgence of growth and consequently metabolic behaviour in the post-confluent cells probably accounts for the differences between P-medt and MEMt, and between the post-confluent and log phase cells. It would appear that stimulation of a post-confluent culture by medium renewal causes greater changes in media sulphydryl compound levels than those associated with a normally encountered log phase culture.

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Intracellular sulphydryl compounds, 5-MeTHF, AdoMet, AdoHcy & amino acids in control & CBS-deficient fibroblasts

Introduction

The aim was to study the levels of intracellular compounds, which included:
(i) the sulphydryl compounds homocysteine, cysteine, cysteinyl-glycine and glutathione;
(ii) 5-MeTHF;
(iii) AdoMet and AdoHcy; and
(iv) free amino acids

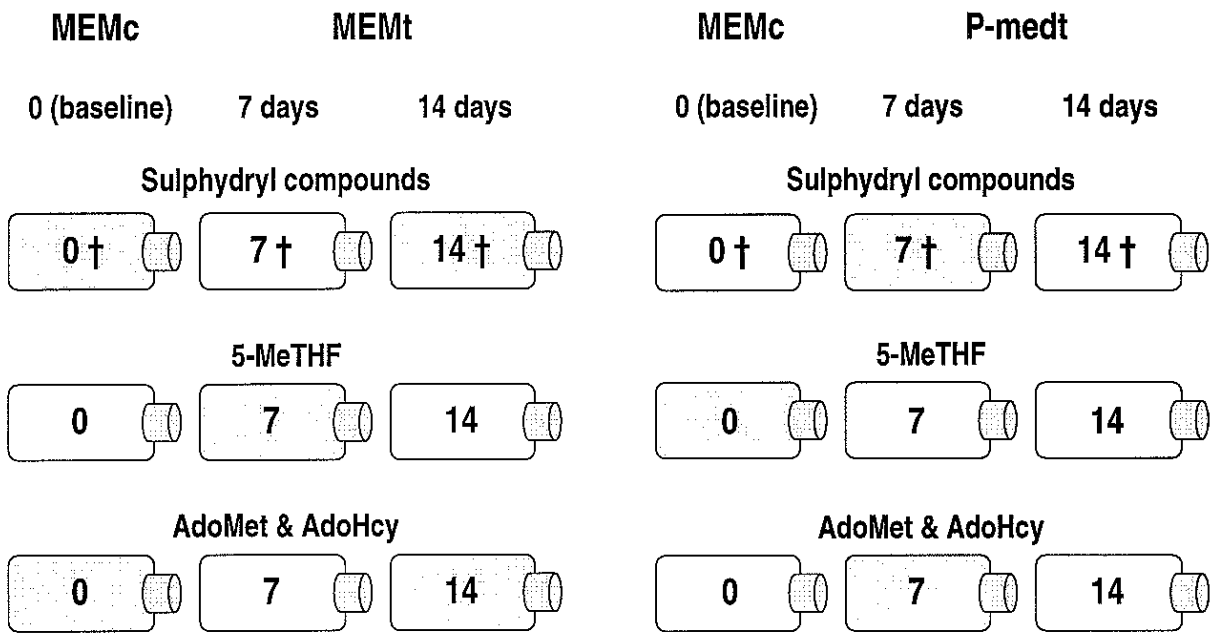
in the light of the previously determined levels of sulphydryl compounds measured the in media i.e. export. This was again performed with cells grown in MEMt and P-medt, but also included CBS-deficient fibroblasts. As with the multi-time point experiments, attempts were made to obtain an optimal cell density : medium ratio to maximise both intracellular and extracellular metabolite concentrations. Preliminary experiments included using high-density plates with seeding areas of up to 450 cm^2, but these proved too complicated and costly to implement effectively. Nevertheless, to mimic the growth characteristics of the cells in the 6-well plates i.e. to obtain both a log and post-confluent phase, it was decided to perform experiments in T_{75} t/c flasks over 14 days, with sampling at 0 (baseline), 7 and 14 days.

Methods

As described in "general aspects" (see Chapter 6, page 117) cells were harvested using trypsin-EDTA and pooled. For experiments, cells were sub-cultured at a split ratio of 1 : 2 in either 20 ml MEMt or P-medt (total flask volume 21 ml). Each metabolite (sulphydryl compounds, 5-MeTHF, adenosyl compounds and amino acids) was determined separately in cells pooled from 3 flasks. Baseline samples were taken from confluent stock cultures grown in MEMc (dilution = 2, to account for the split ratio of 1 : 2). After incubating for 7 and 14 days, cells were harvested using dissociation solution, obtained as a pellet by centrifugation and lysed as described in Chapter 3 (pages 60 & 62). Lysates for the determination of total homocysteine, cysteine, cysteinyl-glycine and glutathione were stored at −70°C. For 5-MeTHF, lysates were treated immediately with γ-glutamyl hydrolase as described in Chapter 4 (page 73). For AdoMet and AdoHcy, lysates were deproteinised by the addition of 56.3 µl 10% (v/v) PCA to a 90 µl lysate as described by Loehrer *et al.*, 1996. Although not a main aim, samples for the determination of other amino acids were also obtained at 7 days and processed as described in Chapter 4 (page 89). Media samples were additionally taken at 0 (baseline, i.e. the plating media), 7 and 14 days and processed as described in Chapter 6 (page 118). A summary of the experiments in this section can be seen in **Figure 7.1**. As described in Chapter 4 (pages 68-90), the sulphydryl compounds, 5-MeTHF, AdoMet and AdoHcy were measured by HPLC, the amino acids by ion-exchange chromatography.

Figure 7.1 Summary of experiments

all flasks x3; † samples also taken for media sulphydryl compounds; sulphydryl compounds included homocysteine, cysteine, cysteinyl-glycine & glutathione



Results

All **intracellular** levels are from single measurements from 3 pooled flasks. All **media** levels are from triplicate measurements, with one measurement from each of the 3 flasks.

Intracellular total sulphydryl compound concentrations (μmol/L) and protein concentrations from the individual cell lines are shown in **Table 7.1. Figure 7.2** shows the mean (±SD) intracellular concentrations of total sulphydryl compounds in the 4 control and 4 CBS-deficient cell lines cultured in either MEMt or P-medt at baseline, 7 and 14 days.

Media sulphydryl compound concentrations (μmol/L) measured as total, free and bound from cultures of control and CBS-deficient cell lines are shown in **Tables 7.2** & **7.3** respectively. **Figures 7.3** & **7.4** show the changes in sulphydryl compound concentrations in media at 7 and 14 days compared to baseline, from control and CBS-deficient cell lines grown in either MEMt (**Figure 7.3**) or P-medt (**Figure 7.4**).

In order to relate these changes with those obtained in the 6-well plate experiments, concentrations are expressed as μmol/L/10^6 cells, where all cell numbers were converted from protein measured by Lowry as described in Chapter 3 (page 62).

Intracellular 5-MeTHF concentrations (nmol/L) and protein concentrations from the individual cell lines are shown in **Table 7.4. Figure 7.5** shows the mean (±SD) intracellular concentrations of 5-MeTHF in the 4 control and 4 CBS-deficient cell lines cultured in either MEMt or P-medt at baseline, 7 and 14 days.

Intracellular AdoMet concentrations (nmol/L) and protein concentrations from the individual cell lines are shown in **Table 7.5. Figure 7.6** shows the mean (±SD) intracellular concentrations of AdoMet in the 4 control and 4 CBS-deficient cell lines cultured in either MEMt or P-medt at baseline, 7 and 14 days. The ratios of intracellular AdoMet : AdoHcy are shown in **Table 7.6**, although the validity of this data is doubtful.

Levels of **intracellular free amino acids** related to homocysteine metabolism are shown in **Table 7.7** as individual values, and as means (±SD) in the 4 control and 4 CBS-deficient cell lines in **Figure 7.7**.

Table 7.1 Intracellular total sulphydryl compound concentrations in control & CBS-deficient cells

cell lines 2, 3, 5 & 6 control; cell lines 13-16 CBS-deficient

Time & media	Cell line	Protein (µg)	Sulphydryl compound concentration (µmol/L)			
			Hcy	Cys	Cys-gly	GSH
0 days MEMc (dilution = 2)	2	174.70	0.43	1.86	0.04	4.33
	3	94.77	0.29	0.99	0.03	2.35
	5	71.94	0.25	0.61	0.02	0.51
	6	131.04	0.31	0.93	0.04	2.00
	13	128.34	0.88	2.96	0.41	6.33
	14	108.88	0.54	2.36	0.48	1.96
	15	176.04	1.00	3.36	0.38	1.37
	16	86.40	1.36	0.99	0.48	0.07
7 days MEMt	2	405.81	2.45	10.88	0.59	15.07
	3	207.00	1.31	3.42	0.14	16.04
	5	348.05	2.38	11.09	0.65	16.28
	6	231.18	1.61	2.19	0.21	17.78
	13	165.36	0.69	1.24	0.66	19.20
	14	252.67	1.38	3.59	1.09	12.65
	15	387.00	2.26	10.03	1.29	5.15
	16	243.27	1.04	7.65	1.57	15.96
14 days MEMt	2	321.18	1.01	3.57	0.14	4.49
	3	270.13	0.86	2.94	0.09	6.94
	5	251.33	0.84	2.16	0.13	2.71
	6	286.25	1.97	5.34	0.40	4.04
	13	256.70	1.76	5.91	0.82	12.66
	14	217.75	1.08	4.72	0.95	3.91
	15	352.08	2.00	6.72	0.76	2.74
	16	172.80	0.71	1.97	0.95	0.14
7 days P-medt	2	274.16	1.29	3.42	0.23	9.74
	3	198.94	1.45	2.14	0.14	7.13
	5	342.67	2.14	9.23	0.56	14.88
	6	200.28	1.42	1.85	0.18	12.61
	13	172.08	0.74	0.90	0.49	6.79
	14	197.60	0.88	1.17	0.68	5.46
	15	211.03	0.70	1.92	0.31	0.95
	16	290.28	1.44	6.50	1.31	5.11
14 days P-medt	2	319.84	1.27	2.57	0.14	5.32
	3	309.09	1.15	2.04	0.11	8.82
	5	254.02	0.78	1.30	0.09	1.32
	6	309.09	1.73	1.90	0.16	3.00
	13	366.85	3.72	10.28	0.63	13.42
	14	196.25	2.57	4.28	0.37	5.22
	15	168.05	0.67	1.08	0.18	0.56
	16	255.36	3.47	3.15	1.48	0.23

values are single measurements from a 90 µl lysate; shaded area = CBS-deficient cells

Figure 7.2 Intracellular total sulphydryl compounds in control & CBS-deficient cells

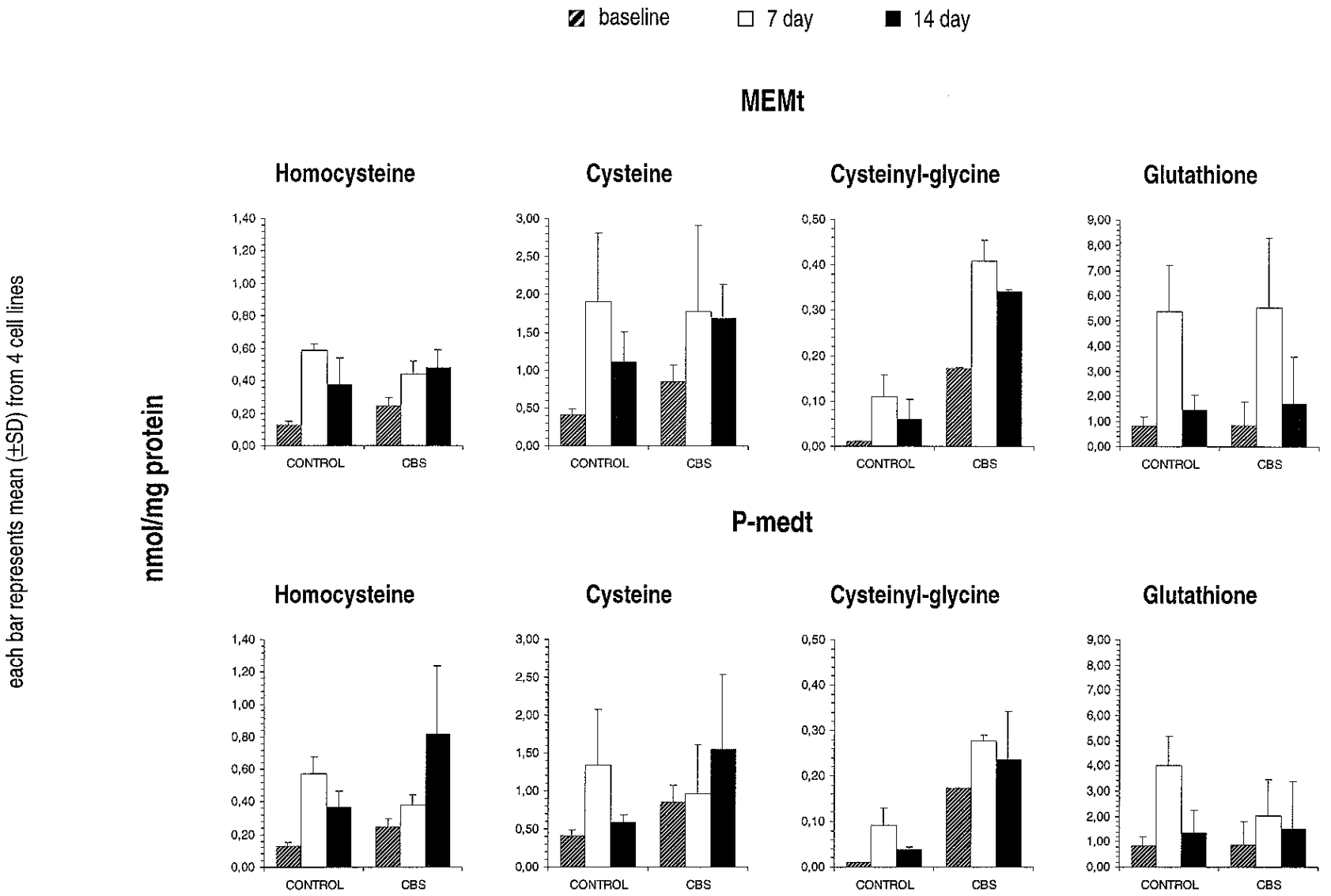


Table 7.2 Sulphydryl compound concentrations (μmol/L) from control cells in culture medium

