Investigation of cystathionine β-synthase as a cause of mild hyperhomocysteinaemia in patients with peripheral vascular disease

Barend J.M. de Wet
B.Sc. (Med) (Hons)

Thesis submitted to the Faculty of Medicine of the University of Cape Town
in fulfillment of the requirements of the degree Master of Science in Medicine

Cape Town
February 1999

DECLARATION

I, Barend J.M. de Wet, hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise), and that neither the whole work nor any part of it has been, or is being, or is to be submitted for another degree in this or any other university.

I empower the University of Cape Town to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

B.J.M. de Wet

11 February 1999

ABSTRACT

Hyperhomocysteinaemia is a recently established risk factor for the development of vascular disease and is caused by a variety of defects in the metabolism of methionine as well as dietary deficiencies of the vitamin cofactors (B6, B12 and folate) of the enzymes involved in methionine metabolism. Cystathionine β-synthase (CBS) is the most common genetic cause of homocystinuria, the severe form of the disease. The incidence of CBS deficiency in a group of 12 young patients of varied ethnic origin, who had peripheral vascular disease (PVD) that could not be ascribed to any of the conventional risk factors and were selected for having hyperhomocysteinaemia, either in the fasting state or after methionine load, was investigated. Nine out of the ten patients tested, showed abnormally elevated plasma homocysteine levels after methionine load, indicating a high incidence of deficient transsulfuration, which may have been caused by defects in CBS.

Very wide variation in the CBS assay has hampered efforts to establish the contribution of CBS deficiency to the hyperhomocysteinaemia observed in this population. Therefore, a major part of this work has focussed on the source of this variation and the data suggests that between experiment variation as a result of changes in enzyme activity during the culture of the fibroblasts makes the biggest contribution. The most appropriate criterion to identify heterozygotes for CBS deficiency under these circumstances is to measure reduced CBS activity on several separate occasions compared to a control group. Only one of the group of 12 PVD patients (patient 1000) was identified as a heterozygote for CBS deficiency using this standard. Heterozygosity for CBS deficiency therefore seems to make only a minor contribution to the observed hyperhomocysteinaemia in this group of patients.

Molecular genetic investigations were performed on selected individuals. Patient 1000 was confirmed to be a heterozygote for CBS deficiency. An A to G transition at nucleotide 695 leading to histidine to arginine substitution at amino acid 232 was found in one allele of this patient. A young homocystinuric female (patient 960) was confirmed to be compound heterozygote for CBS deficiency, with the common Celtic $G_{919}A$ transition on the one allele and a novel duplication of the 7 bases between position 1553 and 1559 on the other allele. This 7bp insertion was identified as coming from the mother (patient 961).

In an attempt to find an alternative or perhaps more sensitive method for the detection of defects in methionine metabolism, dual metabolic labelling of cultured fibroblasts with L-[methyl-^{3}H]-methionine and L-[^{35}S]-methionine was developed to investigate these pathways in homozygotes and heterozygotes for CBS deficiency compared to controls. Although, no differences in the ratio of $^{3}H/^{35}S$ were found that could be used to identify the zygosity of the patient for CBS deficiency, changes in the ratio of $^{3}H/^{35}S$ over time in certain cellular compartments suggest that further development of this approach may prove to be useful.

ACKNOWLEDGEMENTS

I would like to thank everyone who had in some small way made this endeavor possible.

I would especially like to thank:

My supervisors Eric Harley and Tricia Owen for their help and support during this project and for their input during the writing of this thesis.

Tony Marinkaki for his ever-present guidance and pertinent advice and insight and for teaching me the virtues of optimism.

Ingrid Baumgarten for teaching me the rigors of proper tissue culture technique.

Lisa Lambson for the homocysteine assays and her good sense of humour.

Jean Rossouw and Gené Harms for the sequencing at the Core Facility and the use of the scanner.

Di James for the sequencing at the Department of Biochemistry and Microbiology.

Heidi for her never-ending support, understanding and patience.

CONTENTS

ABBREVIATIONS

^{14}C	14 atomic mass isotope of carbon
^{3}H	tritium
^{35}S	35 atomic mass isotope of sulfur
5-MeTHF	5-methyltetrahydrofolate
5,10-MeTHF	5,10-methylenetetrahydrofolate
^{51}Cr	51 atomic mass isotope of chromium
A	adenine
ATP	adenosine triphosphate
bp	base pairs
C	cytosine
C-terminus	carboxyl terminus
CBS	cystathionine β-synthase
cDNA	complementary DNA
Ci	curie
CoA	coenzyme A
CTP	cytidine triphosphate
DEPC	diethyl pyrocarbonate
DMEM	Dulbecco's modification of Eagle's minimal medium
DNA	deoxyribonucleic acid
dATP	deoxy adenosine triphosphate
dCTP	deoxy cytidine triphosphate
dGTP	deoxy guanosine triphosphate
dITP	deoxy inosine triphosphate
dNTP	deoxy nucleotide triphosphates
dpm	disintegrations per minute
dT	deoxy thymidine
DTT	dithiothreitol
dTTP	deoxy tymidine triphosphate
EDTA	ethylenediamine tetraacetic acid
eV	electron volt
FCS	fetal calf serum
G	guanine
GTP	guanosine triphosphate
Hepes	*N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid
IQ	intelligence quotient
K_m	Michaelis constant
M	molar (mole/dm^3)
MAT	ATP:L-methionine *S*-adenosyl transferase

Me	methyl group
MEM	Eagle's minimal medium
MOPS	3-(*N*-morpholino)-propane sulfonic acid
mRNA	messenger RNA
MTHFR	methylenetetrahydrofolate reductase
N-terminus	amino terminus
NAD^+	nicotinamide adenine dinucleotide, oxidized form
NADH	nicotinamide adenine dinucleotide, reduced form
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
PEG	polyethylene glycol
pH	-log $[H^+]$
P_i	phosphate
PP_i	pyrophosphate
PLP	pyridoxal-5-phosphate
PVD	peripheral vascular disease
RNA	ribonucleic acid
SAM	*S*-adensosylmethionine
SDS	sodium dodecylsulphate
T	thymine
Taq	*Thermus aquaticus* polymerase
TCA	trichloracetic acid
THF	tetrahydrofolate
TLC	thin layer chromatography
Tris	tris-(hydroxymethyl)-aminomethane
TTP	thymidine triphosphate
V_{max}	limiting reaction velocity

INTRODUCTION

Methionine metabolism

Homocysteine is an amino acid intermediate of methionine metabolism, which is illustrated in figure 1 and has been reviewed extensively (Mudd et al, 1995 and Finkelstein, 1990). Methionine, ingested through the diet or produced by protein catabolism is combined with the adenosyl moiety of ATP to produce *S*-adenosylmethionine (SAM). This reaction is catalyzed by ATP:L-methionine *S*-adenosyl transferase (EC 2.5.1.6) (MAT) (1) and is irreversible under physiological conditions due to the enzymatic cleavage of the remaining enzyme-bound tripolyphosphate. SAM is utilized in a variety of methylation reactions (2), yielding a methylated acceptor and *S*-adenosylhomocysteine. SAM plays a pivotal role in the C- and N-terminal methylation of proteins, a variety of methylation reactions on RNA and DNA as well as phospholipids (Chiang et al, 1996). *S*-adenosylhomocysteine is cleaved to adenosine and homocysteine by *S*-adenosylhomocysteine hydrolase (EC 3.3.1.1) (3). The equilibrium of this reaction favours the formation of *S*-adenosylhomocysteine, but rapid utilization of both homocysteine and adenosine allows the reaction to proceed in the direction of cleavage under physiological conditions.

Homocysteine can either be remethylated to methionine or utilized in the transsulfuration pathway to produce cysteine. In the liver, flux is divided equally through the remethylation and transsulfuration pathways under steady state conditions (Finkelstein and Martin, 1984), with flux through the transsulfuration pathway being increased in response to excess methionine (Finkelstein and Martin, 1986).

Remethylation of homocysteine can occur from two methyl group donors. Betaine:homocysteine methyltransferase (EC 2.1.1.5) (4) transfers a methyl group from betaine to homocysteine, producing methionine and *N,N*-dimethylglycine. 5-methyl tetrahydrofolate:homocysteine methyltransferase (EC 2.1.1.13) (methionine synthase) (5), a cobalamin dependant enzyme, utilizes 5-methyl tetrahydrofolate as a methyl donor and this reaction links methionine metabolism to the folate cycle. Tetrahydrofolate is produced after the methyl group transfer and is itself methylated by serine hydroxymethyl transferase (6) which transfers the methyl moiety from serine, yielding glycine and 5,10-methylene tetrahydrofolate. 5'-Methyl tetrahydrofolate is reformed by 5,10-methylene tetrahydrofolate reductase (EC 1.1.99.15) (MTHFR) (7).

Homocysteine is committed to the transsulfuration pathway through the condensation of serine with homocysteine by L-serine hydro-lyase (adding homocysteine) (EC 4.2.1.22) (cystathionine β-synthase) (CBS) (8), a reaction that is dependent on pyridoxal 5-phosphate as a cofactor. Cystathionine is subsequently cleaved to cysteine and α-ketobutyrate by

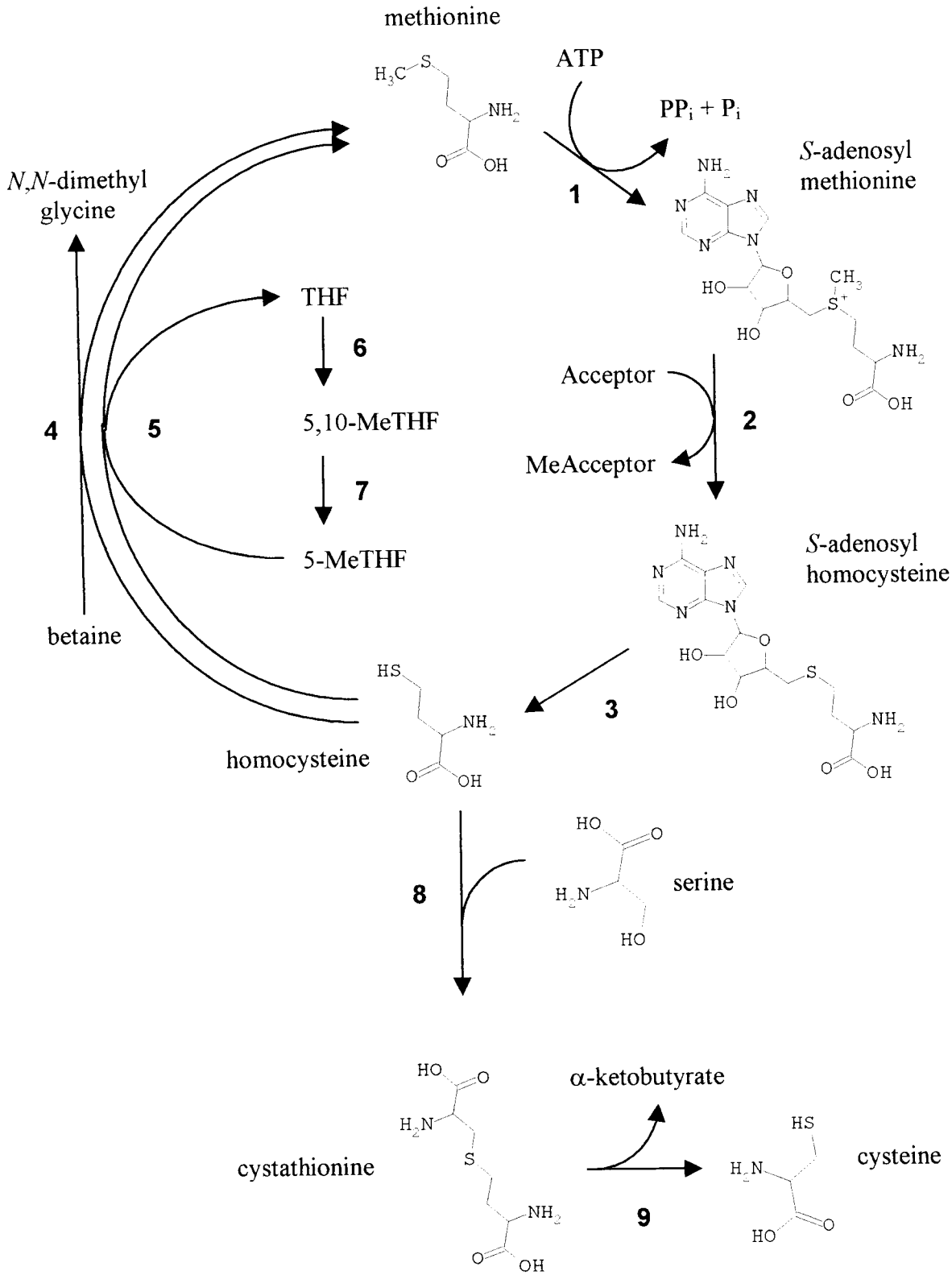


Figure 1. Summary of the pathways of methionine metabolism. The enzymes are numbered as follows: (1) ATP:L-methionine *S*-adenosyl transferase, (2) methyl transferase, (3) S-adenosylhomocysteine hydrolase, (4) betaine:homocysteine methyltransferase, (5) 5-methyl tetrahydrofolate:homocysteine methyltransferase, (6) serine hydroxymethyl transferase, (7) 5,10-methylene tetrahydrofolate reductase, (8) cystathionine β-synthase, (9) γ-cystathionase.

L-cystathionine cysteine-lyase (deaminating) (EC 4.4.1.1) (γ-cystathionase) (9), another vitamin B6 dependant enzyme. Cysteine is utilized for protein anabolism and excess cysteine metabolized to taurine and excreted as sulfate. This allows the transsulfuration pathway to act as a route for the disposal of excess methionine. *S*-adenosylmethionine plays a major regulatory role in the metabolism of methionine and tightly controls its own concentration. SAM acts allosterically on MAT, betaine:homocysteine methyltransferase, CBS and MTHFR. High levels of methionine result in increased levels of SAM which in turn activates MAT and CBS, and inhibits MTHFR and betaine:homocysteine methyltransferase, thereby increasing flux through the transsulfuration pathway and inhibiting the remethylation of homocysteine to methionine. Methionine also regulates its own concentrations by inhibiting methionine synthase and betaine:homocysteine methyltransferase (Finkelstein, 1990 and Selhub and Miller, 1992)

Homocystinuria and hyperhomocysteinaemia

Homocystinuria was first described in 1962 by Gerritsen and coworkers, who observed subjects who excreted homocystine in their urine and had markedly elevated levels of plasma homocystine and methionine. The normal range for homocysteine measured in serum or plasma obtained from subjects in the fasting state has been established by a variety of studies as between 5 and 15μM (Ueland et al, 1993). These measurements reflect the total amount of homocysteine in blood, of which 80% is bound to protein, with the remaining 20% present as disulfides with itself or cysteine, and a minute fraction in the thiolactone form. Increased intracellular homocysteine is managed by export out of cells (Svardal et al, 1986) allowing serum or serum homocysteine levels to be utilized as a measure of the status of intracellular homocysteine metabolism. Mild, intermediate and severe hyperhomocysteinaemia have been defined as 15 to 30μM, 30 to 100μM and >100μM respectively (Kang et al, 1992).