Time (days)	Homocysteine					
	MEMt			P-medt		
	total	free	bound	total	free	bound
0	5.23 ±1.44	3.83 ±0.56	1.92 ±0.65	4.79 ±0.62	3.74 ±0.38	1.77 ±0.45
7	40.96 ±18.46	26.36 ±10.28	14.49 ±5.00	21.17 ±5.59	14.19 ±3.78	14.28 ±3.69
14	75.86 ±8.94	58.37 ±7.26	17.59 ±2.75	59.26 ±14.72	38.30 ±9.85	21.43 ±4.38
Time (days)	**Cyst(e)ine**					
	MEMt			P-medt		
	total	free	bound	total	free	bound
0	71.48 ±7.32	80.55 ±15.06	4.13 ±1.21	33.06 ±3.01	35.26 ±5.61	3.22 ±0.86
7	50.83 ±3.58	55.34 ±4.30	5.77 ±0.66	23.53 ±1.36	20.27 ±1.59	5.03 ±0.43
14	44.30 ±1.65	51.35 ±3.91	4.04 ±0.67	15.41 ±1.23	17.34 ±1.40	3.09 ±0.24
Time (days)	**Cysteinyl-glycine**					
	MEMt			P-medt		
	total	free	bound	total	free	bound
0	0.93 ±0.17	0.58 ±0.03	0.28 ±0.10	0.89 ±0.11	0.60 ±0.09	0.31 ±0.04
7	3.68 ±1.11	1.36 ±0.30	0.11 ±0.02	1.82 ±0.43	1.04 ±0.21	0.20 ±0.04
14	2.73 ±0.39	2.85 ±0.32	0.21 ±0.04	1.56 ±0.30	1.58 ±0.25	0.27 ±0.03
Time (days)	**Glutathione**					
	MEMt			P-medt		
	total	free	bound	total	free	bound
0	2.19 ±0.29	0.71 ±0.18	0.80 ±0.11	2.12 ±0.26	0.78 ±0.15	0.90 ±0.18
7	2.37 ±1.10	2.07 ±0.56	0.14 ±0.01	1.32 ±0.51	1.47 ±0.42	0.19 ±0.03
14	4.51 ±0.63	4.38 ±0.39	0.19 ±0.02	2.45 ±0.37	2.32 ±0.41	0.26 ±0.03

each result represents mean (±SEM) from 4 cell lines, n = 12

Table 7.3 Sulphydryl compound concentrations (μmol/L) from CBS-deficient cells in culture medium

Time (days)	Homocysteine					
	MEMt			P-medt		
	total	free	bound	total	free	bound
0	5.23 ±1.44	3.83 ±0.56	1.92 ±0.65	4.79 ±0.62	3.74 ±0.38	1.77 ±0.45
7	30.27 ±16.42*	22.74 ±12.06*	6.53 ±3.03	25.88 ±10.20	17.91 ±8.41	7.68 ±0.94
14	62.33 ±14.86	47.95 ±10.82	12.50 ±4.01	62.30 ±11.44	39.75 ±8.82	18.15 ±7.06
Time (days)	**Cyst(e)ine**					
	MEMt			P-medt		
	total	free	bound	total	free	bound
0	71.48 ±7.32	80.55 ±15.06	4.13 ±1.21	33.06 ±3.01	35.26 ±5.61	3.22 ±0.86
7	47.29 ±3.45	52.00 ±3.50	3.48 ±0.52	17.97 ±2.73	17.64 ±1.20	2.96 ±1.95*
14	39.76 ±1.86	44.92 ±3.69	2.97 ±0.53	13.85 ±3.44	13.34 ±2.34	1.81 ±1.37*
Time (days)	**Cysteinyl-glycine**					
	MEMt			P-medt		
	total	free	bound	total	free	bound
0	0.93 ±0.17	0.58 ±0.03	0.28 ±0.10	0.89 ±0.11	0.60 ±0.09	0.31 ±0.04
7	2.24 ±0.59	2.35 ±0.71	0.13 ±0.03	1.55 ±0.58	1.51 ±0.60	0.18 ±0.01
14	5.19 ±0.61	5.59 ±0.71	0.34 ±0.09	2.92 ±0.81	2079 ±0.89	0.32 ±0.03
Time (days)	**Glutathione**					
	MEMt			P-medt		
	total	free	bound	total	free	bound
0	2.19 ±0.29	0.71 ±0.18	0.80 ±0.11	2.12 ±0.26	0.78 ±0.15	0.90 ±0.18
7	1.45 ±0.41	1.12 ±0.41	0.08 ±0.03	1.42 ±0.43	1.03 ±0.37	0.09 ±0.02
14	2.49 ±0.58	2.27 ±0.65	0.08 ±0.04	1.94 ±0.57	1.58 ±0.52	0.11 ±0.03

each result represents mean (±SEM) from 4 cell lines, n = 12; * large SEM, reflects wide variation in individual cultures

Figure 7. 3 Sulphydryl compounds from control & CBS-deficient cells in MEMt medium

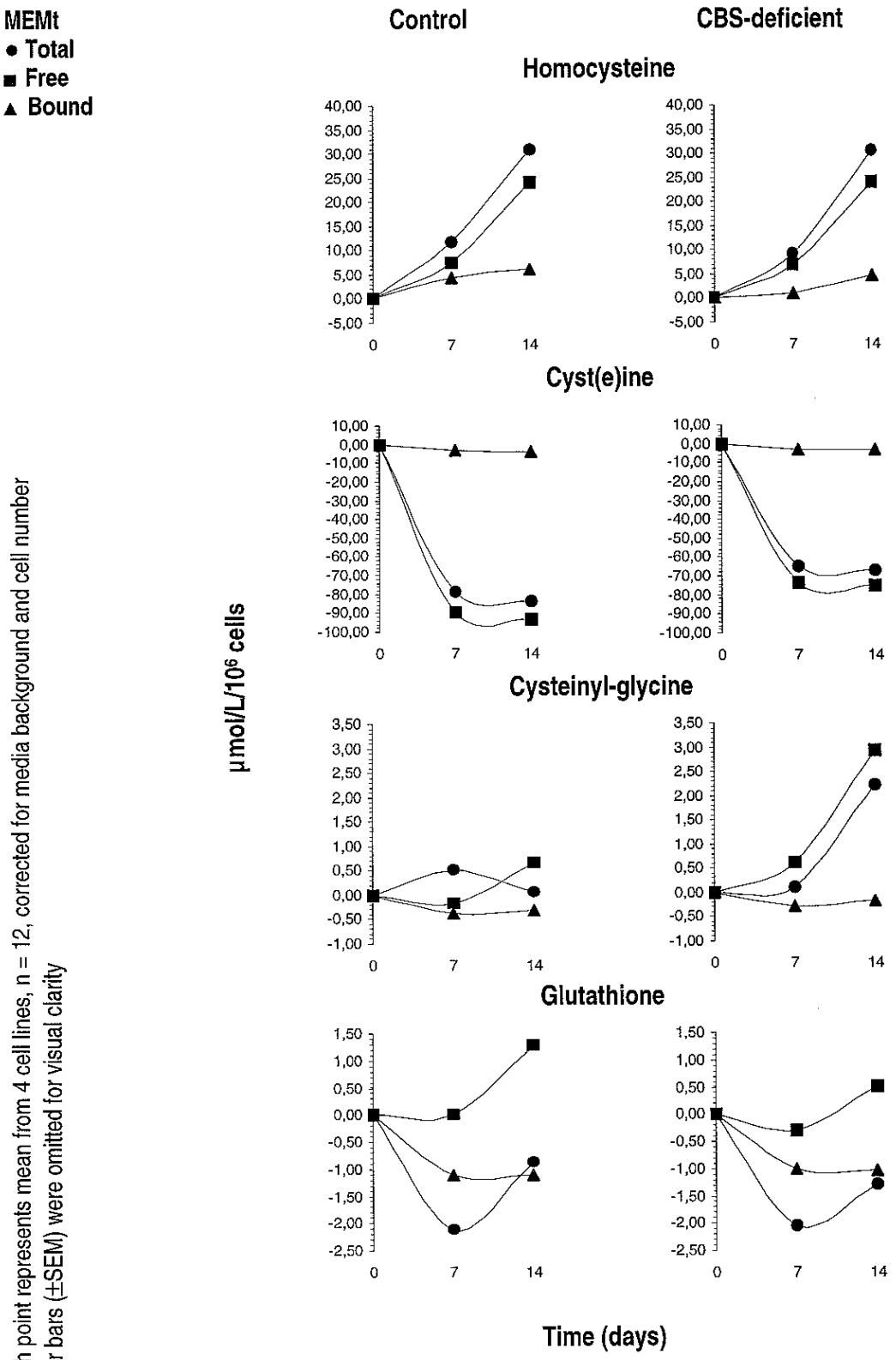


Figure 7. 4 Sulphydryl compounds from control & CBS-deficient cells in P-medt medium

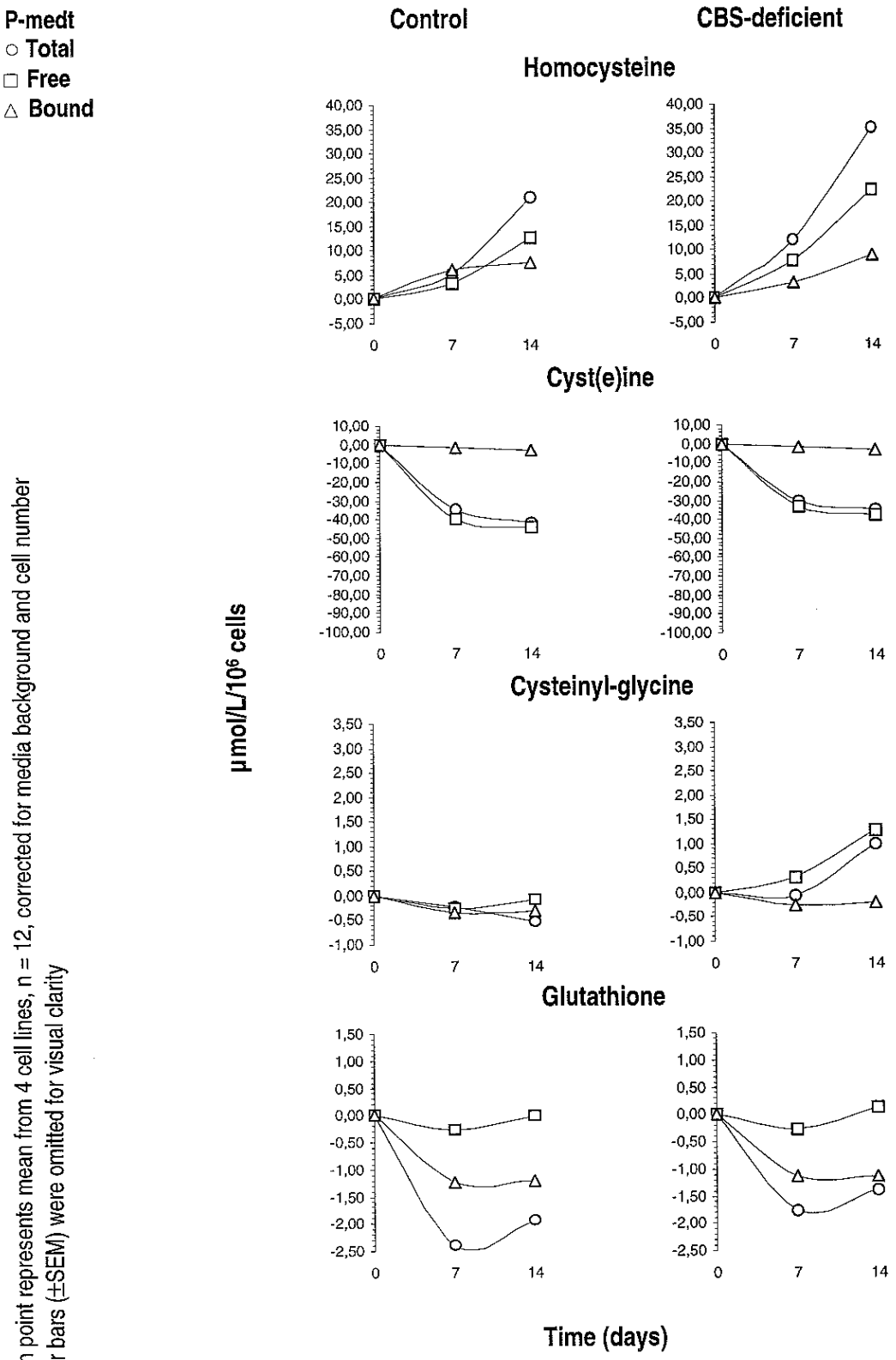


Table 7.4 Intracellular 5-MeTHF concentrations in control & CBS-deficient cells

cell lines 2, 3, 5 & 6 control; cell lines 13-16 CBS-deficient

Time & medium	Cell line	Protein (µg)	5-MeTHF concentration (nmol/L)
7 days MEMt	2	601.93	2.04
	3	205.66	0.16
	5	287.60	0.00
	6	434.01	0.00
	13	108.94	0.13
	14	243.27	0.68
	15	331.93	4.05
	16	249.99	1.88
14 days MEMt	2	368.19	0.39
	3	330.58	0.99
	5	247.30	0.10
	6	236.55	0.00
	13	262.07	1.73
	14	263.42	0.25
	15	365.51	1.14
	16	270.13	1.61
7 days P-medt	2	356.10	0.63
	3	227.15	0.00
	5	212.37	0.00
	6	290.28	0.37
	13	172.07	0.00
	14	141.18	0.00
	15	225.81	0.00
	16	248.64	0.29
14 days P-medt	2	294.18	0.00
	3	120.90	0.00
	5	165.22	0.00
	6	265.97	0.00
	13	333.27	0.00
	14	200.28	0.00
	15	142.52	0.00
	16	201.63	0.00

values are single measurements from a 90 µl lysate;
shaded area = CBS-deficient cells

Figure 7.5 Intracellular 5-MeTHF in control & CBS-deficient cells

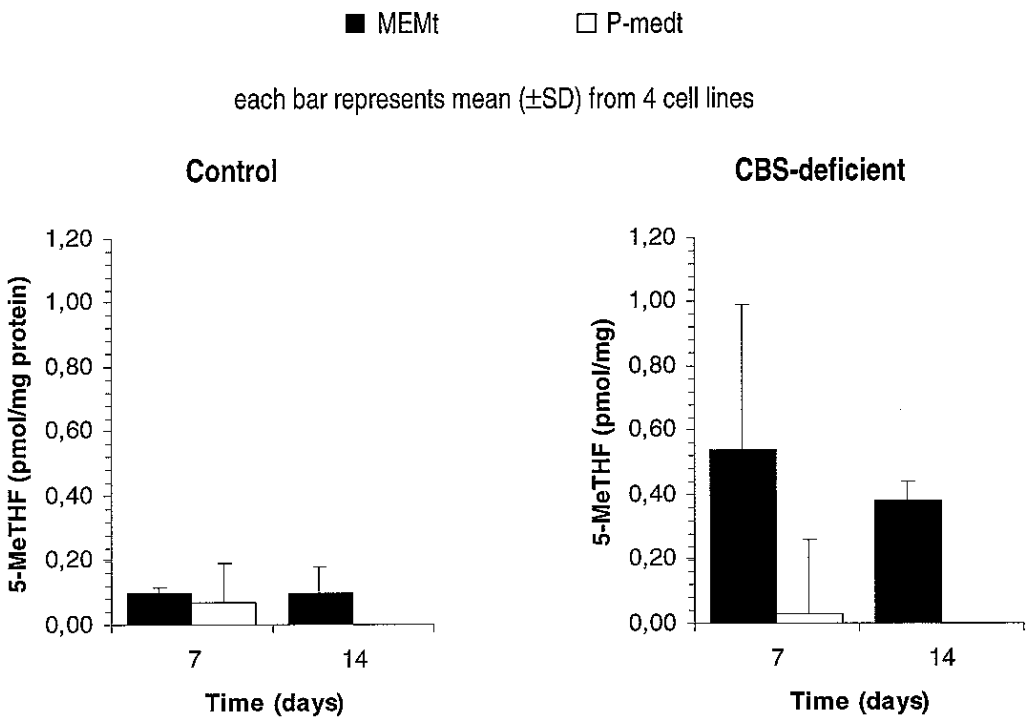


Table 7.5 Intracellular AdoMet concentrations in control & CBS-deficient cells

cell lines 2, 3, 5 & 6 control; cell lines 13-16 CBS-deficient

Time & medium	Cell line	Protein (µg)	AdoMet concentration (nmol/L)
0 days MEMc (dilution = 2)	2	245.89	96.42
	3	165.29	86.70
	5	94.77	7.46
	6	161.94	73.22
	13	171.17	167.98
	14	94.53	130.37
	15	149.17	34.41
	16	135.74	67.70
7 days MEMt	2	181.34	194.56
	3	130.30	26.85
	5	338.51	37.21
	6	210.90	79.89
	13	162.76	158.64
	14	244.61	280.94
	15	357.45	176.93
	16	301.03	298.30
14 days MEMt	2	542.69	105.92
	3	369.40	48.98
	5	153.13	4.20
	6	260.60	58.65
	13	342.33	335.96
	14	189.06	260.73
	15	298.34	68.81
	16	271.48	135.39
7 days P-medt	2	102.09	9.88
	3	52.38	0.50
	5	198.81	7.60
	6	65.82	2.76
	13	174.41	181.48
	14	137.91	169.39
	15	225.81	53.10
	16	244.61	140.98
14 days P-medt	2	158.51	22.84
	3	59.10	0.00
	5	77.91	0.00
	6	228.36	22.77
	13	316.43	349.39
	14	112.14	212.37
	15	168.04	3.53
	16	274.16	99.21

values are single measurements from a 90 µl lysate;
shaded area = CBS-deficient cells

Figure 7.6 Intracellular AdoMet in control & CBS-deficient cells

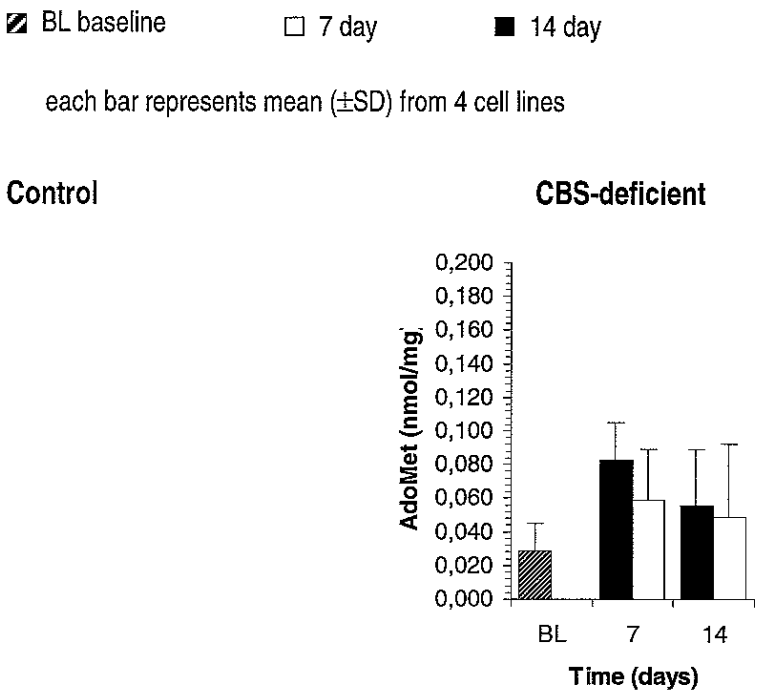


Table 7.6 Ratios of AdoMet : AdoHcy

	Medium	AdoMet : AdoHcy		
		0 days †	7 days	14 days
Control	MEMt	1 : 8.12	1 : 3.17	1 : 4.33
	P-medt		1 : 7.71	1 : 16.67
CBS-deficient	MEMt	1 : 4.03	1 : 2.49	1 : 4.14
	P-medt		1 : 4.32	1 : 5.04

† in MEMc

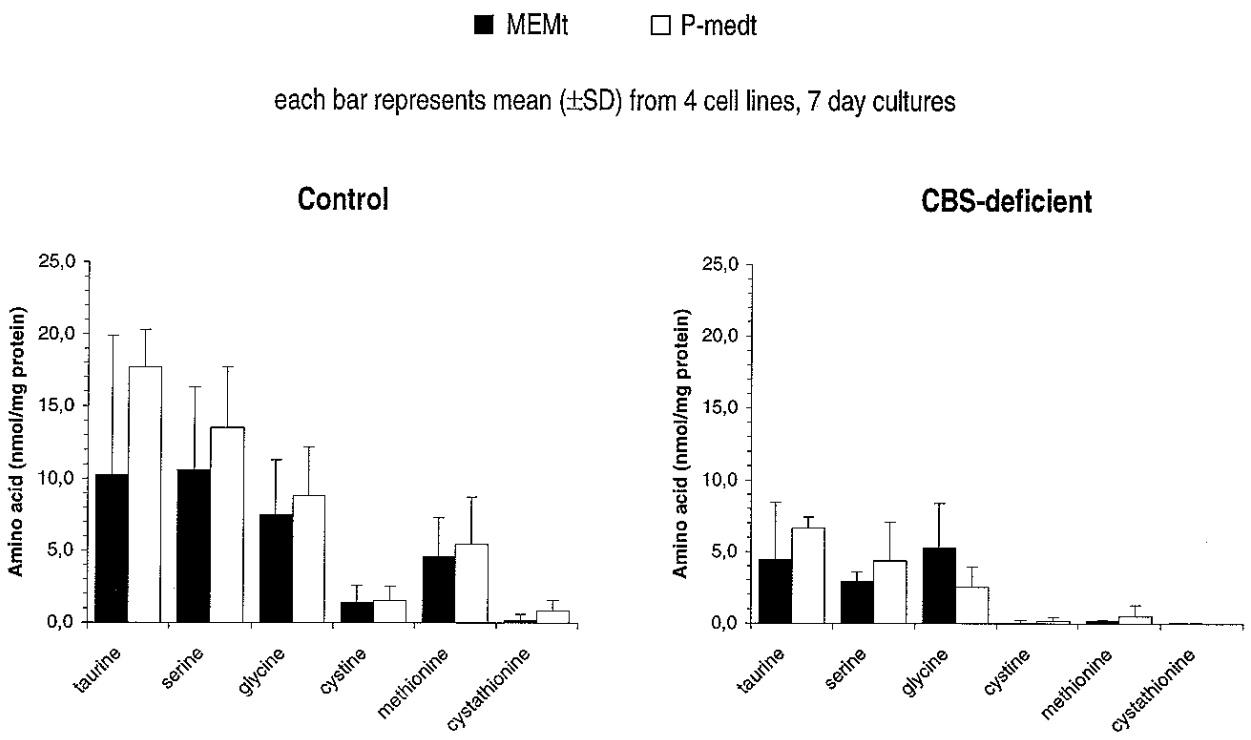
Table 7.7 Intracellular free amino acid concentrations related to homocysteine metabolism in control & CBS-deficient cells

cell lines 2, 3, 5 & 6 control; cell lines 13-16 CBS-deficient

Time & medium	Cell line	Protein (µg)	Amino acid concentration (µmol/L)					
			Taurine	Serine	Glycine	Cystine	Methionine	Cystathionine
7 days MEMt	2	1023.7	6.9	22.5	15.9	0.0	10.1	0.0
	3	1063.4	15.4	46.5	45.3	16.1	22.8	0.0
	5	1514.3	144.1	138.2	97.1	11.9	66.1	0.0
	6	794.0	79.3	52.7	28.2	5.2	17.4	3.1
	13	255.5	12.2	3.5	9.6	0.0	0.0	0.0
	14	819.8	23.4	9.8	33.6	0.0	0.0	0.0
	15	1146.0	11.4	22.7	20.2	0.0	0.0	0.8
	16	927.6	8.4	14.5	13.4	0.0	3.7	0.0
7 days P-medt	2	1052.1	86.8	94.1	62.2	14.1	40.3	8.7
	3	876.3	71.2	45.3	26.9	3.4	15.1	2.4
	5	1199.6	131.3	109.4	77.3	13.6	57.4	8.0
	6	553.0	55.2	28.9	17.4	1.8	6.4	0.0
	13	394.5	5.9	6.1	6.4	0.0	0.0	0.0
	14	400.1	19.7	4.2	7.6	0.0	0.0	0.0
	15	800.0	24.3	10.7	3.3	0.0	0.0	0.0
	16	683.7	47.7	36.1	17.4	2.7	7.6	0.0

values are single measurements from a 190 µl lysate; shaded area = CBS-deficient cells

Figure 7.7 Intracellular free amino acids related to homocysteine metabolism in control & CBS-deficient cells



Sulphydryl compounds

Intracellular total sulphydryl compounds in control and CBS-deficient cells - data shown in Table 7.1 and Figure 7.2.

As shown in **Table 7.1**, homocysteine levels ranged from 0.25-3.72 μmol/L (mean = 1.35 {SD±0.81}), cysteine from 0.61-11.09 μmol/L (mean = 3.78 {SD±3.03}), cysteinyl-glycine from 0.02-1.57 μmol/L (mean = 0.48 {SD±0.42}) and glutathione from 0.07-19.20 μmol/L (mean = 6.78 {SD±5.82}). The wide variation in the measured levels reflects variables such as differences in cell growth and metabolism, export and uptake etc. Notwithstanding these variables, consideration of the data including mean levels reveals a number of clear trends. On comparing the homocysteine levels found here with previously reported data, with the exception of human erythrocytes, levels were much lower than in plasma and tissues from both humans and animals (see Chapter 1, pages 19 & 20). As seen in **Figure 7.1**, the basic changes over time followed approximately the same pattern, irrespective of the media values, being maximal at the end of the growing period, then decreasing with confluence. The only exceptions to this were homocysteine and cysteine in the CBS-deficient cells in P-medt at 14 days, which increased with confluence. Surprisingly, homocysteine levels were very similar in the control cells in MEMt and P-medt from baseline to 14 days, and glutathione levels in the control and CBS-deficient cells in MEMt from baseline to 14 days were almost identical. Other noticeable differences between the metabolites attributable to the media, in relation to MEMt, included:

(i) lower cysteine in control cells in P-medt at 7 and 14 days, and in CBS-deficient cells in P-medt at 7 days;
(ii) lower cysteinyl-glycine in CBS-deficient cells in P-medt at 7 and 14 days; and
(iii) lower glutathione in control and CBS-deficient cells in P-medt at 7 days.

Comparing the CBS-deficient cells with controls, differences were:

(i) higher cysteinyl-glycine in CBS-deficient cells in MEMt and P-medt at baseline, 7 and 14 days; and
(ii) lower glutatione in CBS-deficient cells in P-medt at 7 days.