Factors causing homocystinuria and hyperhomocysteinaemia

Homocystinuria and hyperhomocysteinaemia can arise as a result of a great variety of causes including genetic defects in cystathionine beta-synthase, methionine synthase or methylene tetrahydrofolate reductase, dietary deficiencies of the cofactors involved in the cycle, namely vitamin B6, vitamin B12 and folate as well as defects in the activation and metabolism of these vitamin cofactors and certain disease states and medication (Mudd et al, 1995).

Cystathionine β-synthase deficiency

Homozygosity for cystathionine β-synthase deficiency has been established as the most common cause of homocystinuria, detected with a frequency of 1/300 000 live births by

newborn screening. CBS deficiency leads to an increase of both serum homocysteine and methionine and a decrease in cysteine (Mudd et al, 1995). Not long after homocystinuria was initially described, CBS deficiency was identified as the cause thereof (Mudd et al, 1965). The authors determined CBS activity in human liver obtained post-mortem and by biopsy measuring the conversion of ^{14}C-serine to ^{14}C-cystathionine. Patients with homocystinuria showed virtually no CBS activity and normal methionine synthase and γ-cystathionase activity in extracts of liver biopsy material. CBS activity measured in lysates of cultured fibroblasts obtained from human skin and cells in amniotic fluid was also shown to be decreased in homocystinuric patients compared to controls (Uhlendorf and Mudd, 1968), and fibroblasts are now commonly used to measure CBS activity. The measurement of CBS activity in lysates of phytohaemagglutinin stimulated lymphocytes obtained from blood has also been reported (Goldstein et al, 1972).

The detection of homozygotes for CBS deficiency is easy, as patients have markedly increased plasma homocysteine levels and there is very little measurable enzyme activity in lysates of liver biopsy material or cultured cells. The identification of heterozygotes poses a greater problem as the mutations leading to decreased activity show great heterogeneity causing significant overlap in the ranges of fasting plasma homocysteine levels as well as the CBS activities between heterozygotes and normal subjects. The methionine loading test, in which subjects are fed an oral dose of 0.1mg/kg body weight of L-methionine and homocysteine measured after 4 to 6h, was developed to identify heterozygotes for CBS deficiency by placing a load on the transsulfuration pathway (Fowler et al, 1971). The methionine-loading test however suffers from the same problem of overlap in the ranges of plasma homocysteine levels between heterozygotes and control subjects. Various attempts have been made to optimize the detection of heterozygotes, but none so far have provided satisfactory discrimination (McGill et al, 1990). The problem is further compounded by the fact that subjects heterozygous for the exact same genetic mutation in the CBS gene may show different phenotypes in terms of serum homocysteine levels (Dawson et al, 1996). Obligate heterozygotes for CBS deficiency have between 20 and 45 percent of the mean control activity measured in extracts of liver biopsy material and cultured fibroblasts, indicating negative interactions between normal and mutant subunits of the enzyme (Mudd et al, 1995).

Certain homocystinuric patients show a decrease in plasma and urine homocysteine to normal or near normal levels in response to treatment with high doses of vitamin B6 (Barber and Spaeth, 1967) and this phenomenon is most likely due to stimulation of residual enzyme activity by excess co-factor (Mudd et al, 1970). In a survey of 629 homocystinuric patients, equal numbers appeared to be responsive and non-responsive to vitamin B6 therapy. Fowler et al (1978) provided evidence for variable response to pyridoxine treatment. They measured CBS activity in lysates of cultured fibroblasts obtained from pyridoxine responsive and

non-responsive patients and found that CBS activity was in some cases markedly increased, almost to normal levels, in some case only slightly increased and in some case not at all.

Kraus and coworkers (1978) purified cystathionine β-synthase from human liver and showed that the enzyme exists as dimer with an apparent molecular weight of 94000, consisting of two identical subunits with an apparent molecular weight of 48000. They determined that the enzyme contains pyridoxal 5-phosphate as tightly bound cofactor by showing an absorbance maximum at 427nm, typical of PLP containing enzymes and complete loss of activity upon treatment with hydroxylamine. The apparent K_m values for serine and homocysteine were determined as 1.15mM and 0.59mM respectively and the pH optimum was between 8.4 and 9.0. These findings were refined by Skovby et al (1983), who illustrated that the only transcriptional product of the CBS gene is a larger 63000 molecular weight sub-unit that exists as a homotetramer. The dimer consisting of 48000 molecular weight sub-units is the product of a slow post-translational proteolytic cleavage of the tetramer. The proteolytic cleavage to a smaller subunit occurred only in liver, and could not be detected in fibroblasts. The smaller subunit showed a 60-fold increase in specific activity and incubation of liver extracts at 4°C for seven days led to a threefold increase in total enzyme activity. The increase in enzyme activity by post-translational cleavage is due to an increase in the affinity of the enzyme for homocysteine. Kraus (1987) showed that the apparent K_m for homocysteine of the 63000 molecular weight sub-unit is 25mM, while it is 0.8mM for the 48000 molecular weight sub-unit. Partial trypsin cleavage of the enzyme leads to the formation of the activated dimer with subunits of 45000 molecular weight, which is no longer sensitive to activation by *S*-adenosylmethionine, is less heat-stable than the tetramer and has a three times higher affinity for homocysteine. Trypsin cleaves the CBS enzyme at Arg 413 indicating that residues 414-551 are necessary for maintaining the tetrameric structure of the enzyme (Kery et al, 1998). *S*-adenosylmethionine acts as an allosteric activator of CBS, increasing enzyme activity threefold (Kozich and Kraus, 1992), allowing SAM concentrations to control the flux through the remethylation and transsulfuration pathways (Selhub and Miller, 1992). In addition to the two substrates, and pyridoxal 5-phosphate and *S*-adenosylmethionine, CBS also binds haem as a prosthetic group. The presence of haem is essential for PLP binding and each CBS subunit binds one molecule of haem and one molecule of PLP (Kery et al, 1994). The CBS cDNA has also been cloned and expressed in *E.coli*, and the resulting protein product appears to be identical to the enzyme purified from liver (Kozich and Kraus, 1992).

The human CBS gene has been localized to the subtelomeric region of chromosome 21 by in situ hybridization of a rat cDNA probe (Münke et al, 1988) and the entire human CBS cDNA has been sequenced (Kraus et al, 1993). The 2554bp cDNA coding for 551 amino acids was found to differentially retain a 214bp intron in the 3' noncoding sequence. Subsequently, 4 additional mRNA species differing only in the 5' untranslated regions were described (Chassé

et al, 1995 and Bao et al, 1997). Bao et al (1997) illustrated the differential expression of the different isoforms in different tissues, suggesting a possible mechanism for maintaining different levels of translation between tissues. The rat CBS gene has been characterized, consisting of 17 exons, giving rise to four alternatively spliced mRNA's, two of which are catalytically active. The primary transcripts, type I and III, differ by the presence or absence of the 42bp of exon 16, encoding two isoforms with different half-life (Swaroop et al, 1992 and Roper and Kraus, 1992). Recently, 28046bp encompassing the human CBS gene were sequenced and found to contain 23 exons ranging in length from 42 to 209bp (Kraus et al, 1998). The coding region spans exon 1 through 16, with exon 15 being alternatively spliced, corresponding to the rat exon 16. Eleven out of the fourteen amino acids in exon 15 are conserved between human and rat and inclusion of exon 15 causes a threefold decrease in the half-life of the protein, but no change in kinetic properties, as was found for the rat exon 16.

Molecular analysis of several homocystinuric patients has revealed a great variety of mutations, which appear to be clustered in the amino terminal half of the protein in exons 2 to 10 (Kraus, 1994). Certain mutations have been found to occur more commonly than others do. The G to A transition at position 919 leading to the replacement of glycine 307 by serine (G307S), has been found to occur with a high frequency in patients of Celtic origin (Hu et al, 1993). The mutation completely abolishes enzyme activity, although not affecting its immunoreactivity, and is associated with pyridoxine non-responsiveness *in vivo*. In a subsequent study, the $G_{919}A$ transition was found in 71% of Irish homocystinuric patients (Galagher et al, 1995). The T to C transition at position 833 leading to the substitution of isoleucine 278 for threonine (I278T) (Kozich and Kraus, 1992) was detected in 7 out of 11 pyridoxine responsive and 0 out of 27 pyridoxine non-responsive hyperhomocysteinaemic patients of varied ethnic backgrounds, and appears to be associated with a mild phenotype, even in the homozygous form (Shih et al, 1995). This mutation appears to occur with a high frequency in the Italian population and was found in 7 independent homocystinuric families out 14 tested (Sebastio et al, 1995).

Not all apparently deleterious mutations in the CBS gene have pathological consequences. A 68bp insertion at position 844 in exon 8 described by Sebastio et al (1995) was assumed to introduce a premature stop codon and to cause hyperhomocysteinaemia. This allele was also found to contain the pathogenic $T_{833}C$ transition. In a subsequent study however, this insertion was found in 11.7% of the control population and normal size mRNA was found in individuals carrying the insertion. The authors speculated that the duplication of the intron 7 splice acceptor by the insertion leads to the splicing out of both the insertion and the pathogenic point mutation and the production of functionally normal enzyme (Tsai et al, 1996). Kluijtmans et al (1997) found the incidence of the insertion to be identical between patients with occlusive arterial disease and control subjects and found that the insertion was

not associated with hyperhomocysteinaemia, even in the homozygous form. An extensive study of the incidence of the insertion among several ethnic groups revealed that it occurs in the heterozygous form in 37.7% of blacks, 13.5% of Caucasians, <1% of Native Americans, is absent in Asians and was found to be always associated with the $T_{833}C$ transition (Franco, 1998).

The phenotypic features in hyperhomocysteinaemia have been found to correlate poorly with the genotype of the mutations. Dawson et al (1996) investigated a family, in which both parents of a homozygous child carried the $G_{919}A$ transition. The mother had well defined hyperhomocysteinaemia, ascertained by methionine loading while the father had normal serum homocysteine levels. Kraus (1994) described a case in which two compound heterozygote siblings with identical mutations showed markedly different progression of the disease. The sister was mentally retarded and showed skeletal abnormalities, while the brother was of normal intelligence and only had a single thrombotic episode by the age of 34. Kozich and Kraus (1992) also described a patient who had no appreciable CBS activity in fibroblast but suffered only from mild pyridoxine responsive homocystinuria.

Defects in the synthesis of methionine

Subjects deficient in methionine synthase activity suffer from homocystinuria, megaloblastic anemia and neurological abnormalities. Methionine synthase deficiency has been classified as a cobalamin disorder. The active form of the enzyme has the cobalt atom in the 1+ oxidation state and requires a different gene product to transfer an electron from NADPH to reduce it from the 2+ oxidation state after a number of catalytic cycles. This reduction can be achieved *in vitro* with thiol containing compounds such as dithiothreitol. Two forms of methionine synthase deficiency associated with low methylcobalamin have been characterized, based on complementation studies in cultured fibroblasts. Subjects with cblE display homocystinuria, but not methylmalonic aciduria and decreased methionine synthase activity is measured in fibroblasts only when assayed with low concentrations of reducing thiols. In subjects with cblG, low methionine synthase activity is measured even in the presence of high concentrations of reducing thiols. Deficient intracellular processing of cobalamin also leads to reduced methionine synthase activity and is classified as cblC or cblD. Methionine synthase has been isolated from human placenta and pig liver, exists as a monomer of 151000 to 155000 molecular weight, contains one mole of cobalt per mole of monomer and requires SAM for activity (Fowler, 1997 and Rosenblatt, 1995). The human methionine synthase cDNA has been cloned and the gene was localized to chromosome 1q43 (Leclerc et al, 1996). The cDNA codes for a protein of 1265 amino acids and several mutations in the cDNA of patients in the cblG complementation have been group identified (Leclerc et al, 1996 and Wilson et al, 1998).

5,10-methylenetetrahydrofolate reductase deficiency

The major clinical features associated with 5,10-methylenetetrahydrofolate reductase deficiency are developmental delay, psychiatric disturbances, seizures, abnormalities of gait as well as arteriosclerosis and thrombosis (Rosenblatt, 1995). Homocystinuria due to MTHFR deficiency was described in 1972 (Mudd et al) and subjects typically have low plasma concentrations of methionine and folate. The severity of the clinical features appear to be correlated with the degree of enzyme deficiency (Rosenblatt, 1995). MTHFR has been purified from human liver and was illustrated to exist as a homodimer consisting of subunits of 75000 molecular weight. Each subunit contains one mole of flavin-adenine dinucleotide (FAD) which acts as an electron carrier from NADPH to its substrate (Zhou et al, 1990). The human liver MTHFR cDNA of 2200bp has been cloned and sequenced, coding for a 73600 molecular weight protein which consists of 656 amino acids and the human gene has been mapped to chromosome 1p36.3 (Goyette et al 1994). Several mutations in the coding sequence have been identified leading to varying degrees of enzyme deficiency. Kang et al (1988) have identified a thermolabile variant of MTHFR due to a $C_{677}T$ transition leading to a valine to alanine substitution, that is associated with mild elevations in plasma homocysteine, and occurs in ~10% of the Caucasian population (Rosenblatt, 1995).

Vitamin levels and hyperhomocysteinaemia

In the normal population, plasma levels of all three vitamin cofactors involved in homocysteine metabolism, vitamin B6, B12 and folate appear to influence serum homocysteine levels (Ubbink, 1997). A study of a normal elderly population revealed negative correlations between plasma folate, B12 and pyridoxal 5-phosphate concentrations. Serum homocysteine levels showed the strongest association with plasma folate concentrations and hyperhomocysteinaemia was attributable to deficiency of one or more of the B vitamins in two thirds of the cases (Selhub et al, 1993). A Swedish study of 501 middle-aged subjects also showed a high correlation between serum homocysteine levels and plasma folate and B12 concentrations (Brattstrom, et al 1994). The Hordaland homocysteine study, which investigated 11941 healthy subjects, showed that folate intake is one of the strongest determinants of serum homocysteine concentrations (Nygard et al, 1998). In a study of 44 asymptomatic hyperhomocysteinaemic men 25% had reduced levels of plasma B6, 56.8% had reduced plasma B12 and 59.1% had reduced plasma folate (Ubbink et al, 1993).