Media sulphydryl compounds from control and CBS-deficient cells - data shown in Tables 7.2 & 7.3 and Figures 7.2 & 7.3.

On considering the data shown in **Figures 7.2** & **7.3**, the basic changes were also quite similar between the media, but were less dramatic in P-medt than MEMt. Homocysteine export from control and CBS-deficient cells in MEMt was nearly identical. Sulphydryl compound binding or the freeing of binding sites seemed to occur irrespective of media or cell type. Other noticeable differences between the metabolites caused by the media, in relation to MEMt, included:

(i) lower homocysteine export from control cells in P-medt at 14 days;
(ii) lower cyst(e)ine uptake into control and CBS-deficient cells in P-medt at 7 and 14 days; and
(iii) lower cysteinyl-glycine with CBS-deficient cells in P-medt at 14 days.

5-MeTHF

Intracellular 5-MeTHF in control and CBS-deficient cells - data shown in Table 7.4 and Figure 7.5.

As shown in **Table 7.4**, 5-MeTHF levels ranged from undetectable (i.e. < 0.1 nmol/L) to 4.05 nmol/L (mean = 0.51 {SD±0.90}). In fact this compound was undetectable in > 66% samples so that statistical evaluation of the data is of limited value. Baseline, i.e. endogenous levels of 5-MeTHF, without γ-glutamyl hydrolase addition, from confluent stock cultures in MEMc were also undetectable. The lack of detection reflects the closeness of levels to the detection limit of the method in this system, and less than full recovery of 5-MeTHF due to the heating step. Nevertheless, even though preliminary data, it is clearly apparent from

Figure 7.5 that confluent cells in either media, on the whole, contained more 5-MeTHF than post-confluent cells. Also cells grown in MEMt, whether confluent or post-confluent, contained far more 5-MeTHF than those grown in P-medt.

On considering the actual 5-MeTHF concentration differences obtained from the control and CBS-deficient cells, levels increased in both media over the growing period, but were only maintained in post-confluent cells in MEMt. At the end of log phase, 5-MeTHF levels were similar in the control cells in each media, but considerably different from those in the CBS-deficient cells, as were the levels between the control and CBS-deficient cells. Assuming 5-MeTHF was stable under culture conditions, compared to the media[1] over 14 days, levels were around 2.5- and 10-fold higher in the control and CBS-deficient cells respectively in MEMt, but approximately equal in the cells in P-medt.

AdoMet & AdoHcy

Intracellular AdoMet in control and CBS-deficient cells - data shown in Tables 7.5 & 7.6 and Figure 7.6.

As shown in **Table 7.5**, AdoMet levels showed large variation, especially with the control cells in MEMt at 7 days and the CBS-deficient cells in P-medt at 14 days, and ranged from undetectable (i.e. < 0.1 nmo/L) to 349.39 nmol/L (mean = 105.30 {SD±99.22}). As shown in **Figure 7.6**, the basic changes with time in AdoMet levels roughly followed the same pattern irrespective of the media, and in a similar way to 5-MeTHF, were much higher in CBS-deficient than control cells. In the control cells over the growing period, the levels of AdoMet increased in MEMt, but decreased in P-medt. During confluence, the levels of AdoMet decreased in MEMt and P-medt. In the CBS-deficient cells over the growing period, the levels of AdoMet increased in both MEMt and P-medt. During confluence, like that with the control cells, the levels of AdoMet decreased in both MEMt and P-medt. On comparing the AdoMet levels in control cells in MEMt (0.065 nmol/mg protein at 7 days and 0.021 nmol/mg at 14 days) with previously reported data, levels at 7 days were very similar to those in human plasma, but levels at 14 days were much lower. They were also much lower than the levels in human red blood cells, murine fibrosarcoma L929 cells (0.5-1.0 nmol/mg Hasobe *et al.,* 1989; 0.7 nmol/mg Cools & De Clercq, 1990), but similar to those in rat liver cells (0.087 nmol/mg, see Chapter 1, page 33).

As shown in **Table 7.6**, the ratio of AdoMet : AdoHcy in all cases was considerably lower than the normally expected at least 4 AdoMet : 1 AdoHcy, with the greatest discrepancy in the control cells. Since this finding contradicts all known data in human cells or tissues and it was not possible to confirm the identity of the peak by LC/MS, it was assumed that this was due to an unknown compound interfering with the AdoHcy measurement (see Chapter 4, page 88).

Amino acids

Intracellular homocysteine related amino acids in control and CBS-deficient cells - data shown in Table 7.7 and Figure 7.7.

From the complete profiles of amino acids and their derivatives measured, all were present except α-amino-n-butyric acid, homocystine, tryptophan, Nπ(3)-methyl-histidine and anserine (see Chapter 4, pages 91-93). Phosphoserine, hydroxy-proline, asparagine, citrulline, GABA, ethanolamine and hydoxy-lysine were not present in the media and therefore must be produced by the cells, with glutamic acid being the only consistently high amino acid. Since only taurine, serine, glycine, cystine, methionine and cystathionine have a

[1] 5-MeTHF in FCS-t = 0.404 (SD±0.004, n = 2) pmol/mg protein, therefore 10% (v/v) = 0.040 pmol/mg

direct relationship to either transsulphuration or transmethylation, only these will be considered. Just to reiterate from Chapter 5 (pages 95, 96 & 99-103), these latter amino acids were present as either components of basal media and/or FCS.

As shown in **Table 7.7**, levels of taurine ranged from 5.9-144.1 μmol/L (mean = 46.4 {SD±44.6}), serine from 3.5-138.2 μmol/L (mean = 40.3 {SD±40.5}), glycine from 3.3-97.1 μmol/L (mean = 30.1 {SD±27.2}), cystine from 0-16.1 μmol/L (mean = 4.3 {SD±6.0}), methionine from undetectable to 66.1 μmol/L (mean = 15.4 {SD±21.2}) and cystathionine from undetectable to 8.7 μmol/L (mean = 1.4 {SD±2.9}). The range of values is rather wide, and like that with the other metabolites measured, can be attributed to variables such as differences in cell growth and metabolism, uptake and export etc. Nevertheless, taking average values, some trends are evident.

As shown in **Figure 7.7**, amino acid levels tended to be higher in P-medt than MEMt. Comparing the amino acid levels between the control and CBS-deficient cells, all were lower (by around 2-fold or more) in the CBS-deficient cells, irrespective of media.

Discussion

Media sulphydryl compounds from control cells - comparing the T_{75} flask and multi-time point data from Chapter 6

If the levels of homocysteine, cyst(e)ine, cysteinyl-glycine and glutathione, expressed in μmol/L, as shown in **Tables 7.1** & **7.2** and **Tables 6.6** & **6.7** are compared, both sets of data show remarkable similarity. If the levels expressed in μmol/L/10^6 cells, as shown in **Figures 7.3** & **7.4** and **Figures 6.6** & **6.7** are compared however, both show dissimilarity. Cells in log phase over 7 days exported 10-fold less homocysteine, similar glutathione, but used 2-fold more cyst(e)ine, with media cysteinyl-glycine 4-fold less than previously found. Differences were also apparent in the patterns of change in cysteinyl-glycine and glutathione, as well as approaching confluence, but did not include the patterns of homocysteine export or cyst(e)ine uptake. Although utilising proportionate seeding densities and media volumes, the T_{75} flask and multi-time point experiments were not entirely similar as the growth phases in the latter were discontinuous (cf. Christensen *et al.*, 1994). It was shown by Christensen *et al.*, (1991 & 1994) that homocysteine export is dependent on cell density, a fact that is also likely to apply to cyst(e)ine uptake, cysteinyl-glycine and glutathione export, and could account for the differences.

Intracellular & media sulphydryl compounds and amino acids with control & CBS-deficient cells - differences in transsulphuration

Generally, as cells become confluent, it can be assumed that metabolism is reduced which might lead to decreased intracellular metabolite levels followed by reduced sulphydryl compound export and/or uptake.

Differences in homocysteine levels between CBS-deficient and control cells were only observed in P-medt at 14 days (**Figure 7.2**). This is explained by reduced transsulphuration caused by impaired CBS function, together with reduced transmethylation due to reduced MS activity (Christensen *et al.*, 1994) because of lower medium folate levels. Intracellular cysteine levels in these cells also increased, but was not due to increased uptake and is difficult to explain. In log phase and post-confluent control cells in P-medt and MEMt, homocysteine levels remained similar regardless of the differences in the media levels of for example methionine, folate or vitamin B_6. This reflects the importance of homocysteine as a pivotal metabolite in the salvage of methionine and folate, its strict intracellular control, as well as the role of export in controlling intracellular levels. In log phase and post-confluent control and CBS-deficient cells in MEMt, intracellular glutathione levels, as an essential component of cellular metabolism, remained similar since the medium contained a sufficiently high level of cyst(e)ine, which is the main rate limiting factor in GSH synthesis.

There is little doubt that media and intracellular cysteine, cysteinyl-glycine and glutathione levels are related by transsulphuration and the γ-glutamyl cycle. Interestingly, tissues that posses both CBS and CTH also have the highest turnover of glutathione (Finkelstein *et al.,* 1988). Media methionine, which also gains entry into the cell by the γ-glutamyl transport system, could also affect cysteinyl-glycine and glutathione levels through conversion to homocysteine. The exact influence of glutathione levels will depend on whether homocysteine acts as anti- or pro-oxidant, and therefore the need to maintain the redox status of the cell. From the present data, the probable causes of the lower cysteine and glutathione levels in confluent control cells in P-medt were the lower medium cyst(e)ine levels and reduced uptake. The lower cysteine, cysteinyl-glycine and glutathione levels in confluent CBS-deficient cells in P-medt were probably caused by reduced transsulphuration and consequently lower cysteine synthesis. An increased level of intracellular cysteinyl-glycine may be indicative of increased gluathione recycling, where high cysteinyl-glycine levels relate to low glutathione levels and vice versa through regulation of the sulphydryl compound-exchange system.

Very few studies exist on the measurement of intracellular free amino acids in cultured skin fibroblasts. Those that do have mainly investigated transport of specific amino acids, especially the neutral amino acids proline, alanine and leucine and not the full composition of the free amino acid pool (Gazzola *et al.,* 1980). Other studies are not comparable to the experiments presented here due to differences in cultivation and harvesting. For example, those by Melancon *et al.,* (1972) and Shih *et al.,* (1975) both used MEM as their basal medium but supplemented with 15% (v/v) FCS and non-essential amino acids, together with a respective 2- and 5-day feeding before harvesting. It has been shown that both long periods between feeding and harvesting, excessive washing, prolonged contact with saline and mechanical scraping can result in losses of free amino acids from cells (Piez & Eagle, 1958).

In control cells, the levels of taurine, serine, glycine, cystine, methionine and cystathionine were slightly higher with P-medt than MEMt. This indicates that transport of amino acids is not directly influenced by the excessive amino acid concentrations found in MEMt. Whether the higher levels of sulphur containing amino acids in MEMt cause inhibition of amino acid uptake can only be speculated on. The role of taurine as an antioxidant, its ability to significantly reduce oxidative stress by increasing reduced glutathione and catalase levels, as well as glutathione peroxidase activity are well documented (Hagar, 2004; Oudit *et al.,* 2004), and may explain a link bewteen the higher levels in P-medt and the corresponding lower glutathione levels. In CBS-deficient cells, the levels of taurine, cystine and cystathionine in 7 days old cultures were lower than in control cells, which can be easily explained by impaired CBS function. Changes in the other amino acids are probably not directly related to CBS deficiency. For example, serine has a stronger association to folate metabolism than CBS function and transsulphuration. An unexpected finding was the low methionine level in the CBS-deficient cells. This cannot be due to increased protein synthesis, judging on the protein levels obtained after 7 days, but is more likely due to increased conversion to AdoMet, as the intracellular AdoMet levels were higher than in the controls in both P-medt and MEMt, which may lead to increased methylation or polyamine synthesis.

Intracellular 5-MeTHF & AdoMet in control & CBS-deficient cells - differences in transmethylation

It has been previously shown that folate polyglutamates reach higher levels in log phase human fibroblasts and hepatocytes than in medium, with no equilibrium, and to greater effect when the source is 5-MeTHF. About 95% of folate derivatives are polyglutamates with a chain length of 2-10 glutamate residues, but more specifically 6 residues with folic acid and 7 residues with 5-MeTHF (Foo *et al.*, 1982). Post-confluent foetal rat skin fibroblasts however, do not concentrate polyglutamates when the source is folic acid, uptake proceeding until equilibrium with the medium level is reached (Eilam *et al.*, 1982). It has been suggested that the folate transport systems are probably regulated by media folate levels through changes in the intracellular levels (Kane *et al.*, 1988), as well as by cell proliferation.

The lower intracellular 5-MeTHF levels in the cells in P-medt were unquestionably due to the lower medium folate level. Folic acid enters the cytoplasm via the folate receptor (FR), where it is converted by dihydrofolate reductase firstly to dihydrofolate then tetrahydrofolate, without the need for MS and

homocysteine remethylation. Tetrahydrofolate is then converted to 5, 10-methylenetetrahydrofolate and finally to 5-MeTHF by MTHFR. Low 5-MeTHF reduces the ability of the cell to remethylate homocysteine and increases its export. Compared to the differences in media folic acid levels, media 5-MeTHF levels derived from FCS and therefore the reduced folate carrier (RFC) did not play a significant role. Furthermore, as folate export from human skin fibroblasts is also via RFC (Hilton *et al.,* 1983), loss of folate would be negligible, and this is supported by the finding of consistently undetectable 5-MeTHF in media (see Chapter 4, page 76). Early work suggested that methionine facilitates folate entry into fibroblasts (Grzelakowska-Sztabert, 1976), but seems unlikely here judging on the levels obtained in the cells in both media, especially in the controls, which is in keeping with Christensen & Ueland (1993). So the higher levels of 5-MeTHF in the CBS-deficient cells, were possibly due to increased uptake through increased FR expression or receptor recycling, an adaptive response to reduced transsulphuration.