Subjects deficient in folate, B12 or B6 have abnormal homocysteine metabolism as measured by either fasting serum homocysteine or post-methionine load serum homocysteine levels. Kang et al (1987) found that 84% of subjects with severe folate deficiency and 56% of subjects with low normal folate had higher than normal fasting serum homocysteine levels, while Stabler et al (1988) measured elevated fasting serum homocysteine in 18 out of 19

subjects with confirmed folate deficiency. In a study by Brattstrom et al (1988) total plasma homocysteine in B12 deficient subjects was double that of controls and 40% of B12 deficient subjects had significant hyperhomocysteinaemia, while Stabler et al (1988) measured elevated fasting serum homocysteine in 77 out of 78 cobalamin deficient subjects. Vitamin B6 deficiency does not appear to raise fasting serum homocysteine levels significantly and supplementation with pyridoxine does not appear to be effective in decreasing fasting serum homocysteine levels in the general population (Miller et al, 1992). Deficiency of vitamin B6 does however impair the transsulfuration pathway, as post-methionine load homocysteine levels were significantly higher in a group of theophyline treated asthmatic subjects compared to controls (Ubbink et al, 1996). Pyridoxine treatment lead to significant decreases in post-methionine load serum homocysteine levels in both the control and theophyline treated asthmatics, but did not affect fasting serum homocysteine levels in either group.

Pathological features associated with homocystinuria due to CBS deficiency

The clinical abnormalities associated with homocystinuria are many and involve the eye, skeletal, vascular and central nervous system (Gibson et al, 1964 and Schimke et al, 1965) and have been extensively reviewed by Mudd and coworkers (1995). Mudd and coworkers (1985) conducted an international survey of 629 patients suffering from homocystinuria due to cystathionine β-synthase deficiency in an attempt to define the progression of these abnormalities. Clinical abnormalities appeared on average, much later in untreated B6 responsive patients than in untreated B6 non-responsive patients.

Dislocation of the optic lens is the most common clinical abnormality associated with homocystinuria, a feature that it shares with Marfan syndrome. This is due to breaking of the zonular fibers that hold the lens in place. Mutations in the fibrillin gene, leading to structural instability of the protein have been identified as the cause of Marfan syndrome (Dietz et al, 1992). Fibrillin is one of the main structural components of the zonular fibers and is rich in cysteine residues, which form disulfide bonds that are critical for maintaining the quaternary structure of the protein. The free thiol of homocysteine is thought to interfere with these disulfide bonds and ultimately lead to the destruction of the fibers. In the survey by Mudd (1985) most incidents of ectopia lentis appeared only after the age of 2 years and by age 38, 97% of patients suffered from this condition. By 6 years of age 50% of the vitamin B6 non-responsive patients had had one episode of ectopia lentis while this point was only reached by 10 years of age in the vitamin B6 responsive group.

The skeletal abnormalities that are observed include osteoporosis of the spine and long bones as well as lengthening and thinning of the long bones. Kang and Trelstad (1973) used the cross-linking of purified rat dermal collagen as an experimental system to investigate the effects of homocysteine on connective tissue. Their results indicated that soluble collagen did

not mature to form insoluble fibrils in the presence of homocysteine and that homocysteine interferes with intermolecular cross-link formation by reversibly binding aldehyde groups present in these molecules. They also discovered decreased cross-linking of dermal collagen in skin biopsies obtained from homocystinuric patients compared to controls. These findings could explain the effects of homocysteine on other collagen containing tissues, including bone. Lengthening and thinning of the long bones are typical Marfanoid features and could be explained by the effects of homocysteine on fibrillin, as is the case for ectopia lentis. Fifty percent of the B6 non-responsive patients show radiological evidence of spinal osteoporosis while the 50% mark is only reached by age 20 in the B6 responsive group (Mudd, 1985).

Homocystinuric patients also tend to have decreased mental capabilities. The mean IQ for the B6 responsive group was 79 and for the B6 non-responsive group, 57. Seizures have been reported in about 20% of untreated patients with homocystinuria and appeared at a slightly higher frequency and somewhat earlier in B6 non-responsive patients than in B6 responsive patients (Mudd et al, 1985). Abbott and coworkers (1987) evaluated 63 subjects with homocystinuria and discovered that 51% of them suffered from clinically significant psychiatric disorders, indicating a very high incidence of mental illness among homocystinuric patients. A higher incidence of behavioral disorders was noted in the vitamin B6 non-responsive group compared to the vitamin B6 responsive group. They also reported an overall lower average IQ (80 ± 27) for the homocystinuric patients compared to the general population and lower IQ in the vitamin B6 non-responsive patients compared to the vitamin B6 responsive patients.

Women suffering from homocystinuria appear to have decreased reproductive fitness. Data from the survey by Mudd and coworkers (1985) indicate that 10 out of 55 pregnancies in 32 women responsive to vitamin B6 ended in fetal loss. Male patients appear to have normal reproductive ability and out of 34 offspring produced by 21 males in the survey, 33 showed no clinical abnormalities and only one spontaneously aborted.

Thrombosis and atherosclerosis are two major clinical abnormalities associated with homocystinuria and the greatest number of deaths in homocystinuric patients are as a result of infarcts due to thromboembolism. Mudd and coworkers (1985) examined 253 thromboembolic events in 158 patients in their survey. Fifty one percent of the reported thromboembolic events affected peripheral veins of which 25% resulted in pulmonary embolism, 32% the cerebral vasculature, 11% peripheral arteries, 4% resulted in myocardial infarctions and 2% were in none of the above categories. The cumulative risk for suffering from a thromboembolic episode was 25% by the age of 16 years and 50% by the age of 29 years. The authors however cautioned that the numbers are only indicative of recorded events and that subjects had not been screened for the presence of possible early vascular damage.

The mechanism whereby homocysteine could potentially cause thrombosis and atherosclerosis has been the subject of much experimental attention. The initial observation of a relationship between homocystinuria and vascular damage was made by McCully in 1969, who described premature arterial thrombosis and atherosclerosis similar to what was observed in patients with CBS deficiency in an autopsy of a patient with homocystinuria due to a defect in vitamin B12 metabolism. This patient suffered from hyperhomocysteinaemia but not hypermethionineaemia, which led the author to conclude that increased homocysteine was associated with vascular damage. Subsequent studies have focussed the effect of homocysteine on endothelial cell damage, platelets, the clotting cascade and soluble factors.

Homocysteine may have a direct toxic effect on the vascular endothelium. Wall et al (1980) provided *in vitro* evidence by illustrating the dose dependent release of ^{51}Cr from monolayers of endothelial cell cultures in response to incubation with homocysteine. Similar results were obtained by Dudman et al (1991), who illustrated the detachment of cultured vascular endothelial cells in response to both homocysteine and cysteine. This detachment was however significantly inhibited when the cells were grown on fibronectin coated plates, casting doubt over the relevance of these findings *in vivo*. A possible role for hydrogen peroxide in endothelial damage was described by Starkebaum and Harlan (1986), who observed the copper dependant oxidation of homocysteine in human serum with the concomitant production of peroxide. Incubation of human and bovine endothelia cells with homocysteine in the presence of copper produced dose dependent cell lysis that was prevented by the addition of catalase. Increased homocysteine may also have an influence on the availability of nitric oxide (endothelial-derived relaxing factor), thereby decreasing endothelial response to increases in blood flow, which could eventually lead to atherosclerosis. Bovine aortic endothelial cells cultures showed a dose dependent decrease in nitric oxide in response to homocysteine, leading to the decreased ability of cells to detoxify peroxide. Nitric oxide synthase activity and transcription was unaffected indicating that the production of nitric oxide was not altered (Upchurch et al, 1997).

Endothelial damage and dysfunction has also been observed in several animal models and studies on human subjects. Desquamation of ~10% of the aortic endothelial surface was observed in baboons subsequent to a 3-month infusion of 200μM homocystine in the presence or absence of dipyridamole, an inhibitor of platelet function. A threefold increase in platelet consumption was observed as a consequence of endothelial damage and dipyridamole inhibition of platelet function did not alleviate desquamation. A threefold increase in platelet consumption and shortened platelet survival times was also measured in four homocystinuric patients compared to control subjects (Harker et al, 1974). Intimal lesions containing proliferating smooth muscle cells surrounded by connective tissue was observed in the absence of dipyridamole but not in the presence thereof, suggesting that

platelet activation as a result of endothelial cell damage leads to arteriosclerosis (Harker et al, 1976). The effect of homocysteine on the proliferation of cultured endothelial and smooth muscle was investigated by Tsai et al (1994). They observed a 25% increase in DNA synthesis in rat aortic smooth muscle cells in response to incubation with 100μM homocysteine. mRNA levels of cyclin D1 and cyclin A, both stimulators of smooth muscle cell proliferation, were also increased. DNA synthesis in human umbilical vein endothelial cells however decreased in the presence of homocysteine.

Celermajer and colleagues (1993) illustrated impaired endothelial function in the brachial arteries of 9 homocystinuric children. Using high-resolution ultrasound they found decreased endothelium dependent flow-mediated dilation in the homocystinuric patients but not in their obligate heterozygote parents when compared to control subjects. Nitroglycerin mediated dilation, which is endothelium independent was unaffected. Reduced flow-mediated dilation was confirmed in a group of hyperhomocysteinaemic patients compared to controls using identical methods. Nitroglycerin mediated dilation was as previously reported not affected (Woo et al, 1997). Similar findings were reported in monkeys with diet induced hyperhomocysteinaemia. Using quantitative angiography and Doppler measurement of blood flow, the authors showed a significantly greater decrease in blood flow in response to intra-arterial collagen infusion in homocysteinaemic monkeys compared to controls (Lentz et al, 1996).

McDonald et al (1964) reported increased platelet adhesiveness, but normal platelet counts in 5 homocystinuric patients compared to controls. Harker and coworkers (1974) reported decreased platelet survival times and a threefold increase in platelet consumption, but normal platelet counts in 4 homocystinuric patients compared to controls. Uhleman et al (1976) however, found no difference in platelet survival times or overall morphology using electron microscopy, in 5 homocystinuric patients compared to controls. There is evidence that increased homocysteine may increase platelet production of the prothrombotic thromoboxane A2. Platelet rich plasma incubated in the presence of either 1mM DL-homocysteine or L-homocystine showed a significant increase in the production of thromboxane B2, the stable metabolic end product of thromboxane A2. This increase was not observed in the presence of methionine, cysteine or cystine (Graeber et al, 1982). Di Minno et al (1993) could not confirm these findings *in vitro*, but did find increased urinary excretion of 11-dehydrothromboxane B2, a major metabolic derivative of thromboxane B2 in 11 homocystinuric patients compared to controls.

The influence of homocysteine on various components of the clotting cascade has also been investigated. Rodgers and Kane (1986) illustrated increased factor V activity in cultured endothelial cells treated with homocysteine, which lead to increased activation of prothrombin to thrombin by the Factor Va:Xa complex. Rodgers and Conn (1990) later illustrated that the

increased factor V activation was due to decreased activation of protein C by the direct inhibition of thrombin, reducing its ability to inactivate factor Va. Lentz and Sadler (1991) however, showed that the inhibition of protein C activation by thrombin appears to effected by diminished levels of thrombomodulin, which acts as a cofactor in this activation. Decreased thrombomodulin levels appear to be due to inhibition of the post-translational modification and transport of the protein along the secretory pathway. Their findings have been supported by Hayashi et al (1992), who illustrated a negative correlation between the cofactor activity and thrombin binding ability of thrombomodulin and homocysteine concentration. Antithrombin appears to be another component affected by increased homocysteine. Antithrombin levels in seven homocystinuric patients were shown to be about 50% of the control levels (Giannini et al, 1975). Decreased antithrombin III activity in homocystinuric patients has also been reported by Palareti and Coccheri (1989) and Brattstrom et al (1989). All of the factors discussed above eventually lead to a more thrombotic environment.

Mild hyperhomocysteinaemia and vascular disease

In 1969, McCully postulated that homocysteine was responsible for the precocious vascular damage he observed in autopsy material obtained from a homocystinuric patient with deficient cobalamin metabolism. A great deal of work since then has focused on the association between elevated serum homocysteine and the development of vascular disease. Wilcken and Wilcken (1976) measured homocysteine after methionine load in a group of 25 patients with angiographically proven coronary artery disease compared to a healthy control group. A significantly higher number of patients showed raised post methionine load levels of homocysteine compared to the control group. Boers et al (1985) investigated 75 young patients with vascular disease and confirmed hyperhomocysteinaemia due to CBS deficiency in 7/25 patients with peripheral vascular disease, 7/25 patients with cerebrovascular disease and 0/25 patients with myocardial infarction. Clarke et al (1991) illustrated hyperhomocysteinaemia in 16/38 patients with cerebrovascular disease, in 7/25 patients with peripheral vascular disease and in contrast to Boers, in 18/60 patients with coronary artery disease. CBS deficiency was identified in 18/23 hyperhomocysteinaemic patients who suffered from vascular disease. A recent study by Den Heijer et al (1996) of 269 patients with clinically confirmed deep-vein thrombosis showed that subjects with fasting plasma homocysteine >22μM had an odds ratio of 4:1 to suffer from deep-vein thrombosis and that plasma homocysteine levels had to reach a certain threshold before exerting a thrombogenic effect.

A European study consisting of 750 patients with atherosclerosis and 800 controls illustrated that the relative risk for the development of vascular disease was 2.2 for subjects with plasma homocysteine in the top quintile of the distribution. This study also investigated other risk factors and found that homocysteine was an independent risk factor for the development of

vascular disease and that plasma homocysteine levels have a graded dose dependant effect on the risk. An additional 27% of the subjects were identified as suffering from hyperhomocysteinaemia by methionine load (Graham et al, 1997). A meta-analysis of 27 studies investigating the link between plasma homocysteine and vascular disease, consistently showed that homocysteine is an independent risk factor for the development of vascular disease, even when using different methods and studying different populations (Boushey et al, 1995). The results obtained by the case-control studies have been supported by prospective studies, which record the incidence of vascular disease after an initial homocysteine measurement. The most notable of these, a study of 14916 US physicians, showed that the homocysteine levels in the subjects who did develop heart disease was significantly higher than in controls. The relative risk for vascular disease for subjects with plasma homocysteine in the highest 5% of the range was 3.4 after correcting for the standard risk factors (Stampfer, 1992).