AdoMet levels were higher in CBS-deficient cells in both media compared to controls, but especially in P-medt. This reflects the situation observed in hyperhomocystinuria due to CBS deficiency, where it is thought that high homocysteine leads to both high AdoHcy and AdoMet.

Conclusions

This part of the work answered the important question, could the model be used to reliably measure levels of intracellular metabolites? Indeed, total homocysteine, cysteine, cysteinyl-glycine, glutathione, AdoMet and the free amino acids could be measured, but 5-MeTHF and AdoHcy could not. Although the failure to measure these latter metabolites was disappointing, it was not strictly speaking a direct failing of the model, but of the methods of detection. Using sephadex fractionation for 5-MeTHF and isocratic elution with a Hypersil® ODS C18 column for AdoHcy, perhaps may have given complete data on the pathway.

In searching for additional information, the study not only highlighted the complexity of the metabolism, but the importance of export in the control of intracellular homocysteine, folate receptors and folate in homocysteine remethylation in post-confluent CBS-deficient cells, transsulphuration in glutathione synthesis, and the unrelated nature of media amino acid concentrations in amino acid transport.

This added to the existing work by obtaining more comprehensive data on the main metabolite levels in media and cells under similar conditions, and gave support and validation to our knowledge of export from previous studies as applicable to the human physiological situation.

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Effect of methionine & folate on the sulphydryl compounds, 5-MeTHF & AdoMet in control fibroblasts

Introduction

These experiments were designed to evaluate the effect of methionine and folate on:
(i) the sulphydryl compounds homocysteine, cysteine, cysteinyl-glycine and glutathione;
(ii) 5-MeTHF; and
(iii) AdoMet
in control fibroblasts grown in P-medt. Concentrations of methionine and folate were chosen to favour conditions of methionine conservation (remethylation) or catabolism (transulphuration). Comparisons could then be made between control cells grown in P-medt and P-medt supplemented with methionine or folate.

Methods

As described in Chapter 6 (page 117), cells were harvested using trypsin-EDTA and pooled. For experiments, cells were sub-cultured at a split ratio of 1 : 2 into T_{75} t/c flasks containing 20 ml P-medt supplemented with 100 µl of L-methionine (Mx10 and Mx50, Sigma) or folic acid (Fx10, Fx100 and Fx1000, Sigma) made up in sterile PBS pH 7.3 to equal the concentrations given in **Table 8.1**, or P-medt supplemented with 100 µl sterile PBS pH 7.3 (baseline, BL), where the total flask volume was 21.1 ml in each case.

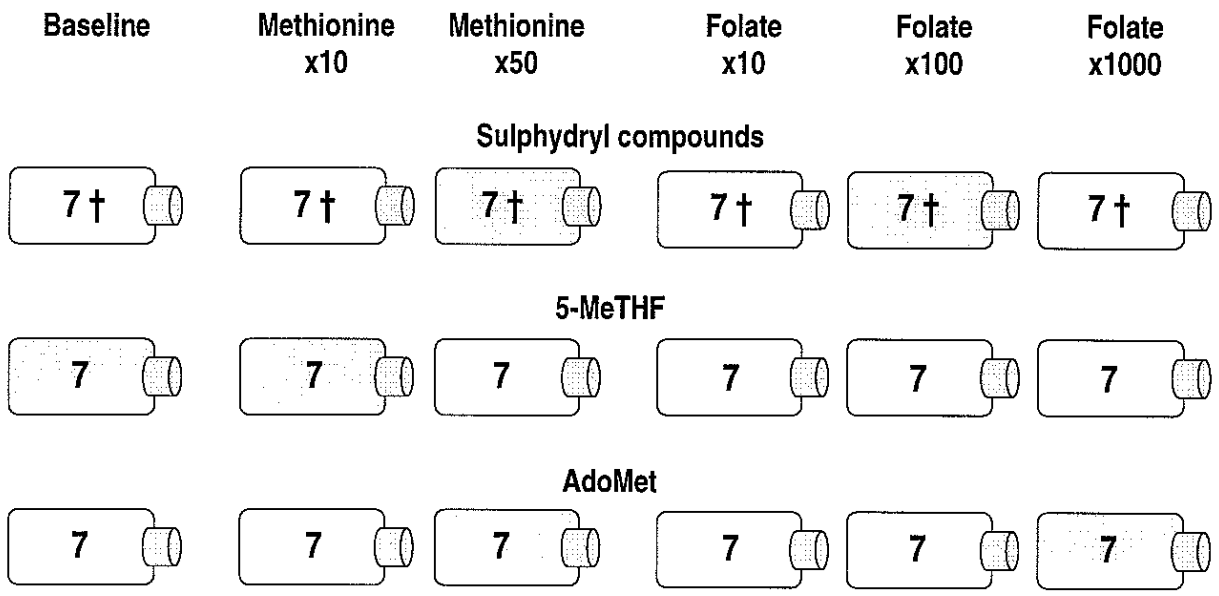
Each metabolite was determined separately from pooled cells from 3 flasks. After incubating for 7 days, cells were harvested using dissociation solution, obtained as a pellet by centrifugation and lysed as described in Chapter 3 (pages 60 & 62). Lysates for the determination of total homocysteine, cysteine, cysteinyl-glycine and glutathione were stored at –70°C. For 5-MeTHF, lysates were treated immediately with γ-glutamyl hydrolase as described in Chapter 4 (page 73). For AdoMet, lysates were deproteinised by the addition of 56.3 µl 10% (v/v) PCA to a 90 µl lysate. Media samples of 150 µl were additionally taken daily, which was below the 174 µl that would cause a flask volume change of 5%, and processed as described in Chapter 6 (page 118). A summary of the experiments in this section can be seen in **Figure 8.1**. The sulphydryl compounds, 5-MeTHF and AdoMet were measured by HPLC as described in Chapter 4 (pages 68-90).

Table 8.1 Medium levels of methionine & folate

Treatment	Concentration added to flask (μmol/L)	Final concentration (μmol/L)	Relation to physiological level
Baseline	-	Methionine 35.52; folate 0.02	x1
Methionine	319.68	355.20	x10
"	1740.48	1776.00	x50
Folate	0.21	0.23	x10
"	2.25	2.27	x100
"	22.64	22.66	x1000

Figure 8.1 Summary of experiments

all flasks x3; † samples also taken for media sulphydryl compounds; sulphydryl compounds included homocysteine, cysteine, cysteinyl-glycine & glutathione; P-medt used with (methionine & folate) or without (baseline) supplementation as shown for all cultures



Results

All **intracellular** levels are from single measurements from 3 pooled flasks. All **media** levels are from triplicate measurements, with one measurement from each of the 3 flasks.

Intracellular total sulphydryl compound concentrations (μmol/L) and protein concentrations from the individual cell lines are shown in **Table 8.2. Figure 8.2** shows the mean (±SD) intracellular concentrations of total sulphydryl compounds in the control cell lines cultured in P-medt, P-medt supplemented with methionine and P-medt supplemented with folate at 7 days.

Medium sulphydryl compound concentrations (μmol/L) measured as total, free and bound from cultures of control cell lines are shown in **Table 8.3** (homocysteine), **Table 8.4** (cyst{e}ine), **Table 8.5**

(cysteinyl-glycine) & **Table 8.6** (glutathione). **Figure 8.3** (homocysteine and cyst{e}ine) & **Figure 8.4** (cysteinyl-glycine and glutathione) show the changes in sulphydryl compound concentrations at daily intervals up to 7 days compared to baseline at the corresponding time, in control cell lines grown in P-medt and supplemented P-medt.

Intracellular 5-MeTHF concentrations (nmol/L) and protein concentrations from the individual cell lines are shown in **Table 8.7. Figure 8.5** shows the mean (±SD) intracellular concentrations of 5-MeTHF in the control cell lines cultured in P-medt and supplemented P-medt at 7 days.

Intracellular AdoMet concentrations (nmol/L) and protein concentrations from the individual cell lines are shown in **Table 8.8. Figure 8.6** shows the mean (±SD) intracellular concentrations of AdoMet in the control cell lines cultured in P-medt and supplemented P-medt at 7 days.

Table 8.2 Intracellular total sulphydryl compound concentrations (μmol/L)

Treatment	Cell line	Protein (μg)	Sulphydryl compound concentration (μmol/L)			
			Hcy	Cys	Cys-gly	GSH
Baseline	2	385.66	3.61	10.41	1.71	5.75
Methionine x10		321.18	2.82	6.42	1.35	3.48
Methionine x50		264.76	2.49	3.94	0.84	2.57
Folate x10		292.97	2.62	6.53	1.27	3.08
Folate x100		239.24	1.96	2.59	0.84	2.17
Folate x1000		337.30	1.91	5.47	1.04	2.04
Baseline	3	251.33	1.84	3.16	0.59	5.63
Methionine x10		174.76	1.12	1.98	0.47	2.62
Methionine x50		217.75	1.60	2.47	0.50	4.84
Folate x10		235.21	1.57	3.51	0.06	4.10
Folate x100		213.72	1.21	3.35	0.61	2.42
Folate x1000		284.91	2.70	7.57	0.98	4.73
Baseline	5	107.60	0.00	0.63	0.06	2.27
Methionine x10		137.15	0.66	0.91	0.09	3.78
Methionine x50		150.58	0.65	0.91	0.10	3.93
Folate x10		137.15	0.62	0.97	0.10	4.49
Folate x100		115.66	0.50	0.69	0.06	2.78
Folate x1000		288.94	1.58	6.27	0.53	7.07
Baseline	6	306.40	2.48	5.74	1.26	3.61
Methionine x10		311.78	2.77	6.50	1.47	3.46
Methionine x50		309.09	3.03	6.25	1.29	6.12
Folate x10		40.43	0.47	0.30	0.02	0.64
Folate x100		275.51	1.83	3.50	1.19	3.75
Folate x1000		279.54	1.79	5.86	1.22	2.35

values are single measurements from a 90 μl lysate

Figure 8.2 Intracellular total sulphydryl compound concentrations (nmol/mg protein)

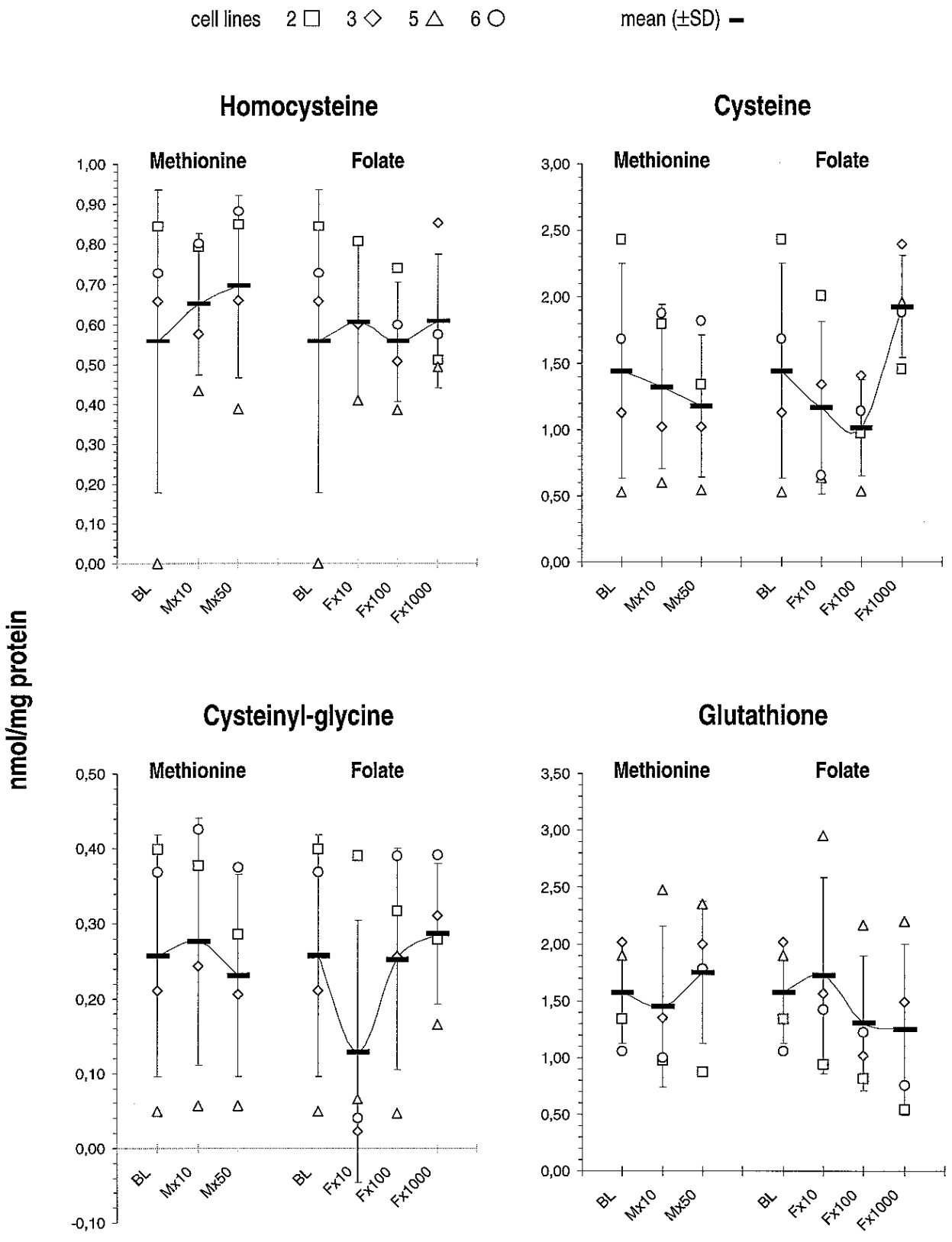


Table 8.3 Medium homocysteine concentrations (μmol/L) measured as total, free and bound from cultures of control cell lines