The incidence of vascular disease in obligate heterozygotes for CBS deficiency has also been investigated. High-resolution ultrasound has been employed as a non-invasive method of assessing vascular damage. Celemajer et al (1993) found impaired flow-mediated dilation in the brachial arteries of homocystinuric patients compared to controls, but not in their obligate heterozygote parents. Rubba et al (1990), however, using similar techniques reported a higher incidence of arterial wall damage and stenoses in obligate heterozygotes for CBS deficiency compared to control subjects. The thermolabile variant of MTHFR has been illustrated to occur with a high frequency in the general population and to be a cause of mild hyperhomocysteinaemia. A recent analysis of 23 case control studies however, could not confirm any association of this variant with an increased risk for the development of vascular disease (Brattstrom et al, 1998). Increased risk for vascular disease has also been ascribed to decreased plasma vitamin B6 and folate levels. Verhoef et al (1996) measured lower plasma vitamin B6 and folate, but not vitamin B12 in patients who had suffered from myocardial infarction compared to controls. This finding has been confirmed by a European multi-center case control study consisting of 750 patients with vascular disease and 800 control subjects. They illustrated that plasma folate levels below the first decile and plasma vitamin B6 levels below the second decile were associated with increased risk for the development of vascular disease in the control subjects and that this was independent of plasma homocysteine in the case of vitamin B6 (Robinson et al, 1998).

The scope of this study

The aim of the work presented here was to investigate the contribution of CBS deficiency to hyperhomocysteinaemia in patients suffering from PVD without any of the conventional risk factors for the development of PVD. CBS deficiency was investigated as a cause of hyperhomocysteinaemia as it is the most common cause of the homocystinuria the homozygous and severe form of the disease. The specific objectives of this study were:

1. To standardise the CBS assay, and to investigate factors influencing CBS activity as measured in lysates of cultured fibroblast cells. The extent to which each of the different factors affect the measured CBS activity was also determined. This aspect was especially important, as the activity had been shown to be highly variable from one occasion to the next. This study aimed to compare enzyme activity between a control group and a group of patients with hyperhomocysteinaemia. Such a comparison would be difficult if the enzyme assay were not reproducible. Additionally, the ranges of CBS activity measured in control subjects and patients known to be heterozygous for CBS deficiency has been shown to overlap significantly by several researchers. The kinetic constants of the enzyme in the crude lysate were also determined to establish conditions under which maximal activity could be obtained. Assay under conditions of maximal activity was compared to assay under conditions of sub-maximal activity to determine the most effective conditions to distinguish control subjects and subjects heterozygous for CBS deficiency.

2. To measure the CBS activity in the group of hyperhomocysteinaemic patients suffering from PVD compared to an age and sex matched control group as well as known homozygotes and heterozygotes for CBS deficiency. The aim of this section was to determine the contribution of CBS deficiency to the hyperhomocysteinaemia observed in the PVD patients. A control group as well as known heterozygotes and homozygotes for CBS deficiency were assayed in conjunction with PVD group as a basis for comparison. An additional patient who exhibited a classical homozygous CBS deficiency phenotype and her mother were also investigated.

3. To confirm CBS deficiency in patients with reduced enzyme activity by employing molecular genetic methods to identify the genetic mutations in the coding sequence of the CBS gene. Messenger RNA was extracted from fibroblast cultures of the subjects and reversed transcribed to cDNA. The cDNA was used as a template for two rounds of nested PCR with two primer pairs. The PCR products were then sequenced directly using internal primers or ligated into pGEM-T vectors and used to transform competent *E.coli*. Insert containing plasmids were isolated and sequenced using primers to the

plasmid sequence. Where possible, base changes were confirmed by restriction enzyme digest of PCR products of cDNA and insert containing plasmids.

4. Preliminary investigations into the use of two differentially radiolabelled isotopes of methionine to study flux through the transsulfuration and remethylation pathways were performed. Confluent cultured fibroblast were incubated in different media with L-[methyl-^{3}H]-methionine and L-[^{35}S]-methionine and the uptake and incorporation of the labels into acid precipitable material determined. The amount of each label associated with the medium and the acid soluble intracellular fraction was also determined. These studies were undertaken in an attempt to find a new and perhaps more reliable method to identify heterozygotes for CBS deficiency and to investigate the flux through and products of methionine metabolism.

MATERIALS

L-[3-^{14}C]-serine was purchased from Amersham Life Science (Buckinghamshire, England). Ampicillin, Avian Maloney virus (AMV) reverse transcriptase, dithiothreitol, and NADH were obtained from Boehringer Mannheim (Germany). Fast-Link DNA Ligation kit was purchased from Epicentre Technologies (Madison, WI 53713, USA). *Thermus aquaticus* polymerase (Taq) was obtained from Gibco Life Technologies (Paisley, Scotland). L-[^{35}S]-methionine and L-[methyl-^{3}H]-methionine was purchased from NEN Life Science Products, Inc. (Boston, MA 02118, USA). RNase Inhibitor, Wizard Plus SV Minipreps and pGEM-T Vector were obtained from Promega (Madison, WI 53711-5399, USA). Qiaex II gel extraction kit was obtained from Qiagen (Santa Clarita, CA 91355, USA). Diethyl pyrocarbonate, homocysteine-*S*-thiolactone, propargylglycine, pyridoxal-5-phosphate, pyruvate, *S*-adenosyl methionine and 20×20cm cellulose TLC plates were obtained from Sigma-Aldrich (St. Louis, MO 63178, USA). Fetal calf serum (FCS) was obtained from Highveld Biological Supplies (Kelvin, South Africa). Dulbecco's modification of Eagle's minimal medium (DMEM) was obtained from Gibco Life Technologies (Paisley, Scotland) and BioWhitaker (Walkersville, Maryland 21793, USA).

Diethyl pyrocarbonate (DEPC) treated water was made up by adding 200µl DEPC to 100ml water, mixing well, incubating at room temperature for 15 minutes and autoclaving for 30 minutes.

LB medium contained 10g/L tryptone, 5g/L yeast extract and 10g/L NaCl. The medium was adjusted to pH 7 with 5M NaOH and was autoclaved. Solid media contained an additional 15g/L of Agar. Selective media contained an additional 100µg/L Ampicillin, added after the media had been autoclaved and had cooled down to ~50°C. For the purpose of blue/white screening, agar plates were coated with 40µl of a 10:1 mixture of 20mg/ml Xgal in dimethyl formamide and 100mM IPTG in sterile distilled water, both stored at -20°C.

Medium Z contained 130mM NaCl, 5mM KCl, 1mM $CaCl_2$, 1mM $MgCl_2$, 15mM Hepes and 4mg/L phenol red.

Solution D consisted of 4M guanidinium thiocyanate, 25mM sodium citrate pH 7, 0.5% sarcosyl and 100mM 2-mercaptoethanol. A stock solution that is stable at room temperature for three months was prepared by adding 58.6ml water, 3.5ml 750mM sodium citrate pH 7, and 5.3ml 10% sarcosyl to 50g guanidinium thiocyanate in the manufacturer's bottle. To obtain a working solution 72µl 2-mercaptoethanol was added to 10ml stock solution.

Ψ-Broth contained 20g/L tryptone, 5g/L yeast extract, 20mM Mg_2SO_4, 10mM NaCl and 5mM KCl and was autoclaved. Transformation buffer I (TFBI) contained 30mM potassium acetate,

100mM $RbCl_2$, 10mM $CaCl_2$, 50mM $MnCl_2$ and 15% glycerol. The buffer was adjusted to pH 5.8 with 0.2M acetic acid and filter sterilized. Transformation buffer II (TFBII) contained 10mM MOPS, 75mM $CaCl_2$, 10mM $RbCl_2$ and 15% glycerol. The buffer was adjusted to pH 6.8 with 0.1M HCl and filter sterilized.

2×TY medium contained 16g/L tryptone, 10g/L yeast extract and 5g/L NaCl and was autoclaved.

Glucose/Tris/EDTA (GTE) contained 50mM glucose, 25mM Tris, pH 8.0 and 10mM EDTA, was autoclaved and stored refrigerated. 5M potassium acetate solution was made up by adding KOH to 29.5ml glacial acetic acid until the solution reached pH 4.8 and the volume made up to 100ml with distilled de-ionized water.

Tris/EDTA (TE) contained 25mM Tris, pH 8.0 and 10mM EDTA and was autoclaved.

Primers

The following primers were used to amplify sections of the cystathionine beta-synthase cDNA or in sequencing reactions. The primers are numbered according to the human CBS cDNA sequence, starting with position 1 at the ATG translation start site (Kraus et al, 1993). The letter "f" indicates a forward primer while "r" indicates a reverse primer. The length as well as the theoretical melting temperature of each primer is also given.

Primer	Sequence	Length (bp)	T_m(°C)
CBS f-107	5'-GACCACAAGGCTACGACAAAATGA-3'	24	54
CBS f-57	5'-GTGGCGAGTTTGAGACATTCCTCTG-3'	25	55
CBS f186	5'-GTCCCCACATCACCACACTGC-3'	21	55
CBS f382	5'-GAGGATGCTGAGCGCGAC-3'	18	51
CBS f570	5'-GACGCCCACCAATGCCAGG-3'	19	58
CBS f665	5'-AACGACCGCAACGCCATCA-3'	19	55
CBS f769	5'-ACGGGCGGCACCATCACG-3'	18	60
CBS f1106	5'-GCTGCGTGGTCATTCTGCCCG-3'	21	62
CBS f1295	5'-GGCACACCATCGAGATCCTCC-3'	21	55
CBS f1487	5'-TGGGCAGGCTCTCGCACATCC-3'	21	62
CBS r1212	5'-TTCTTCGGGACCCACCACCG-3'	19	54
CBS r1928	5'-CATCCTACTTGTGTCCGTTACTGC-3'	25	56
CBS r1955	5'-CGTCTCACCTAATTTCCGTTTTCTCT-3'	26	54

Patients

Twelve patients suffering from peripheral vascular disease (patients 972 to 977, 979 to 983 and 1000) were selected. The group consisted of non-diabetic, non-smokers to whom no known clinical reason for their condition could be ascribed. The group of 9 males and 3 females were aged between 32 and 60 years (average age 42.5 $\pm$ 7.4) and were of varied ethnic origin. A complete list of the patients' information is given in table 1 in Results. Patient 960 is an 11-year old, homocystinuric female and her mother, patient 961 showed no clinical abnormalities. Two siblings who share the same compound heterozygote phenotype for CBS deficiency, patients 488 and 489 along with patient 960 where used as a reference for homozygous CBS deficiency. Their confirmed heterozygote parents where used along with patient 961 as a reference for heterozygous CBS deficiency.

METHODS

Plasma homocysteine measurement

Plasma homocysteine concentrations were determined in our laboratory as part of a routine service using a method adapted from Brattström et al (1988). Subjects were screened by measuring plasma homocysteine in the fasting state, as well as six hours after an oral methionine load of.1mg per kg of bodyweight. Methionine was taken orally dissolved in orange juice and subjects were fed a diet free from protein for the six hour period. EDTA venous blood samples were obtained from the subjects and separated by centrifugation, within one hour of collection and stored frozen until assay. Disulfide bonds were reduced by adding 25μl 1M DTT to 500μl plasma and incubating at 37°C for 1h. Norleucine was added as an internal standard. Protein was denatured with 100μl 50% SSA, centrifuged and de-proteinized plasma filtered through a 0.22μ filter. Samples were separated on a Beckman System 6300 amino acid analyzer using a cation-exchange column with lithium citrate buffers of increasing pH. Amino acids were detected spectrophotometrically by post-column ninhydrin derivatization. Homocysteine was quantified by measuring the height of the homocysteine and the norleucine internal standard peaks, as well as a known homocysteine standard separated during the same run.

Tissue culture

Human fibroblast cultures were established from skin biopsies, cut from the forearms of the patients. Biopsy material was sliced into approximately $1mm^3$ pieces, covered with 0.5ml DMEM containing 10% FCS and 30mg/ml penicillin, 50mg/ml streptomycin and 25mg/ml neomycin. Fragments were dispensed into several tissue culture dishes and covered with sterile coverslips. An additional 2.5ml of the above mentioned medium was added to the dishes and the dishes subsequently incubated in an atmosphere of 10% CO_2 at 37°C in a humidified incubator. Medium was changed twice a week. Once a reasonable amount of cells had grown from out from the fragments, the coverslips were turned over and placed in a new tissue culture dish. Both of the cultures were then grown to confluence before trypsinizing and transfer to a tissue culture flask.

All cultures were maintained in DMEM containing 10% fetal calf serum, in an atmosphere of 10% CO_2 at 37°C and omitting antibiotics. This results in avoidance of infections by enforcing a high standard of aseptic technique, and is highly valuable in guarding against mycoplasma infection. Cultures were fed twice a week by replacing the culture medium using the standard precautions to maintain sterility (Freshney, 1994). Cells were periodically examined for the presence of mycoplasma infection (Chen, 1977). Once fibroblast cultures had reached confluence, they were trypsinized and subcultered. Fibroblast culture monolayers were

released by treating with trypsin solution (0.25% trypsin, 1mM EDTA in sterile distilled water) for 5 to 10 minutes at 37°C. A quarter of the original culture was reseeded into the same flask. For the purposes of enzyme assays, the remaining three quarters of the trypsin suspension was cooled on ice and washed once with ice cold complete medium to inhibit trypsin activity and twice with ice cold PBS. Cells were sedimented for 5 minutes at 1000rpm in a Sigma 3E-1 bench-top centrifuge.

Cell lysis

Trypsinized, washed fibroblast suspensions were lysed using three methods: sonication using a Branson sonicator, freezing and thawing in a dry ice and ethanol mixture and treatment with Digitonin (Mackall et al, 1979). The protein concentrations of the extracts were determined using the Bio-Rad method (Bradford, 1976) with bovine serum albumin as a standard.

Digitonin treatment

The trypsinized, washed cell pellets were re-suspended in 100µl Digitonin solution (3mg/ml Digitonin, 250mM sucrose and 3mM EDTA in sterile distilled water, freshly prepared) and incubated at 4°C for 5 minutes. Cell debris was sedimented by centrifugation at 10000rpm in a Sigma 2MK Eppendorf centrifuge at 4°C. The supernatant containing the material released from the cells was transferred to a clean Eppendorf tube and kept at 4°C until use.