Time (hours)	Homocysteine (μmol/L)								
	Baseline			Methionine x10			Methionine x50		
	total	free	bound	total	free	bound	total	free	bound
0	4.55 ±0.59	3.55 ±0.36	1.68 ±0.43						
24	12.26 ±1.48	5.07 ±1.05	1.75 ±0.42	14.01 ±2.15	6.70 ±1.41	2.50 ±0.84	15.14 ±2.39	7.27 ±1.61	2.51 ±0.71
48	15.43 ±4.01	7.59 ±2.02	2.78 ±1.06	19.13 ±5.26	10.16 ±3.13	4.11 ±1.77	20.92 ±5.78	11.13 ±3.59	4.36 ±1.56
72	18.74 ±5.29	9.71 ±3.15	3.97 ±1.69	24.80 ±6.98	13.99 ±4.67	5.98 ±2.08	26.91 ±7.53	15.25 ±4.67	6.43 ±2.45
96	22.44 ±7.91	12.54 ±4.76	5.44 ±2.35	30.48 ±10.51	17.82 ±6.74	8.02 ±2.93	36.51 ±10.70	20.12 ±6.98	8.51 ±3.23
120	29.52 ±9.38	15.63 ±5.42	6.99 ±2.83	42.03 ±12.58	23.26 ±8.31	10.52 ±3.32	46.22 ±13.27	25.58 ±8.32	11.55 ±4.04
144	35.41 ±10.65	19.85 ±7.54	8.83 ±3.42	52.22 ±17.78	28.87 ±10.55	13.26 ±4.58	54.98 ±17.17	31.67 ±10.60	14.13 ±5.22
168	36.77 ±15.37	19.49 ±10.63	9.49 ±4.50	52.35 ±21.24	28.77 ±14.54	14.72 ±5.29	57.17 ±23.55	32.67 ±16.10	15.22 ±5.39

Time (hours)	Homocysteine (μmol/L)								
	Folate x10			Folate x100			Folate x1000		
	total	free	bound	total	free	bound	total	free	bound
0									
24	12.30 ±1.42	5.19 ±0.90	1.91 ±0.48	11.89 ±1.28	4.84 ±1.07	1.77 ±0.34	11.73 ±1.41	4.56 ±0.97	2.59 ±1.24
48	15.39 ±3.48	7.25 ±2.50	3.05 ±0.99	14.75 ±3.38	7.05 ±2.39	2.65 ±0.92	15.66 ±2.09	6.11 ±1.38	3.29 ±0.55
72	18.74 ±18.74	9.70 ± 3.18	3.80 ±1.29	17.13 ±4.06	8.50 ±2.75	3.77 ±1.20	19.26 ±3.98	8.06 ±2.38	4.44 ±1.03
96	24.00 ±24.00	11.24 ±4.85	5.43 ±2.21	21.18 ±6.39	10.26 ±3.85	4.71 ±1.93	22.50 ±5.12	9.24 ± 3.42	5.38 ±1.34
120	28.39 ±8.61	13.89 ±5.29	6.89 ±3.07	24.35 ±7.77	12.02 ±4.62	6.07 ±2.63	26.16 ±6.11	11.20 ±3.89	6.40 ±1.99
144	33.86 ±11.64	17.51 ±7.04	8.25 ±3.35	28.21 ±9.71	14.23 ±5.63	7.19 ±3.35	29.39 ±7.62	13.67 ±5.15	7.55 ±2.01
168	33.60 ±12.14	17.46 ±8.70	9.17 ±3.91	28.17 ±10.94	14.06 ±7.33	7.40 ±3.35	32.55 ±9.21	14.99 ±5.27	9.49 ±2.19

each result represents mean (±SEM) of 3 flasks from 4 cell lines, n = 12

Table 8.4 Medium cyst(e)ine concentrations (μmol/L) measured as total, free and bound from cultures of control cell lines

Time (hours)	Cyst(e)ine (μmol/L)								
	Baseline			Methionine x10			Methionine x50		
	total	free	bound	total	free	bound	total	free	bound
0	33.06 ±3.01	35.26 ±5.61	3.22 ±0.86						
24	23.86 ±1.62	23.33 ±1.64	2.01 ±0.89	23.28 ±2.17	23.10 ±1.94	2.12 ±0.70	24.46 ±1.43	22.06 ±0.96	2.46 ±0.15
48	21.15 ±0.26	20.22 ±1.59	1.83 ±0.76	21.07 ±1.71	20.18 ±1.51	2.39 ±0.55	22.32 ±1.61	20.03 ±0.44	2.38 ±0.28
72	19.75 ±0.75	18.58 ±1.20	1.86 ±0.70	20.91 ±0.82	19.43 ±1.41	2.65 ±0.20	20.43 ±1.31	18.57 ±0.58	2.24 ±0.37
96	19.31 ±0.37	17.63 ±0.90	1.91 ±0.90	19.25 ±0.38	18.01 ±0.72	2.59 ±0.43	19.73 ±0.84	17.70 ±0.15	2.23 ±0.43
120	17.96 ±0.92	16.43 ±0.34	1.50 ±0.80	18.79 ±1.68	17.22 ±0.59	2.32 ±0.58	18.77 ±1.02	17.38 ±0.36	2.13 ±0.39
144	17.40 ±0.64	16.07 ±1.63	1.77 ±0.85	18.20 ±1.35	15.95 ±0.25	2.03 ±0.44	16.95 ±1.33	15.97 ±1.21	1.82 ±0.48
168	16.65 ±1.30	15.44 ±0.84	1.48 ±0.76	16.45 ±1.03	15.49 ±0.44	1.95 ±0.40	16.04 ±0.91	15.54 ±0.17	1.63 ±0.47

Time (hours)	Cyst(e)ine (μmol/L)								
	Folate x10			Folate x100			Folate x1000		
	total	free	bound	total	free	bound	total	free	bound
0									
24	24.15 ±1.28	21.74 ±0.34	2.44 ±0.34	22.86 ±1.84	22.03 ±0.95	1.55 ±0.31	25.88 ±0.00	26.21 ±0.00	3.94 ±0.00
48	21.39 ±1.08	19.35 ±0.25	2.30 ±0.45	14.53 ±10.79	19.71 ±0.73	1.58 ±0.43	24.66 ±2.09	22.23 ±1.48	3.29 ±0.77
72	20.17 ±0.98	18.05 ±0.45	2.08 ±0.69	18.72 ±1.45	18.35 ±0.40	1.80 ±0.17	23.33 ±2.22	20.84 ±1.28	3.24 ±0.57
96	18.64 ±1.67	17.05 ±0.98	2.15 ±0.86	19.33 ±0.94	17.54 ±0.64	1.80 ±0.07	22.19 ±2.23	18374 ±0.85	3.25 ±0.65
120	18.07 ±0.96	15.93 ±0.52	1.59 ±0.17	18.05 ±0.31	17.44 ±0.49	1.75 ±0.15	21.63 ±2.23	18.13 ±1.38	3.24 ±0.56
144	17.20 ±0.44	15.16±0.80	1.50 ±0.35	17.62 ±0.43	16.32 ±0.44	1.67 ±0.12	19.99 ±2.53	17.58 ±1.07	3.08 ±0.31
168	16.20 ±0.47	14.48 ±0.58	1.33 ±0.25	16.04 ±0.65	15.25 ±0.74	1.54 ±0.29	19.11 ±1.78	16.36 ±1.69	2.82 ±0.94

each result represents mean (±SEM) of 3 flasks from 4 cell lines, n = 12

Table 8.5 Medium cysteinyl-glycine concentrations (μmol/L) measured as total, free and bound from cultures of control cell lines

Time (hours)	Cysteinyl-glycine (μmol/L)								
	Baseline			Methionine x10			Methionine x50		
	total	free	bound	total	free	bound	total	free	bound
0	0.89 ±0.11	0.60 ±0.09	0.31 ±0.04						
24	1.08 ±0.03	1.01 ±0.05	0.10 ±0.05	1.07 ±0.03	1.04 ±0.03	0.09 ±0.02	1.16 ±0.08	0.95 ±0.13	0.12 ±0.03
48	1.35 ±0.17	1.20 ±0.20	0.10 ±0.05	1.34 ±0.17	1.30 ±0.20	0.12 ±0.04	1.46 ±0.09	1.32 ±0.11	0.14 ±0.02
72	1.50 ±0.32	1.35 ±0.30	0.13 ±0.05	1.59 ±0.32	1.50 ±0.36	0.17 ±0.03	1.62 ±0.33	1.49 ±0.27	0.17 ±0.01
96	1.74 ±0.49	1.54 ±0.46	0.15 ±0.09	1.78 ±0.52	1.64 ±0.48	0.22 ±0.02	1.83 ±0.47	1.64 ±0.41	0.19 ±0.01
120	1.81 ± 0.55	1.64 ±0.62	0.15 ±0.11	1.94 ±0.62	1.81 ±0.64	0.22 ±0.01	1.93 ±0.60	1.79 ±0.57	0.20 ±0.02
144	2.05 ±0.67	1.80 ±0.74	0.19 ±0.14	2.15 ±0.80	1.94 ±0.72	0.24 ±0.03	1.99 ±0.61	1.83 ±0.60	0.20 ±0.01
168	2.16 ±0.77	1.95 ±0.82	0.18 ±0.15	2.24 ±0.84	2.09 ±0.81	0.27 ±0.05	2.12 ±0.86	1.90 ±0.74	0.20 ±0.01

Time (hours)	Cysteinyl-glycine (μmol/L)								
	Folate x10			Folate x100			Folate x1000		
	total	free	bound	total	free	bound	total	free	bound
0									
24	1.09 ±0.04	0.98 ±0.07	0.12 ±0.04	1.04 ±0.02	0.94 ±0.06	0.07 ±.0.01	1.11 ±0.00	0.95 ±0.00	0.17 ±0.00
48	1.33 ±0.16	1.22 ±0.14	0.13 ±0.04	1.31 ±0.20	1.20 ±0.12	0.08 ±0.01	1.38 ±0.11	1.22 ±0.06	0.14 ±0.05
72	1.53 ±0.30	1.37 ±0.25	0.13 ±0.04	1.45 ±0.33	1.38 ±0.29	0.11 ±0.01	1.65 ±0.15	1.47 ±0.14	0.17 ±0.03
96	1.69 ±0.44	1.54 ±0.45	0.16 ±0.03	1.74 ±0.55	1.56 ±0.44	0.14 ±0.04	1.31 ±1.01	1.49 ±0.29	0.20 ±0.04
120	1.87 ±0.69	1.61 ±0.63	0.14 ±0.04	1.84 ±0.65	1.74 ±0.64	0.16 ±0.05	1.91 ±0.31	1.57 ±0.31	0.23 ±0.05
144	2.05 ±0.84	1.75 ±0.78	0.15 ±0.05	2.05 ±0.80	1.89 ±0.77	0.18 ±0.06	1.94 ±0.53	1.68 ±0.42	0.24 ±0.05
168	2.16 ±0.90	1.88 ±0.87	0.16 ±0.06	2.12 ±0.80	1.98 ±0.86	0.20 ±0.09	2.06 ±0.64	1.74 ±0.50	0.24 ±0.08

each result represents mean (±SEM) of 3 flasks from 4 cell lines, n = 12

Table 8.6 Medium glutathione concentrations (μmol/L) measured as total, free and bound from cultures of control cell lines

Time (hours)	Glutathione (μmol/L)								
	Baseline			Methionine x10			Methionine x50		
	total	free	bound	total	free	bound	total	free	bound
0	2.12 ±0.26	0.78 ±0.15	0.90 ±0.18						
24	1.70 ±0.26	1.48 ±0.25	0.40 ±0.45	1.74 ±0.40	1.47 ±0.24	0.19 ±0.05	1.76 ±0.25	1.32 ±0.12	0.17 ±0.02
48	1.70 ±0.45	1.50 ±0.53	0.13 ±0.07	1.68 ±0.49	1.58 ±0.49	0.15 ±0.04	1.75 ±0.42	1.27 ±0.17	0.13 ±0.04
72	1.65 ±0.62	1.48 ±0.57	0.13 ±0.05	1.72 ±0.64	1.60 ±0.58	0.16 ±0.03	1.68 ±0.61	1.28 ±0.53	0.13 ±0.04
96	1.62 ±0.59	1.45 ±0.59	0.11 ±0.06	1.73 ±0.73	1.57 ±0.66	0.17 ±0.04	1.65 ±0.58	1.09 ±0.63	0.10 ±0.00
120	1.45 ±0.53	1.39 ±0.61	0.10 ±0.06	1.66 ±0.70	1.50 ±0.63	0.14 ±0.02	1.46 ±0.56	1.02 ±0.59	0.09 ±0.01
144	1.60 ±0.56	1.42 ±0.62	0.11 ±0.07	1.75 ±0.75	1.52 ±0.61	0.13 ±0.02	1.43 ±0.49	1.08 ±0.48	0.11 ±0.02
168	1.68 ±0.61	1.53 ±0.66	0.11 ±0.06	1.81 ±0.72	1.64 ±0.65	0.15 ±0.04	1.51 ±0.61	0.98 ±0.67	0.08 ±0.01