Sonication

The trypsinized, washed cell pellets were re-suspended in 100µl PBS and sonicated, on ice with a Branson sonicator, for 10 cycles and 10% of the duty cycle at 10% of the maximal output. Cell debris was sedimented by centrifugation at 10000rpm in a Sigma 2MK Eppendorf centrifuge at 4°C. The supernatant containing the material released from the cells was transferred to a clean Eppendorf tube and kept at 4°C until use.

Freezing and thawing

The trypsinized, washed cell pellets were re-suspended in 100µl PBS and frozen in dry ice ethanol and thawed in a water bath at 37°C successively six times. Cell debris was sedimented by centrifugation at 10000rpm in a Sigma 2MK Eppendorf centrifuge at 4°C. The supernatant containing the material released from the cells was transferred to a clean Eppendorf tube and kept at 4°C until use.

Lactate dehydrogenase assay

Cell lysates were assayed for lactate dehydrogenase activity by measuring the conversion of NADH to NAD^+ (decrease in absorbance at 340nm) as a result of the reduction of pyruvate to lactate (Pesce et al, 1964). The reaction mixture contained 1.6mM pyruvate, 0.175mM NADH and 100mM NaH_2PO_4 pH 6.8, in a volume of 1ml at 37°C in a quartz cuvet. To this mixture, 5μl of the lysate was added and the contents mixed by inversion. The cuvet was placed in a thermocell at 37°C in a Beckman DU-62 spectrophotometer and the absorbance at 340 nm measured at 20 second intervals for three minutes. The slope of the absorbance as a function of time was used to calculate the reaction rate.

Citrate synthase assay

Citrate synthase activity in cell lysates was measured by the production of citrate from acetyl-CoA and oxaloacetate. Dithionitrobenzoate was used as a chromogenic indicator of citrate production (Lowenstein, 1969). Included in the reaction mixture, in a volume of 1ml was 300μM acetyl-CoA, 500μM oxaloacetate, 100μM dithionitrobenzoate, 50mM Tris, pH 8.1. Acetyl-CoA and dithionitrobenzoate were mixed and allowed to equilibrate at 25°C for 1 minute. Fifty microlitres of the cell lysate was added and the reaction mixture incubated at 25°C for 3 minutes to allow residual oxaloacetate in the lysate to be utilized. The absorbance of the mixture was measured at 412nm to obtain the starting value. Subsequently oxaloacetate was added and the absorbance at 412nm measured after three minutes to obtain the final value. The reaction rate was calculated from the difference between the final and initial absorbance values.

Cystathionine beta-synthase assay

Cystathionine β-synthase irreversibly condenses homocysteine and serine to produce cystathionine. The assay for enzyme activity is based on the conversion of ^{14}C-[3]-serine to ^{14}C-cystathionine in lysates of fibroblast and lymphoblast cell lines derived from patients and control subjects. Reaction products are separated by chromatographic methods and the radioactive cystathionine quantified as a measure of the enzyme activity. One unit of enzyme activity is defined as the amount of enzyme that catalyses the formation of 1 nanomole of cystathionine per hour. Results are expressed as units of activity per milligram of protein measured in the lysate.

Homocysteine preparation

Homocysteine was prepared by incubating 7.7mg homocysteine-*S*-thiolactone in 100μl 5M NaOH at 37°C for 5 minutes. The pH was adjusted to 8.6 with ~220μl 2M HCl and 50μl

100mM Tris pH 8.6. Dithiothreitol was added to a final concentration of 20mM and the volume made up to 500μl with distilled water.

Measurement of cystathionine beta-synthase activity

CBS was essentially assayed under two sets of conditions. Under conditions of maximal enzyme activity, the reaction mixture contained 20mM L-homocysteine (freshly prepared from its thiolactone form), 40mM L-serine, ^{14}C-serine to a known specific activity, 2mM pyridoxal-5-phosphate, 1mM *S*-adenosyl methionine, 1mM propargylglycine, 100mM Tris pH 8.6 and 0.05 to 0.10mg cell protein. The conditions of sub-maximal enzyme activity were essentially based on Fowler et al, (1978) and the reaction mixture usually consisted of 10mM L-homocysteine (freshly prepared from its thiolactone form), 5mM L-serine, ^{14}C-serine to a known specific activity, 100mM Tris pH 8.6 and 0.05 to 0.10mg cell protein. Assay conditions are specified where they differed from the above. The reaction was initiated by the addition of the cell extract and incubated at 37°C for 4 hours. The reaction was terminated by precipitating protein by the addition of 10μl 12.5% sulphosalicylic acid. Precipitated protein components were sedimented by centrifugation in an Eppendorf centrifuge for 10 minutes and the supernatant transferred to a clean Eppendorf tube. The solution was adjusted to pH 2 with the addition of 2μl 2.5M NaOH.

Separation of the reaction mixture was achieved using a Beckman System 6300 amino acid analyzer coupled to a Gilson FC-203 fraction collector. The amino acid analyzer was set up to run a standard program for the separation of total amino acids, using a cation-exchange column with lithium citrate buffers of increasing pH. The fractions containing cystathionine were collected in scintillation vials, dissolved in 10ml Packard Hionic-Fluor scintillation fluid and counted in a Beckman LS6000IC liquid scintillation counter using an open window. An aliquot of original reaction mixture was also counted to determine the specific activity of the serine.

Alternatively the reaction mixture was separated using cellulose thin layer chromatography. Aliquots of the reaction mixture were spotted on cellulose TLC plates and resolved for 48 to 72 hours in a mixture of 2-propanol/acetic acid/water (80/6/20). An aliquot of the original reaction mixture was applied to the resolved and dried TLC plates to determine the specific activity of the serine, and the plates counted in a Packard InstantImager electronic autoradiography system.

RNA isolation

RNA was isolated using the acid guanidinium thiocyanate-phenol-chloroform method of Chomczynski and Sacchi (1987). Lymphoblast cultures were centrifuged at 1000rpm for 5 minutes in a Sigma 3E-1 centrifuge. Cells were washed twice with sterile saline. One ml solution D was added to the cell pellet, the suspension mixed by inversion and transferred to a clean 8ml polypropylene tube. Sequentially 0.1ml 2M sodium acetate, pH 4, 1ml water saturated phenol and 0.2ml chloroform/isoamyl alcohol (49/1) was added to the tubes. Tubes were mixed by inversion after each addition and vigorously mixed for 15 seconds after the final step. The mixture was incubated on ice for 15 minutes.

Fibroblast cultures were washed by rinsing tissue culture flasks twice with saline. One ml solution D was added directly to the flasks and incubated on ice for 15 minutes. A rubber policeman was used to detach cells from the inside surface of the flask and the contents transferred to a clean 8ml polypropylene tube. The remaining reagents were added exactly as for lymphoblasts and the tubes incubated for a further 5 minutes after the final step.

Tubes were centrifuged at 10000g for 15 minutes at 4°C in a Heraeus Sepatech Varifuge 20RS. The aqueous phase containing the RNA was transferred to a fresh tube and the DNA and protein contained in the interface and organic phase discarded. RNA was precipitated by incubating in 1 volume of isopropanol at –80°C for 1 hour, followed by a 20 minute centrifugation step at 10000g and 4°C. The pellet was dissolved in 0.3ml solution D, transferred to a clean Eppendorf tube and RNA precipitated in 1 volume isopropanol at –80°C for 1 hour. Tubes were centrifuged in an Eppendorf centrifuge at 4°C to sediment RNA. Supernatant was discarded and the RNA pellet washed three times with 75% ethanol (made up with DEPC treated water). Finally RNA was dried under vacuum and dissolved in 100µl DEPC treated water. The quality of the RNA preparation was assessed by calculating the ratio of absorbance at 260nm to 280nm. The nucleic acid concentration was determined from the absorbance at 260nm.

First strand cDNA synthesis

cDNA was reverse transcribed from the extracted total RNA using Avian Maloney virus (AMV) reverse transcriptase and an oligo dT primer to the 3' polyadenosine tail of the message. Included in the reaction mixture was 1 to 5µg total RNA, 5µg oligo dT primer, dNTP's at 0.5mM each, 80U AMV reverse transcriptase, 5U RNase inhibitor, 1× manufacturer's buffer and sterile distilled water to 100µl. To ensure effective priming of the mRNA template, oligo dT, mRNA and water was combined, heated to 65°C for 5 minutes and then cooled on ice. The remaining components of the reaction were subsequently added and the reaction allowed to proceed at 42°C for 1 hour.

Polymerase chain reaction (PCR)

PCR amplification of cDNA was performed using a Techne PHC-2 thermocycler. The PCR reaction mixture contained the following components: forward and reverse primers at 0.5μM each, dNTP's at 200μM each, 1 to 4mM $MgCl_2$, 1× manufacturer's buffer, 25mU/μl *Thermus aquaticus* (Taq) polymerase, 1-5μl of the cDNA template and sterile distilled water to a total volume of either 50 or 100μl. Eighty microlitres of mineral oil was layered on top of the reaction mixture to prevent evaporation.

Amplification of cystathionine beta-synthase cDNA

For the purpose of direct sequencing the CBS cDNA was amplified in two overlapping segments of about 1000bp each. A semi-nested approach using three primers in two rounds of amplification was followed in both cases. The 5' section was amplified using the primers CBS f-107 and CBS r1212 for 40 cycles in the initial round of amplification. The PCR products from the initial amplification was subsequently further amplified using CBS f-57 and CBS r1212 for 30 cycles in a second round of amplification. The temperature profile consisted of denaturing at 94°C for 45s, annealing at 50°C for 45s and extension at 72°C. Extension time was increased in a stepwise manner and was 120s during the first thirteen cycles, 150s during the middle thirteen cycles and 180s during the last thirteen cycles in the first round of amplification. During the second round of amplification the extension time was increased in a similar fashion every ten cycles. The 3' section of the CBS cDNA was amplified in a similar fashion using the primers CBS f1106, r1955 and r1928 and an annealing temperature of 54°C. The reactions took place in 2mM $MgCl_2$. In all cases 1μl of the first PCR reaction was used as template for the semi-nested PCR. A reaction excluding the template DNA was used in both rounds of amplification to detect the possible erroneous amplification of contaminating template.

For the purpose of cloning, a ~2000 base pair segment of the CBS cDNA, spanning the entire coding region, was amplified. A nested approach was again utilized. The primers CBS f-107 and CBS r1955 were used for 40 cycles in the initial round of amplification. The PCR products from the initial amplification was subsequently further amplified using CBS f-57 and CBS r1928 for 30 cycles in a second round of amplification. The temperature profile consisted of denaturing at 94°C for 45s, annealing at 54°C for 45s and extension at 72°C. Extension time was increased in a stepwise manner and was 120s during the first thirteen cycles, 180s during the middle thirteen cycles and 240s during the last thirteen cycles in the first round of amplification. During the second round of amplification the extension time was increased in a similar fashion every ten cycles. The optimal $MgCl_2$ concentration was determined to be 1.5mM. Gel purification was again employed to clean the PCR products.

Gel purification of PCR products

Products from four identical 100μl PCR reactions of each template were pooled and mineral oil removed by organic extraction with 1 volume of chloroform. The organic and aqueous phases were separated by centrifugation in an Eppendorf centrifuge for 1 minute. The top aqueous layer was transferred to a clean Eppendorf tube and DNA precipitated by adding 1/10 volume 3M sodium acetate, pH 5.2 and 2 volumes of ethanol. This was mixed well and incubated at -80°C for 15 minutes. DNA was sedimented by centrifugation in an Eppendorf centrifuge for 10 minutes, the supernatant removed and DNA washed by adding 1ml of 70% ethanol. Tubes were centrifuged in an Eppendorf centrifuge for 1 minute, supernatant discarded and DNA dried under vacuum. DNA was dissolved in 20μl sterile distilled water.

The pooled and concentrated PCR product was resolved using agarose gel (1.5 to 2%) electrophoresis. The appropriate bands were excised from the gel using a scalpel blade and transferred to a clean Eppendorf tube, taking care to minimize exposure of the DNA to ultraviolet light. DNA was extracted from the agarose gel using the Qiaex II gel (Qiagen) extraction kit following the manufacturer's protocol. DNA was recovered in sterile distilled water.

Cloning of CBS cDNA

Purified CBS PCR products were ligated into the plasmid cloning vector pGEM-T. Ligation into this vector is based on the terminal transferase activity of *Taq* polymerase, which adds a single 3' overhanging adenine to the PCR product. The vector is supplied as a linear molecule with single 5'-overhanging thymidines, which are complimentary to the 3' single adenines in the PCR product. This feature facilitates the direct ligation of PCR products into this vector without any further manipulations. The multiple cloning site occurs within the coding region of the *lacZ* gene, coding for the enzyme β-galactosidase. Successful ligation of the insert into the vector leads to inactivation of the *lacZ* gene, providing a marker for the detection of colonies containing recombinant vectors. This is achieved using solid media containing IPTG, which acts as an inducer for the transcription of the *lacZ* gene and Xgal, a synthetic, colourless substrate of β-galactosidase yielding a blue product. The vector also contains an Ampicillin resistance gene allowing for the selection of transformed bacteria using antibiotic containing media.

Ligation of CBS cDNA into pGEM-T

Ligation of purified PCR products into the plasmid vector was carried out according to the manufacturer's protocol for T-vectors. The reaction mixture contained 200ng plasmid DNA,

50ng PCR product (2:5 molar ratio of plasmid to insert), 1× manufacturer's ligation buffer, 0.5mM ATP and 2U of DNA ligase in a final volume of 15µl. Ligation was allowed to proceed in a water bath at 16°C for 1 hour and ligase was inactivated by heating to 70°C for 15 minutes.

Preparation of competent cells using $RbCl_2$

Competent *E.coli* cultures were prepared by $RbCl_2$ treatment (Ausubel et al, 1995). Frozen glycerol stocks of a commercial strain of *E.coli*, XL1 Blue, were streaked onto LB plates and allowed to grow overnight at 37°C. A single colony was selected from the plate and used to inoculate 40ml Ψ-broth and grown overnight at 37°C, with shaking. Two pre-warmed 200ml volumes of Ψ-broth were each inoculated with 4ml of the overnight culture and grown with agitation at 37°C until the cell suspension reached an absorbance of between 0.5 and 0.6 at 600nm. Flasks were cooled on ice and the bacteria sedimented at 4°C at 5000g for 10 minutes in pre-chilled centrifugation tubes. Cell pellets were re-suspended in 40ml of ice-cold TFBI and incubated on ice for 30 minutes. Bacteria were again sedimented at 4°C at 5000g for 10 minutes, re-suspended in 8ml of ice-cold TFBII and incubated on ice for 15 minutes. The cell suspensions were aliquoted into 250µl volumes using chilled pipette tips, frozen in liquid nitrogen and stored at -70°C. Transformation efficiency was assayed using 1ng of pUC18 plasmid vector.