Time (hours)	Glutathione (μmol/L)								
	Folate x10			Folate x100			Folate x1000		
	total	free	bound	total	free	bound	total	free	bound
0									
24	1.69 ±0.29	1.42 ±0.27	0.18 ±0.01	1.70 ±0.23	1.42 ±0.22	0.14 ±0.02	2.21 ±0.00	1.61 ±0.00	0.44 ±0.00
48	1.68 ±0.49	1.52 ±0.48	0.13 ±0.02	1.72 ±0.40	1.48 ±0.40	0.11 ±0.00	1.85 ±0.09	1.49 ±0.11	0.19 ±0.07
72	1.72 ±0.69	1.49 ±0.62	0.12 ±0.00	1.76 ±0.67	1.54 ±0.61	0.11 ±0.01	1.81 ±0.14	1.49 ±0.21	0.16 ±0.06
96	1.57 ±0.62	1.45 ±0.60	0.12 ±0.00	1.72 ±0.70	1.44 ±0.58	0.11 ±0.03	1.70 ±0.19	1.34 ±0.27	0.16 ±0.05
120	1.59 ±0.59	1.37 ±0.60	0.10 ±0.02	1.55 ±0.59	1.40 ±0.63	0.11 ±0.03	1.63 ±0.25	1.28 ±0.31	0.14 ±0.06
144	1.68 ±0.64	1.41 ±0.65	0.09 ±0.01	1.71 ±0.72	1.46 ±0.66	0.11 ±0.03	1.58 ±0.33	1.26 ±0.34	0.14 ±0.05
168	1.82 ±0.73	1.53 ±0.64	0.10 ±0.03	1.79 ±0.73	1.59 ±0.68	0.12 ±.0.04	1.62 ±0.43	1.26 ±0.37	0.12 ±0.06

each result represents mean (±SEM) of 3 flasks from 4 cell lines, n = 12

Figure 8.3 Changes in medium homocysteine & cyst(e)ine compared to baseline

each point represents mean (±SEM, n = 12) from 4 control cell lines 2, 3, 5 & 6 ○ total □ free △ bound

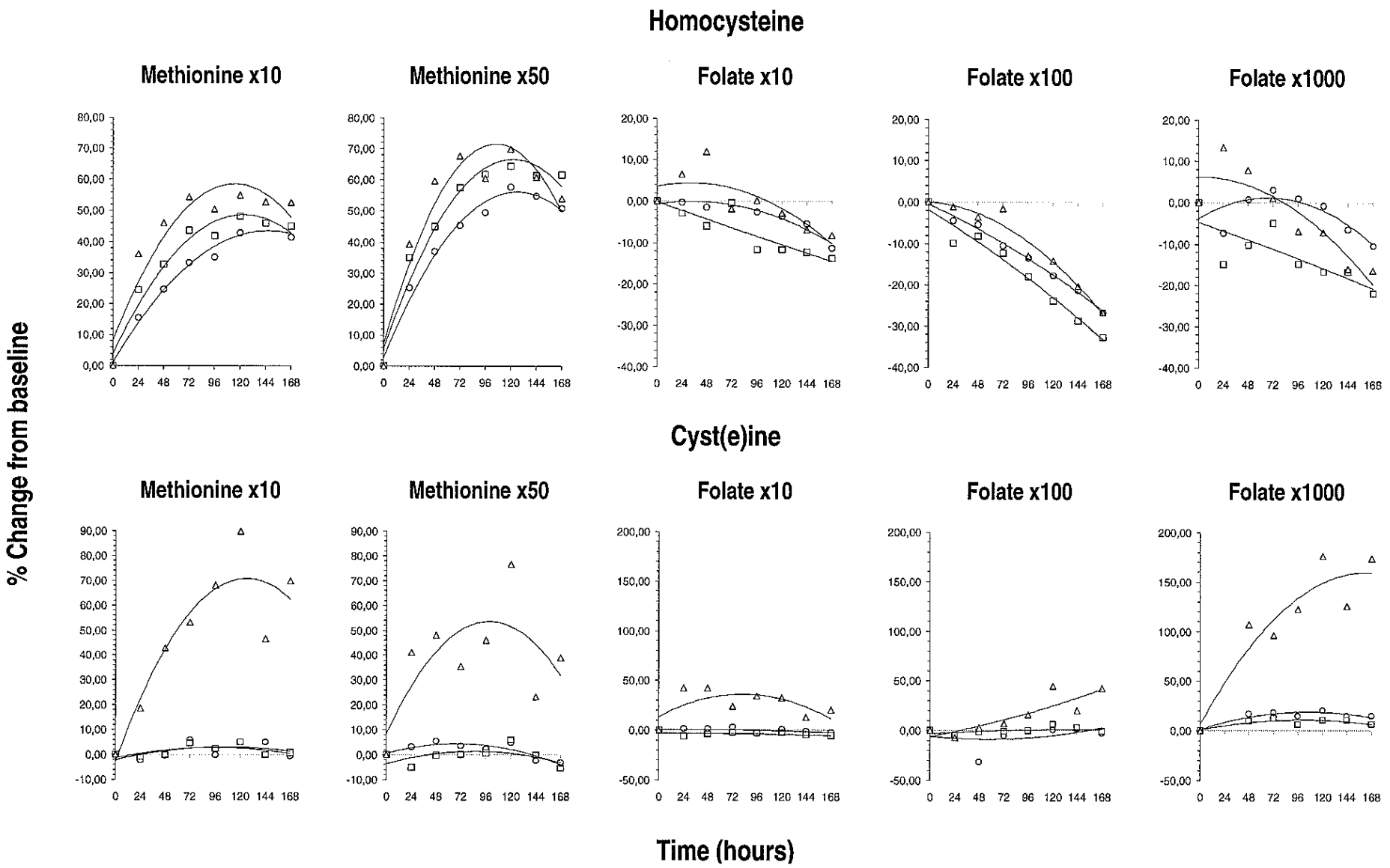


Figure 8.4 Changes in medium cysteinyl-glycine & glutathione compared to baseline

each point represents mean (±SEM, n = 12) from 4 control cell lines 2, 3, 5 & 6 ○ total □ free △ bound

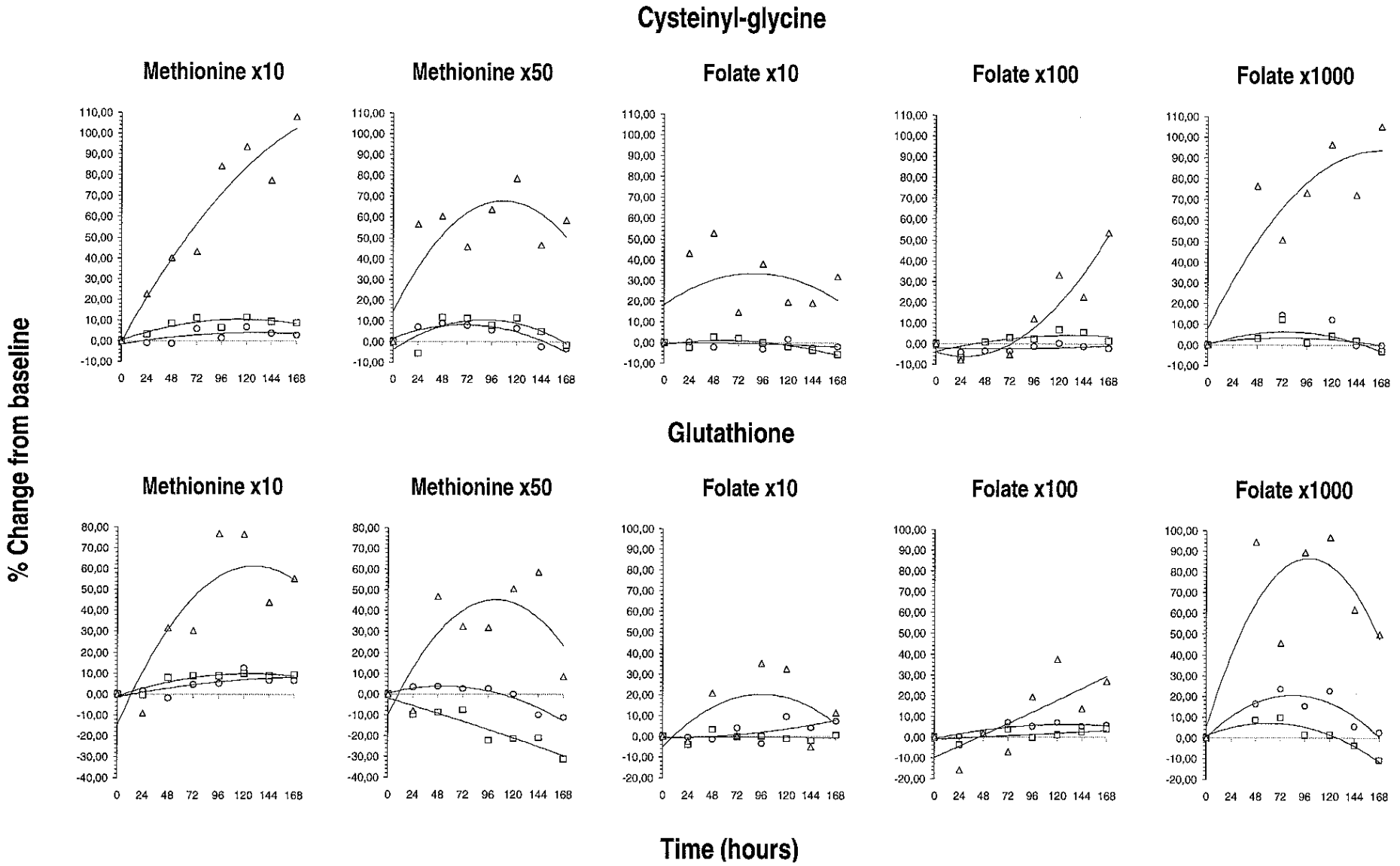


Table 8.7 Intracellular 5-MeTHF concentrations (nmol/L)

Treatment	Cell line	Protein (µg)	5-MeTHF concentration (nmol/L)
Baseline	2	356.10	0.16
Methionine x10		311.78	0.20
Methionine x50		275.51	0.00
Folate x10		297.00	0.35
Folate x100		275.51	1.40
Folate x1000		338.64	1.06
Baseline	3	227.15	0.00
Methionine x10		220.43	0.00
Methionine x50		209.69	0.00
Folate x10		243.27	0.08
Folate x100		229.84	0.58
Folate x1000		322.52	1.76
Baseline	5	212.37	0.00
Methionine x10		153.27	0.00
Methionine x50		177.45	0.00
Folate x10		162.67	0.00
Folate x100		134.46	0.00
Folate x1000		315.81	1.26
Baseline	6	290.28	0.12
Methionine x10		267.45	0.13
Methionine x50		248.64	0.11
Folate x10		278.19	0.21
Folate x100		295.66	0.64
Folate x1000		292.97	2.72

values are single measurements from a 90 µl lysate

Figure 8.5 Intracellular 5-MeTHF concentrations (pmol/mg protein)

each bar represents mean (±SD); paired student's t-test * Fx1000 vs. BL $p < 0.05$ (0.02)

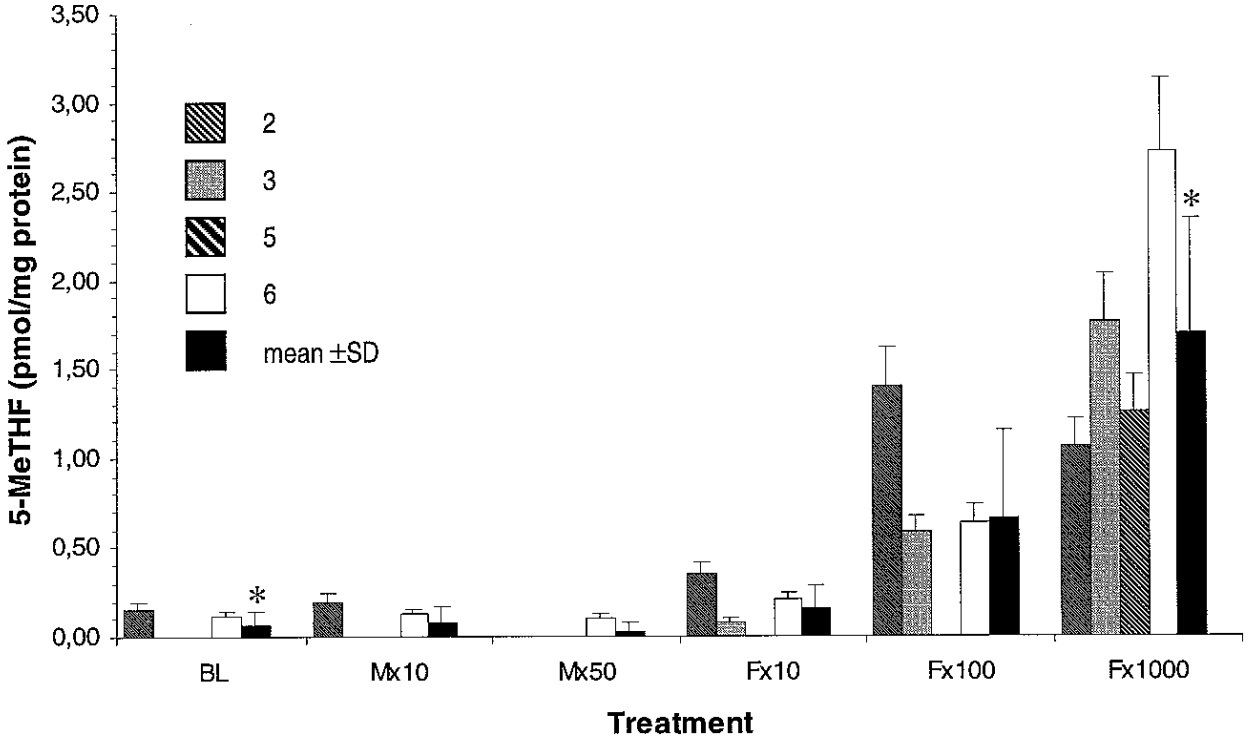
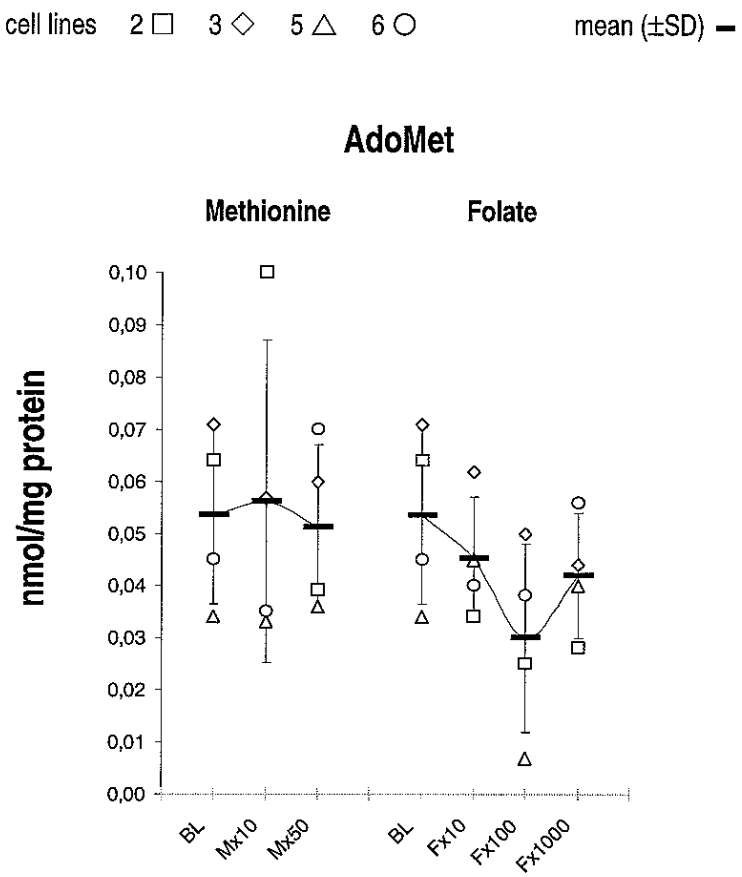


Table 8.8 Intracellular AdoMet concentrations (nmol/L)

Treatment	Cell line	Protein (μg)	AdoMet concentration (nmol/L)
Baseline	2	310.43	220.45
Methionine x10		323.87	358.93
Methionine x50		272.82	119.58
Folate x10		344.01	129.44
Folate x100		270.13	74.38
Folate x1000		401.78	126.70
Baseline	3	221.78	175.46
Methionine x10		205.66	130.10
Methionine x50		217.75	145.40
Folate x10		217.75	148.87
Folate x100		232.52	128.77
Folate x1000		370.88	182.67
Baseline	5	157.30	59.46
Methionine x10		165.36	60.88
Methionine x50		141.18	55.74
Folate x10		158.64	79.37
Folate x100		102.22	8.12
Folate x1000		373.57	164.60
Baseline	6	291.63	144.46
Methionine x10		626.10	244.60
Methionine x50		254.01	197.83
Folate x10		331.93	148.41
Folate x100		298.34	124.31
Folate x1000		334.61	208.69

values are single measurements from a 90 μl lysate

Figure 8.6 Intracellular AdoMet concentrations (pmol/mg protein)



Sulphydryl compounds

Changes in relation to medium methionine and folate levels - intracellular total sulphydryl compounds

As shown in **Table 8.2**, overall levels of homocysteine regardless of medium, ranged from undetectable (i.e. < 0.01 μmol/L) to 3.61 μmol/L (mean = 1.74 {SD±0.95}), cysteine from 0.30-10.41 μmol/L (mean = 4.00 {SD±2.68}), cysteinyl-glycine from 0.06-1.71 μmol/L (mean = 0.74 {SD±0.54}) and glutathione from 0.64-7.07 μmol/L (mean = 3.65 {SD±1.50}). With additional methionine and folate in the medium, these levels were generally similar to baseline (**Table 8.9**).

Table 8.9 Mean intracellular total sulphydryl compound levels (μmol/L) with increasing medium methionine & folate

Treatment	Mean (±SD) sulphydryl compound concentration (μmol/L)			
	Hcy	Cys	Cys-gly	GSH
Baseline	1.98 ±1.51	4.99 ±4.18	0.91 ±0.73	4.32 ±1.68
Methionine x10	1.84 ±1.12	3.95 ±2.93	0.85 ±0.67	3.34 ±0.50
Methionine x50	1.94 ±1.04	3.39 ±2.27	0.68 ±0.51	4.37 ±1.50
Folate x10	1.32 ±0.99	2.83 ±2.83	0.36 ±0.61	3.08 ±1.73
Folate x100	1.38 ±0.67	2.53 ±1.29	0.68 ±0.47	2.78 ±0.69
Folate x1000	2.00 ±0.49	6.29 ±0.91	0.94 ±0.29	4.05 ±2.35

The wide variation in the measured levels reflects variables such as differences in cell growth and metabolism, export and uptake etc. Notwithstanding these variables, consideration of the data including the mean levels revealed a number of clear trends. As shown in **Figure 8.2**, the most noticeable changes were:

(i) homocysteine increased with increasing medium methionine, but changed very little with increasing medium folate;

(ii) cysteine decreased with increasing medium methionine and folate; and

(ii) cysteinyl-glycine and glutathione changed very little with increasing medium methionine, but decreased with increasing medium folate.

- medium sulphydryl compounds

On considering the data shown in **Figures 8.3** & **8.4**, the most obvious changes were:

(i) total, free and bound homocysteine increased with increasing medium methionine, but decreased with increasing medium folate;

(ii) cyst(e)ine, cysteinyl-glycine and glutathione showed similar changes in relation to higher to medium methionine and folate levels;

(iii) total and free cyst(e)ine and cysteinyl-glycine changed very little with increasing medium methionine and folate, but their bound fractions increased with increasing medium methionine and folate; and

(iv) total and free glutathione changed very little with 10-fold higher methionine and folate, but decreased with 50-fold higher methionine. Bound glutathione increased with increasing medium methionine and folate.

5-MeTHF

Changes in relation to medium methionine and folate levels - intracellular 5-MeTHF

5-MeTHF levels (**Table 8.7**) ranged from undetectable (i.e. < 0.1 nmol/L) to 2.72 nmol/L, so that the data is again not statistically strong with > 33% of values undetectable. Nevertheless, it is clear that the mean level of 5-MeTHF decreased with increasing medium methionine, but increased exponentially with increasing medium folate (**Figure 8.5**).

AdoMet

Changes in relation to medium methionine and folate levels - intracellular AdoMet

As shown in **Table 8.8**, overall levels of AdoMet ranged from 8.12-358.93 nmol/L, with detectable levels in all samples. Mean levels were 0.054 (SD±0.017) nmol/mg protein in un-supplemented medium, compared with 0.056 (SD±0.031) and 0.051 (SD±0.016) nmol/mg protein in medium supplemented with 10- and 50-fold higher methionine respectively, and compared with 0.045 (SD±0.012), 0.030 (SD±0.018) and 0.042 (SD±0.012) nmol/mg protein in medium supplemented with 10-, 100- and 1000-fold higher folate respectively. Thus, as shown in **Figure 8.6**, mean levels of AdoMet changed very little with increasing medium methionine, but decreased with increasing medium folate.

Discussion

Sulphydryl compounds

Exported total, free and bound and intracellular homocysteine levels exhibited a clear dose-dependent increase to increasing medium methionine. Exported total, free and bound homocysteine, except with 1000x physiological levels, exhibited a clear dose-dependent decrease to increasing medium folate. Intracellular homocysteine however, was unchanged by increasing medium folate, but since remethylation is also dependent on levels of vitamin B_{12}, AdoMet and the synthesis of active folate forms, which additionally requires several enzymes, adequate supplies of vitamin B_2, B_3, B_6, zinc and serine, so that it is not surprising that an effect was not observed. Results for homocysteine were mainly as expected, although levels and the extent of changes were lower than in previous reports, probably due to differences in experimental design i.e. seeding density and media volume etc. Overall changes however, were in direct agreement with both earlier *in vitro* and *in vivo* work.

Intracellular cysteine levels decreased with increasing medium methionine and folate, explained by reduced transsulphuration through increased homocysteine export from increasing methionine, or remethylation from increasing folate. From the folate levels, it is unlikely that it was by remethylation, but in support of reduced transsulphuration, AdoMet that stimulates CBS activity, decreased with increasing medium folate (see page 159).

The unchanged intracellular cysteinyl-glycine and glutathione levels in medium with increased methionine probably reflect the strict intracellular control of glutathione synthesis (as discussed in Chapter 7, pages 142 & 143). The lower levels observed with increasing folate, can be explained by reduced transsulphuration and increased remethylation, in parallel to cysteine levels, as discussed previously.

5-MeTHF

Methionine loading has been shown to inhibit MS but not folate levels in normal human fibroblasts (Christensen & Ueland, 1993; Christensen *et al.,* 1994). The decrease in 5-MeTHF must therefore be due to something other than changes in the remethylation of homocysteine. As discussed in Chapter 7 (pages 143 & 144) folic acid on entering the cell is converted to THF, which is the preferred substrate for polyglutamate formation by folylpolyglutamate synthetase. It has been previously shown that the major derivatives from folic acid are hexa-polyglutamates, and which, along with dihydrofolate, may be more susceptible to folate-utilising enzymes.

The exponential increase in 5-MeTHF with increasing medium folate is purely a consequence of uptake via FR. As discussed in Chapter 7 (page 143), when the folate source is folic acid, cells concentrate hexa-polyglutamates without equilibrium with the media.

AdoMet

AdoMet levels changed little in relation to increasing medium methionine. This is supported by the work of Christensen & Ueland (1993) on normal human fibroblasts, but contrasts that by Finkelstein *et al.,* (1988) on rats and Loehrer *et al.,* (1996) on humans. The role of liver MAT III in the conversion of methionine to AdoMet being an obvious difference between the *in vitro* and *in vivo* situation.

AdoMet levels decreased in relation to increasing medium folate. If the bulk of the cellular folate is THF, a decrease in AdoMet would release inhibition of MTHFR producing an increased diversion of other folate co-factors, including THF into the synthesis of 5-MeTHF.

Conclusions

Increasing medium methionine and folate resulted in methionine catabolism (transsulphuration) and conservation (remethylation) respectively, but the effects were not as great as expected or from previous studies with commercial media, which was disappointing. Although this approach has been extensively applied to a variety of cell types in various commercial media (see Chapter 1, page 22), it may still have scope for examining the effects of additional substrates, for example arginine, taurine and the antioxidant vitamins A, C and E, that have not always given conclusive data from studies with animals or humans on aspects of homocysteine metabolism, including its export or its modification of LDL.

General discussion, conclusions & future perspectives

Cardiovascular disease, principally heart disease and stroke, is the leading cause of death for both men and women among all racial and ethnic groups in the western world. In recent years, a great number of studies have provided evidence identifying elevated plasma homocysteine as a potential risk factor for the development of cardiovascular disease. Nevertheless, a satisfactory explanatory pathological mechanism is yet to be established. Since homocysteine levels are fundamental in the disease process, understanding of those factors which modulate its concentration in tissues and physiological fluids, such as metabolism, its relationship to other metabolites and its transport across membranes measured as uptake and specifically export, is a prerequisite to explaining disease causing mechanisms. Although many *in vitro* studies investigating homocysteine have been performed, the great majority suffer from the use of physiologically irrelevant concentrations or forms of homocysteine, as well as using non-physiological levels of substrates and vitamins in media, in particular methionine and folate.

The main objectives of this work therefore were to develop an *in vitro* model for homocysteine metabolism using human skin fibroblasts grown in a physiologically based medium, and to measure the levels of the main metabolites of homocysteine, 5-MeTHF, AdoMet and AdoHcy in media and cells. Except for the measurement of AdoHcy and that 5-MeTHF suffered from low recovery, all other metabolite measurements were accomplished. Additional measurements included those of cyst(e)ine, cysteinyl-glycine and glutathione, the free (non-protein bound) and protein-bound sulphydryl compound levels, the related amino acids of taurine, serine, glycine, cystine, methionine and cystathionine, as well as any sulphydryl compound losses through oxidation.

The findings clearly confirmed export of homocysteine in controlling its intracellular levels and emphasised the role of protein binding in export. Similarities in the levels and time course of homocysteine export between the physiological based and commercial media were in part explained by the complexity of the metabolism, but importantly serve to validate our present understanding of export based on studies performed in previous work using non-physiological conditions. Studies also highlighted the importance of folate uptake and methionine conservation (homocysteine remethylation by MS) in CBS-deficient cells, cysteine formation (homocysteine catabolism by CBS and CTH) in glutathione synthesis, and the independent nature of media amino acid concentrations and their uptake.

Due to difficulties in recreating atherosclerotic processes or the interrelationships between sulphydryl compounds in short term studies, *in vitro* models have been somewhat overshadowed by the use of animal models and epidemiological studies, and consequently homocysteine export has gained relatively little attention. It is clear that *in vitro* models are unlikely to provide complete answers to open questions of the pathophysiology of homocystinaemia. It is also clear however, that animal models such as murine CBS- and Apolipoprotein (Apo)E-null models together with retrospective, cross-sectional and prospective studies have compounded homocysteine's role and increased the controversy over its atherogenic properties. Furthermore, two very basic and important questions remain unanswered. First, how is homocysteine transported across cell membranes? And second, in what form (reduced or {non-}protein bound) is homocysteine involved in the disease process of atherosclerosis and/or thrombosis and by which aetiological pathway? Thus, the studies described in this thesis provide the basis for studies of transport and toxicity of homocysteine in other tissues such as endothelial and/or vascular smooth muscle cells. The use of more physiologically based *in vitro* models will undoubtedly increase the relevance of studies on questions pertaining to transport and form, in attempting to understand whether homocysteine really is a causative agent in the multifactorial disease with which it has been associated.