Transformation

Ligated plasmid vectors were used to transform the $RbCl_2$ competent XL1 Blue *E.coli* strain. Aliquots of the competent cells were thawed on ice and distributed into 50µl volumes in Eppendorf tubes. One µl of each ligation mixture was added to the competent cell suspension and incubated at 4°C for 45 minutes. The suspensions were subsequently exposed to a heat shock at 37°C for 30 seconds to allow for the entry of the plasmid vectors into the bacteria, and transferred to 1ml ice cold 2×TY medium. Bacterial cells were stimulated to recover by incubating cultures with shaking at 37°C for 1 hour. Cultures were centrifuged in an Eppendorf centrifuge for 30 seconds, resuspended in 100µl of 2×TY medium and two equal volumes plated onto Ampicillin containing LB agar plates coated with Xgal an IPTG. The plates were incubated overnight at 37°C. White colonies, carrying insert containing vector, as well as blue colonies, containing self-ligated vector, were streaked onto a fresh Ampicillin containing LB agar plate and grown overnight at 37°C.

Minipreps

The alkaline lysis method of preparing plasmid DNA was performed using either the method of Birnboim and Doly (1979) or a commercial Wizard Plus SV miniprep kit. In these procedures, bacteria are lysed in the presence of sodium dodecyl sulfate and NaOH, which denature bacterial proteins and chromosomal and plasmid DNA respectively. Upon the addition of potassium acetate the mixture is neutralized, resulting in the rapid re-annealing of plasmid DNA. SDS, bacterial proteins and chromosomal DNA precipitate and can be removed by centrifugation.

A single colony from each plate was grown overnight in 5ml Ampicillin containing LB medium with shaking at 37°C. Bacterial cells were sedimented by successive centrifugation in an Eppendorf centrifuge. The pellets were re-suspended completely in 100μl GTE by vortexing and subsequently incubated at room temperature for 5 minutes. Bacterial cells were lysed and DNA and protein denatured by adding 200μl of a freshly prepared solution containing 1% SDS and 0.2M NaOH to the cell suspension, mixing by inversion and incubating, on ice, for 5 minutes. The solution was neutralized with 150μl 5M potassium acetate, pH 4.8, vortexed for 5 seconds and centrifuged in an Eppendorf centrifuge for 3 minutes. The supernatants were transferred to fresh tubes, RNase A added to 50μg/ml and the solutions incubated at room temperature for 30 minutes.

Residual protein contaminants were extracted by adding 500μl TE saturated phenol/chloroform/isoamyl alcohol (50/49/1), vortexing and centrifuging in an Eppendorf centrifuge for 2 minutes. The top aqueous layer was transferred to a fresh tube and any remaining phenol and protein contaminants extracted with 500μl chloroform. After centrifugation the aqueous phase was again transferred to a fresh tube. DNA was precipitated by adding 1ml ice cold 100% ethanol, incubating at -70°C for 15 minutes and pelleted by centrifuging in an Eppendorf centrifuge for 5 minutes. The DNA pellets were rinsed with 70% ethanol, centrifuged for 1 minute, ethanol aspirated and the pellets dried under vacuum. Further purification was achieved using PEG precipitation at high salt concentration and elution in 70% ethanol. Dried DNA pellets were dissolved in 16.8μl de-ionized water, 3.2 μl 5M NaCl was added and the solution mixed well. Twenty μl of 13% PEG was added, the solutions mixed well and incubated on ice overnight. Samples were centrifuged in an Eppendorf centrifuge for 10 minutes, supernatants discarded and the pellets rinsed with 70% ethanol. Pellets were dried under vacuum and dissolved in 20μl de-ionized water. The quantity of DNA was estimated by comparing the band intensities of the samples to a standard on an agarose gel stained visualized with ethidium bromide.

The manufacturer's protocol was followed exactly when the Wizard Plus SV minipreps were used. In this method however, plasmid DNA was purified by binding to a silica gel matrix at low pH and elution with sterile distilled water.

DNA Sequencing

Sequencing of PCR products and cloned material was performed using three methods. Manual sequencing was performed using a Sequenase DNA sequencing kit (Amersham Life Science). Automated sequencing was performed by the Core Facility at UCT using a ThermoSequenase dye terminator cycle sequencing kit (Amersham Life Science) with an ABI 373 DNA Sequencer (Perkin Elmer) and also at the Department of Biochemistry and Microbiology at UCT using a ThermoSequenase fluorescent labeled primer cycle sequencing kit (Amersham Life Science) with an ALFexpress DNA Automated Sequencer (Amersham Pharmacia Biotech AB).

Manual Sequencing

The Sequenase manual sequencing protocol is based on the Sanger dideoxy nucleotide sequencing method (Sanger et al, 1977). The manufacturer's protocol was followed with some modifications (Kraft et al, 1988).

Double-stranded plasmid DNA was denatured by adding 2μl of a freshly prepared solution containing 2M NaOH and 2mM EDTA to 20μl of the purified mini-preps, containing 3 to 5μg DNA, and incubating for 5 minutes at room temperature. Intra-strand secondary structure formation was promoted by sequentially adding, on ice, 8μl 1M Tris, pH 4.5 and 3μl 3M sodium acetate and DNA precipitated by adding 75μl ice cold 100% ethanol and incubating at -70°C for 30 minutes. DNA was sedimented by centrifugation in an Eppendorf centrifuge for 5 minutes at 4°C and the pellets were washed with 200μl ice cold 70% ethanol. Tubes were centrifuged in an Eppendorf centrifuge for 2 minutes at 4°C and the pellets dried under vacuum after the supernatant was aspirated. The appropriate sequencing primer was annealed to the single stranded template by dissolving the dried DNA pellets in 7μl sterile de-ionized water and 2μl 5× Sequenase Sequencing Buffer (200mM Tris, pH 7.5, 100mM $MgCl_2$, 250mM NaCl), adding 0.5pmol primer and incubating at 37°C for 30 minutes. The primed templates were subsequently centrifuged and chilled on ice. Extension and labeling of the complementary strand was achieved by adding 1μl 0.1M DTT, 2μl diluted Labeling Mix (7.5μM each of dGTP, dCTP and dTTP), 5μCi [α-^{35}S]-dATP and 2μl diluted Sequenase Polymerase and incubating at room temperature for 3 minutes. The reactions were terminated by adding the respective dideoxy nucleotides. Three and a half μl of the reaction mixture was added to tubes containing 2.5μl pre-warmed (37°C) Termination Mixture (80μM

each of dATP, dGTP, dCTP and dTTP and 8μM of the specific dideoxy nucleotide) for each nucleotide and incubated at 37°C for 5 minutes. Termination reactions were stopped by adding 4μl Stop Solution (95% formamide, 20mM EDTA, 0.05% bromo-phenol blue and 0.05% xylene cyanol).

Each of the terminated sequencing reactions was resolved at 60W on a 6% denaturing polyacrylamide gel. The gel contained 8M urea and was prepared in the running buffer containing 134mM Tris, 45mM boric acid and 2.5mM EDTA. The terminated sequencing reactions were loaded three times successively, each new loading occurring after the xylene cyanol had been run off the gel. The resolved gel was adhered to a sheet of filter paper, covered with cling-wrap and dried at 80°C under vacuum. The dried gel was exposed to autoradiography film for 72 hours, which was subsequently developed and analyzed.

Automated sequencing at the Core Facility at UCT

The Core Facility at UCT performed automated DNA sequencing using a ThermoSequenase dye terminator cycle sequencing kit with an ABI 373 DNA sequencer. The following is a brief description of the manufacturer's protocol used. To ~1μg of DNA and ~5pmol primer in a volume of 12μl, 8μl of Sequencing Reagent Pre-mix was added. The reagent pre-mix contained 125mM Tris, pH9.5, 5mM $MgCl_2$, T7 DNA polymerase, *Thermoplasma acidophilum* thermostable inorganic pyrophosphatase, Nonidet P40, Tween 20, 6.25% glycerol, 1.25mM dITP, 0.25mM each of dATP, dCTP, dTTP and dGTP, and uniquely dye-labeled dideoxy ATP, dideoxy CTP, dideoxy TTP and dideoxy GTP. The sequencing reactions were allowed to proceed using 30 cycles of the following temperature profile: 30s at 96°C, 15s at 45°C and 4min at 60°C. The completed sequencing reactions were precipitated with 70μl 5mM $MgCl_2$ in 70% ethanol, re-suspended in 4μl formamide loading buffer and separated on a 4.75% PAGE gel and each uniquely labeled dideoxy terminator detected by its fluorescence.

Automated sequencing at the Department of Biochemistry and Microbiology at UCT

Automated sequencing at the Department of Biochemistry and Microbiology at UCT was performed using a ThermoSequenase fluorescent labeled primer cycle sequencing kit (Amersham Life Science) with an ALFexpress DNA Automated Sequencer (Amersham Pharmacia Biotech AB). The sequencing reaction is based on the Sanger chain termination technique using 7-deaza-dGTP with dye labeled M13 –40 sequencing primer at 2pmol/μl. The manufacturer's instructions were followed exactly.

Metabolic labeling

The metabolism of methionine in cultured fibroblasts was investigated using two radiolabeled isotopes of methionine. L-[^{35}S]-methionine was used to follow methionine through the transsulfuration and remethylation pathways, while L-[methyl-^{3}H]-methionine was used as a marker for methyl transfer reactions. Figure 21 shows the metabolism of methionine with the metabolites that the ^{35}S and tritium labels can be incorporated into in yellow and blue respectively. The ^{35}S radiolabel can be incorporated into protein directly as methionine or after being metabolized through the transsulfuration pathway, as cysteine. The tritium radiolabel, however is on the methyl group of methionine, and is transferred to a methylated acceptor, mainly nucleic acid and protein. Changes in the flux through the transsulfuration and remethylation pathways, such as caused by enzyme deficiencies, could therefore affect the ratio of tritium to ^{35}S incorporated into acid precipitable material.

Confluent fibroblast cultures were incubated in pre-warmed buffer or tissue culture media containing the isotopes. At various time intervals culture medium was removed, protein and DNA precipitated with 5% trichloracetic acid (TCA) and the precipitated material dissolved in 0.5M NaOH. The medium acid soluble and acid precipitable fractions at each time point were counted in a scintillation counter. A 0 to 200keV window was used to detect tritium (which includes a defined contribution from ^{35}S) and a 400 to 600keV window was used to detect only ^{35}S. The amount of each isotope was calculated for the medium, the TCA soluble fraction containing all the small molecular components and the TCA precipitated macromolecules.

On the day preceding each experiment fibroblast cultures were seeded into 3cm polystyrene tissue culture dishes at a density of 3×10^5 cells per well. This number of cells had been determined to yield a confluent culture the following day (Baumgarten, 1984). Fibroblast cultures were trypsinized and re-suspended in complete medium and washed once with complete medium. An aliquot of the suspension, diluted 1/100 in filtered Isoton III was enumerated in a Coulter Counter. A volume of the suspension corresponding to 3×10^5 cells was seeded into each dish, 1ml of complete medium added and the dishes incubated overnight at 37°C and an atmosphere of 10% CO_2. On the day of the experiment, cells were visually checked for confluence.

The medium was aspirated and the cultures washed once with Medium Z. The time course was initiated by adding 1ml preheated (37°C) incubation medium containing ^{35}S-methionine at ~1μCi and [methyl-^{3}H]-methionine at ~10μCi. At various times a dish was processed and the incubation medium removed and retained. The cultures were washed twice with ice-cold saline and subsequently twice with ice cold 5% TCA to precipitate protein and nucleic acids. The first TCA wash, containing the soluble cytoplasmic constituents, was also retained. The

precipitated material was released from the cultures by incubating with shaking in 0.1M NaOH for 10 minutes. Of each fraction, 500μl was dissolved in 10ml Packard Hionic Fluor, an alkaline resistant scintillation fluid and counted in a Beckman LS6000IC liquid scintillation counter. The TCA fraction was adjusted to pH 10 by adding15μl 10M NaOH, before adding scintillation fluid. A 0 to 200keV window was used to detect tritium and ^{35}S and a 400 to 600keV window was used to detect only ^{35}S.

RESULTS

Plasma homocysteine measurements

Peripheral vascular disease patients were screened by measuring fasting plasma homocysteine as well as 6 hours after an oral methionine load. Fasting and post methionine load homocysteine levels as well as the change in plasma homocysteine measured in the subjects of this study are shown in table 1.

Patient	Age	Sex	Fasting [15.7μM]	Post methionine load [42.2μM]	Increase after loading [27.7μM]
972	39	M	***16.2***	39.5	23.3
973	49	M	14.1	***46.5***	***32.4***
974	32	M	11.0	***49.0***	***38.0***
975	39	M	***17.7***	-	-
976	42	M	15.1	***44.2***	***29.1***
977	45	M	14.8	***45.9***	***31.1***
979	37	F	10.6	***54.5***	***43.9***
980	60	M	***17.3***	-	-
981	47	F	***19.7***	***52.1***	***32.4***
982	45	M	6.7	41.7	***35.0***
983	36	F	***28.2***	***69.0***	***40.8***
1000	39	M	***28.0***	***67.9***	***39.9***

Table 1. Fasting plasma homocysteine and plasma homocysteine 6h after an oral methionine load. The increase in plasma homocysteine from the fasting level to the 6h post methionine load level is also shown. The upper limit of normal for each measurement established in our laboratory is given in square brackets and abnormally high values printed in bold italic lettering.

Six patients in the PVD group showed fasting homocysteine levels above the upper limit of normal (patients 972, 975, 980, 981, 983 and 1000), two of whom were not available for methionine loading (patients 975 and 980). Three (patients 981, 983 and 1000) of the remaining four showed abnormally high post methionine load homocysteine as well as increase in homocysteine following the methionine load. Patient 972 showed only an abnormally high fasting homocysteine level. All six subjects with normal fasting homocysteine levels showed hyperhomocysteinaemia as ascertained by post methionine load peak or the increase in plasma homocysteine after loading (patients 973, 974, 976, 977, 979 and 982).

Cystathionine β-synthase activity in fibroblasts

Method of cell lysis

Three methods of cell lysis were investigated to determine their effectiveness in the release of cytoplasmic constituents from cultured fibroblasts. The aim of this investigation was to find a method that preferentially releases cytoplasmic components while leaving organelles intact, as it has been illustrated that post-translational proteolytic cleavage of CBS leads to a 60-fold increase in enzyme activity (Skovby et al, 1983). Release of lysosomal material may therefore lead to proteolytic cleavage of the enzyme and erroneously high and/or variable activity.

Digitonin treatment of cultured cells has been proposed as a rapid and mild procedure for the preferential release of cytoplasmic constituents (Mackall et al, 1979), while freezing and thawing and sonication have been used more frequently as standard methods of lysis. Lactate dehydrogenase was used as a marker for the release of cytoplasmic components, while citrate synthase, occurring in the mitochondria was used as a measure of organellar disruption. A trypsinized control fibroblast cell line was resuspended in PBS and divided into three equal parts. The suspensions were lysed with digitonin, by freezing and thawing in dry ice/ethanol or by sonication. Lactate dehydrogenase and citrate synthase assays were subsequently performed on each supernatant. Both the activities were measured in the linear range with respect to time and the amount of extract added.

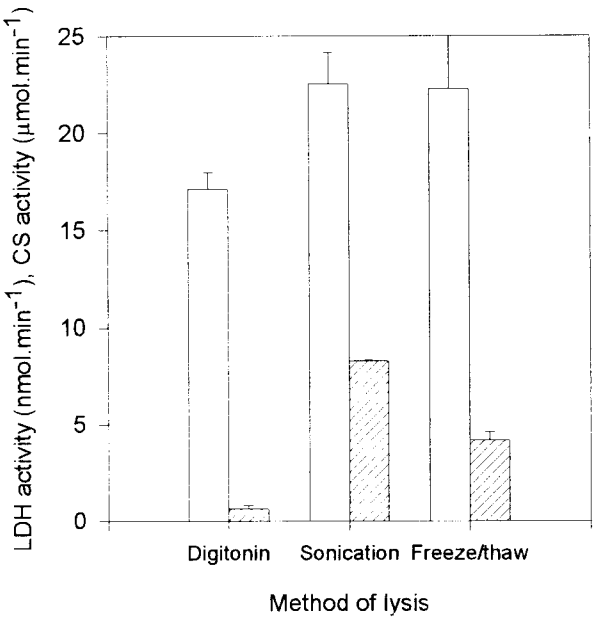


Figure 2. Lactate dehydrogenase (open bars) and citrate synthase (hashed bars) activity released from identical suspensions of a trypsinized fibroblast culture lysed by either sonication, freezing and thawing or treatment with digitonin.

Figure 2 shows the absolute amount of both enzyme activities that was released by each method of lysis. Sonication and freeze/thawing appears to release very similar amounts of lactate dehydrogenase while digitonin treatment releases about 25% less. Sonication, being

the more disruptive method, released twice the citrate synthase activity compared to freeze/thawing and ten times the amount released by digitonin treatment. Digitonin was therefore chosen as a method that preferentially releases cytoplasmic components from trypsinized fibroblast cultures. Digitonin treatment also appeared to be more reproducible than sonication in releasing CBS activity from trypsinized fibroblast cultures in previous studies in our laboratory (L. Human, personal communication).

Cystathionine β-synthase assay

Cystathionine β-synthase irreversibly condenses homocysteine and serine to produce cystathionine. The assay for enzyme activity is based on the conversion of L-[^{14}C-3]-serine to ^{14}C-cystathionine by lysates of fibroblast cultures. Reaction products are separated by chromatographic methods and the amount of radioactive cystathionine formed determined as a measure of the enzyme activity. One unit of enzyme activity is defined as the amount of enzyme that catalyses the formation of 1 nanomole of cystathionine per hour. Results are expressed as units of activity per milligram of protein measured in the lysate. The initial conditions for the assay were modified from Fowler et al (1978). Reaction mixtures contained 15mM L-homocysteine (freshly prepared), 5mM L-serine, L-[^{14}C-3]-serine to a specific activity of ~6300dpm/nmol, 1mM pyridoxal-5-phosphate and 100mM Tris, pH 8.6 in a final volume of 50μl and were incubated at 37°C for 4h. Assay blanks, in which the cell lysates were replaced with sterile distilled water, were included in the initial experiments, and showed no activity.

Separation of reaction products by amino acid analyser and TLC

The assay was standardised in our laboratory using a Beckman System 6300 amino acid analyser coupled to a Gilson FC-203 fraction collector to separate the reaction mixture and to collect the cystathionine containing fractions. The appropriate fractions were counted in a liquid scintillation counter to measure enzyme activity. This process is time consuming and very labour intensive. Separation of the reaction mixture using thin layer chromatography was compared to the amino acid analyser method.

Paper chromatography with isopropanol/formic acid/water (80/6/20) as mobile phase has been widely used to separate the reaction mixture of the CBS assay (Fowler et al, 1978). TLC using a cellulose stationary phase and a development time of ~24 hours in the same mobile phase was found to be successful in separating the components of the reaction mixture. Reaction products were identified on the basis of their relative mobilities compared to that of known standards visualised with ninhydrin. Radiolabelled cystathionine was quantified on a Packard flat bed counter. The specific activity of the radiolabelled serine was determined by applying an aliquot of the reaction mixture to each developed TLC before counting. Nine specimens were divided in half and analysed by both methods, the results of which are given in table 2. The measured values were on average 10% less when TLC separated the reaction mixtures were compared to the amino acid analyser separated the reaction mixtures. As shown in Figure 3, values measured by both methods correlated well (r^2=0.373). This is in spite of the fact that a narrow range of activities was present in these sets of data, amplifying the negative effect that the inherent variation in the measurements

has on the correlation between the two sets of data. TLC was therefore chosen as a convenient method of resolving the reaction mixtures.

Specimen	A	B	C	D	E	F	G	H	I
AAA	21.8	22.0	21.8	22.3	18.7	22.5	24.2	23.1	24.1
TLC	19.2	20.0	19.2	19.2	20.8	20.2	20.9	19.9	19.6

Table 2. CBS activity (U/mg) measured in identical reaction mixtures separated either on an amino acid analyser (AAA) or by cellulose thin layer chromatography (TLC).

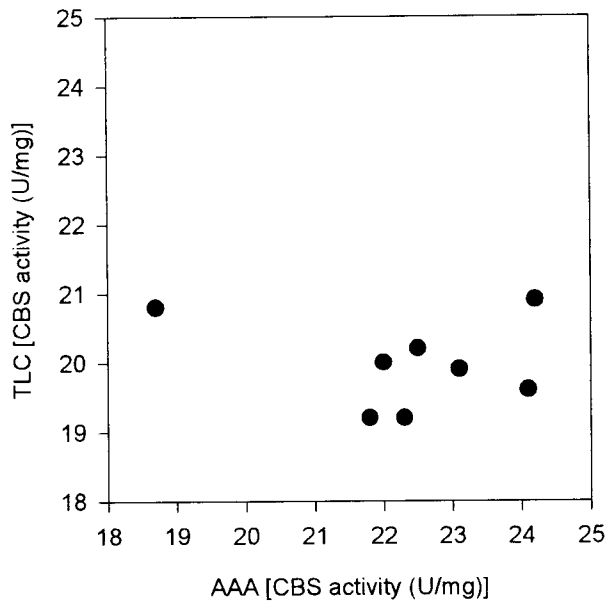


Figure 3. Scatter plot showing CBS activity (U/mg) measured on specimens separated by TLC versus the identical specimens separated by amino acid analyzer.

Variability of the CBS activity measured in lysates of cultured cells

Preliminary studies in our laboratory (L. Human, personal communication) have indicated that cystathionine β-synthase activity measured in lysates of cultured fibroblasts and lymphoblasts is highly variable from one occasion to the next. In order to quantify this variation a control fibroblast culture (F958) at the third passage was seeded into three tissue culture flasks and maintained in the same culture medium. The cultured cells were assayed weekly in triplicate for a period of five weeks. Cells were fed with tissue culture medium two days prior to each assay. Substrate solutions were prepared independently for every assay.

The result of this five-week experiment is shown in Figure 4. The enzyme activity, normalised for protein content, showed a fourfold increase between the fourth and seventh passages from 5.5 ± 1.29U/mg to 22.3 ± 1.62U/mg as illustrated in Figure 4a. Notably, the enzyme activity released from each of the three separate cultures changed in tandem. The variation between the replicates of each flask (average 8.0%) was less than the variation between the three identical flasks (average 14.6%). The protein concentration in each lysate is shown in Figure 4b and the absolute enzyme activity, not corrected for protein content in Figure 4c. The amount of protein released from each culture remained relatively constant over the course of the five-week period, while the absolute activity of each replicate shows the same variation as the normalised enzyme activity. Additionally, higher protein concentrations could not be correlated with lower or higher normalised enzyme activities. These observations suggest that the large between-experiment variation is likely due to differences in the enzyme activity produced by the cells rather than changes in the total protein in each lysate.

Various factors that may contribute to the variation in enzyme activity were investigated, including the effect of changing substrate solutions from one assay to the next. A single lysate of a control fibroblast line (F974) was assayed five times with different substrate solutions of the same composition. Separately made up reagents were L-serine, L-homocysteine, pyridoxal-5-phosphate and the Tris reaction buffer. L-[^{14}C-3]-serine could not be varied as it was obtained from a commercial source. The enzyme activity measured for the five different sets of substrate solutions was 15.9 ± 2.03 (12.7%) U/mg. This variation was very similar to the variation seen within the triplicates of one flask (average 8.0%) as well as between different flasks (average 14.6%) in the previous experiment. Slight variations in substrate and co-factor concentrations were therefore excluded as the main source of the large changes in enzyme activity seen over the five-week period in the previous experiment.

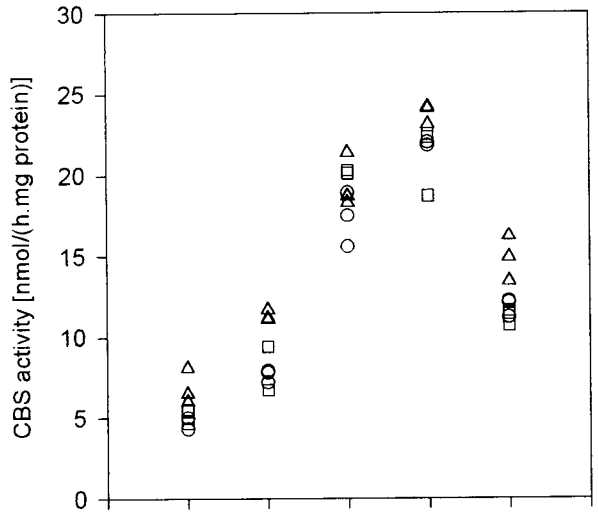


Figure 4a. Normalized CBS activity released from three replicate flasks of control fibroblast line F958 over a period of five weeks. Each symbol represents a different flask and each point a single replicate thereof.

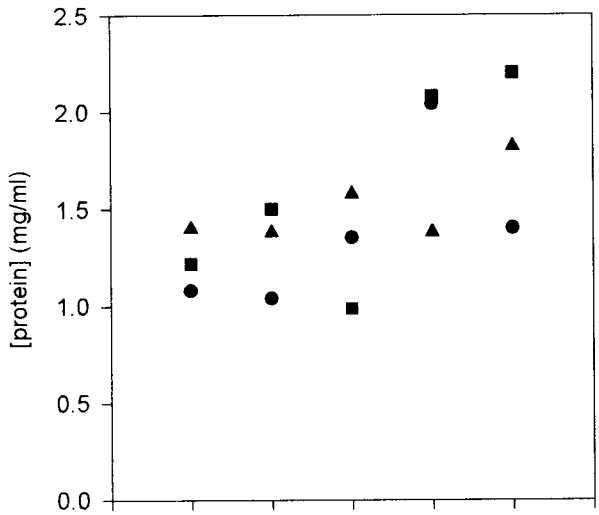


Figure 4b. Total protein concentration released from three replicate flasks of control fibroblast line F958 over a period of five weeks. Each symbol represents a different flask.

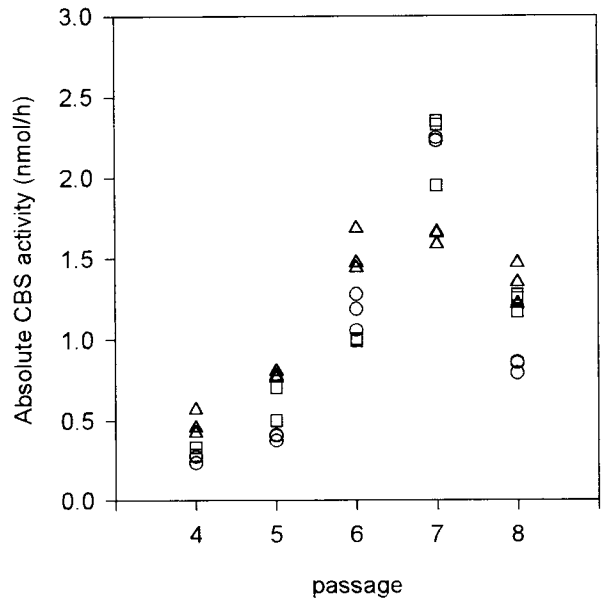


Figure 4c. Absolute CBS activity released from three replicate flasks of control fibroblast line F958 over a period of five weeks. Each symbol represents a different flask and each point a single replicate thereof.

Variable release of either total protein or CBS activity from fibroblast cultures by digitonin was investigated as a possible factor contributing to the observed variation. A cell pellet of a trypsinized control fibroblast line (F992) was divided into four equal parts, and each part was treated with a separately prepared digitonin solution. Enzyme activity was assayed for each lysate at 25mM serine, 20mM homocysteine and 1mM PLP. ^{14}C-serine was added to a specific activity of ~630dpm/nmol. The amount of enzyme activity that was not released from the cells but remained associated with the cell debris was estimated by washing the cell pellet with 50µl PBS and assayed for CBS activity under exactly the same conditions. CBS activity, protein concentration and the percentage of the absolute enzyme activity not released from the cells are given in table 3.

CBS activity (U/mg)	[Protein] (mg/ml)	% activity remaining
35.1	1.93	11.3
36.5	2.00	10.5
37.2	1.83	9.1
41.8	1.78	10.1

Table 3. CBS activity (U/mg), protein concentration (mg/ml) and percentage of absolute activity remaining in the cell pellet after lysis of four identical cell pellets treated with four individual digitonin solutions.

The average CBS activity for the four flasks was 37.7 ± 2.9 (7.7%) U/mg. The variation in the activity released with each of the digitonin solutions was similar to the observed within replicate variation. The total protein released from the cells by each digitonin treatment similarly showed little variation with an average of 1.89 ± 0.10 (5.2%) mg/ml being released. Additionally, an almost constant fraction of the total enzyme activity remained associated with the cell debris after each digitonin treatment. Taken together, this data indicates that lysis of the fibroblast cells with digitonin released a reproducible amount of the total protein as well as enzyme activity and did not contribute greatly towards the observed variation.

After trypsinization cells were resuspended in complete medium to inhibit trypsin activity and washed twice with ice cold PBS. Any foetal calf serum protein remaining after washing would affect the calculated enzyme activity. A control fibroblast line (F992) was trypsinized in the usual manner divided into six equal parts. Pairs of the suspensions were either directly lysed with digitonin, or washed either once or twice with ice cold PBS and subsequently lysed with digitonin. Enzyme activity was assayed as using ~263dpm/nmol ^{14}C-serine under conditions of maximal activity described in the kinetics section. The enzyme activity obtained for each duplicate treatment is given in table 4.

Number of washes	none	one	two
Replicate A	153.2	144.0	146.2
Replicate B	144.9	149.7	157.7

Table 4. The effect of washing cell pellets with PBS on CBS activity (U/mg). Pairs of the suspensions were either directly lysed with digitonin, or washed either once or twice with ice cold PBS and subsequently lysed with digitonin. Lysates were subsequently assayed for CBS activity as described in the text.

The calculated enzyme activity remained constant, irrespective of the number of washes the cell pellet was subjected to before lysis. A gradual almost linear reduction was observed in the amount of protein measured in the lysates. In view of the constant enzyme activity, this is most likely due to loss of intact cells in each wash step.

CBS activity measured in the lysates of fibroblast cultures is normalised to the protein content of each lysate. Variability in the protein assay will directly influence the calculated enzyme activity. The reproducibility of the Biorad protein assay was investigated by assaying five replicates of a lysate of a control fibroblast line on three consecutive days. The standard deviation between experiments was 5.0% of the mean and 2.5% of the mean within each experiment. The variation in protein measurement was too little to affect the calculated enzyme activity drastically. The presence of digitonin did not interfere with the Biorad assay.

The reproducibility of the enzyme assay itself was investigated by freezing aliquots of a cell pellet as well as aliquots of a lysate obtained from the same cell pellet at -70°C and assaying CBS activity every five days. The identical cultured cells were also trypsinized, reseeded and assayed on each occasion. The enzyme activity obtained from the lysates of the growing cultures and the frozen cell pellets as well as the frozen lysates is illustrated in Figure 5. CBS activity measured in lysates of the frozen cell pellets and the frozen lysates showed little change when assayed repeatedly over a period of two weeks, indicating that the enzyme assay itself is reproducible. There was no loss of activity after two weeks at -70°C. The enzyme activity measured in lysates of the identical cell culture grown in parallel with the assays varied markedly. This suggests that factors during the culture of the fibroblast cells most likely have the greatest contribution to the large variation when measuring the enzyme activity released from cultured fibroblasts at different times. The activity released from the frozen cell pellets was also constantly about 10% higher than that of the frozen lysates, suggesting that the freezing and thawing of the cell pellets had released additional enzyme activity.

In an attempt to determine the influence that cell density may have on the measured enzyme activity, equal amounts of a control fibroblast line (F992) was seeded into eight tissue culture flasks. A pair of flasks was subsequently assayed for CBS activity every week for four weeks.

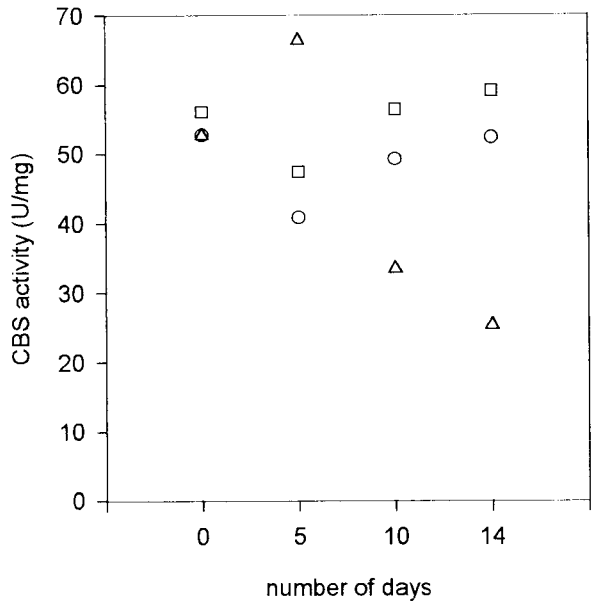


Figure 5. CBS activity assayed on four occasions over a two week period in cells grown in culture (triangles), cells pellets of the same origin frozen at -70°C (squares) and lysates of identical origin frozen at -70°C (circles).

Tissue culture conditions were kept constant by feeding cells twice a week with one change of medium occurring two days before each assay. The first pair of flasks that were assayed had already reached confluence by the time of the assay. Enzyme activity measured on lysates of the duplicate flasks over the four-week period is shown in Figure 6. Very little change in enzyme activity could be detected during the entire experiment, suggesting that the fluctuations in activity might be associated with the state of the cultures prior to reaching confluence. Once the cultures have attained confluence the enzyme activity seems to remain stable.

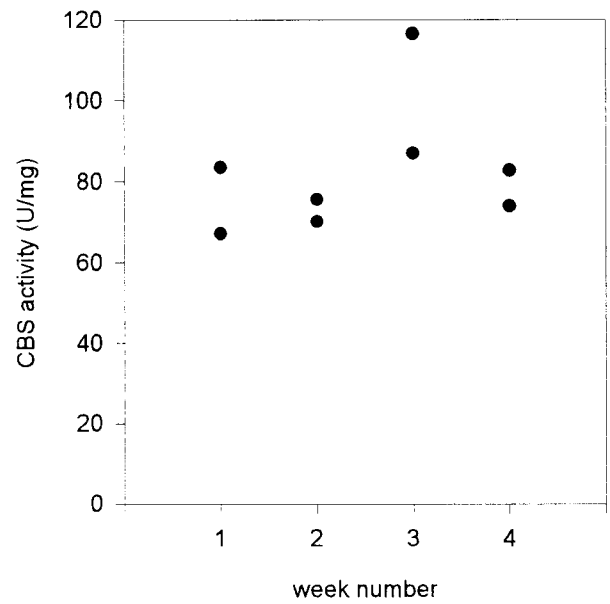


Figure 6. CBS activity measured over a period of four weeks in identical cultures that were started at the same time and fed twice a week. The cultures had reached confluence when the experiment was started and were allowed to grow more confluent with time. A pair of flasks was sacrificed and assayed on each occasion.

Table 5 gives a summary of the factors investigated and the variation measured in each experiment. Variation is expressed as the standard deviation of the replicate measurements as a percentage of the mean of the measurements.

Source of variation	Variation
Replicates of the same lysate	8.0%
Replicates of different flasks of identical cultures	14.6%
Between identical cultures on different occasions A	49.7%
Between substrate solutions	12.7%
Between lysis solutions	7.7%
Between wash procedures	1.7%
Between protein determinations	5.0%
Between identical lysates on different occasions	11.3%
Between identical pellets lysed on different occasions	9.3%
Between identical cultures on different occasions B	52.1%
Between identical, highly confluent cultures on different occasions	16.2%

Table 5. Summary of the factors influencing measured CBS activity and the variation measured in each experiment. Variation is expressed as the standard deviation of the replicate measurements as a percentage of the mean of the measurements.

Kinetic properties of CBS in lysates of cultured fibroblasts

The kinetic properties of CBS in lysates of cultured fibroblasts were investigated in an attempt to define the conditions under which maximal enzyme activity could be achieved. Titrations were performed for serine, homocysteine and pyridoxal-5-phosphate. Lysates of a control cell line (F992) were used as a source of CBS activity for the assays.

The serine titration was performed at 20mM homocysteine and 1mM PLP. The serine concentration was varied between 2.5 and 15mM. The results represent the mean of duplicate measurements. Figure 7 shows CBS activity plotted as a function of serine concentration. A Michaelis-Menten curve was fitted to the data (Figure 7a) using non-linear regression. A Lineweaver-Burk plot was drawn from the reciprocal of the reaction rate and substrate concentration (Figure 7b). The apparent K_m of the enzyme for serine was 10.2mM determined with the Michaelis-Menten fit and 7.2mM using the reciprocal plot. These values correspond well with the previously published 4mM obtained for cystathionine beta-synthase purified from human liver (Kraus, 1987). The maximal enzyme activity (V_{max}) was estimated as 97 U/mg using the Michaelis-Menten fit and 81 U/mg using the Lineweaver-Burk plot.

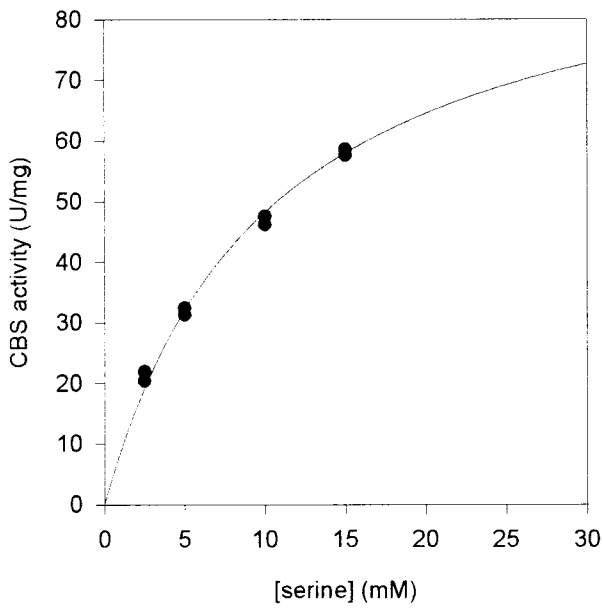


Figure 7a. CBS activity as a function of serine concentration. Assays were performed in duplicate and the fitted curve is described by the Michaelis-Menten equation.

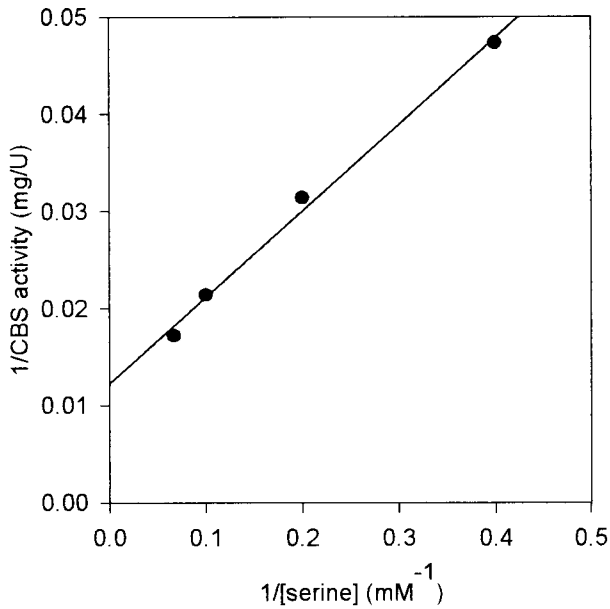


Figure 7b. Lineweaver-Burk plot showing the inverse of the reaction rate plotted as a function of the inverse of the substrate concentration. Each point is the average of duplicate measurements.

The homocysteine titration was performed using 15mM serine, ~2100dpm/nmol ^{14}C-serine and 1mM PLP. Homocysteine was varied between 0 and 60mM. Each point represents the mean of triplicate measurements and the experiment was performed twice to verify the results. CBS activity is plotted as a function of homocysteine concentration in Figure 8. Maximal CBS activity was observed at 20mM homocysteine. The curve is fitted to the data according to an equation describing substrate inhibition: $v=V_{max}\cdot S/(K_m+S+S^2/K_i)$, where v is the reaction rate, V_{max} the maximal velocity, K_m the apparent Michaelis constant, K_i the inhibition constant and S the substrate concentration. The apparent K_m obtained for the fitted data was 8.4mM, V_{max} 84U/mg and K_i 39.0mM. The apparent K_m agreed with the measured value of 25mM reported by Kraus for CBS purified from human liver (1987). Product inhibition was excluded by performing the assay at 10mM homocysteine and cystathionine between 0 and 5mM. No inhibition of CBS activity was noted as a function of cystathionine concentration. Additionally, only about 1% of the radiolabelled serine is converted to cystathionine in the 4 hour period of the assay, making it unlikely that product could accumulate to levels sufficient to inhibit enzyme activity. Fleisher et al (1973) illustrated similar pattern of inhibition of CBS activity in response to increasing homocysteine, but did not comment further.

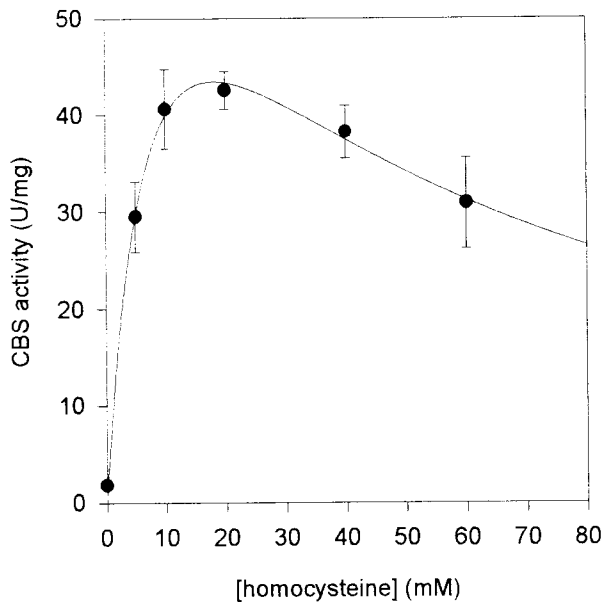


Figure 8. CBS activity as a function of homocysteine concentration in a lysate of control fibroblast line F992. Each point represents the mean of 3 measurements with the error bars showing the standard deviation from the mean. The fitted curve was drawn from an equation describing substrate inhibition.

The pyridoxal 5-phosphate titration was performed at 20mM homocysteine, 5mM serine with the ^{14}C-labelled serine at a specific activity of ~6300dpm/nmol. Pyridoxal 5-phosphate is a tightly bound co-factor and the presence of 0.02mM pyridoxal in the tissue culture medium could allow the enzyme to be saturated with its co-factor. No change in enzyme activity was apparent when the PLP concentration was increased from 0.25 to 2.00mM. This finding disagrees with a previous report (Fleisher et al, 1973) in which a threefold increase in enzyme activity was measured upon the addition of 1.25mM PLP.

The conditions of maximal activity were defined as 40mM serine, 2mM pyridoxal-5-phosphate, 100mM Tris, pH 8.6. Further activation of CBS activity was achieved by the addition of 5mM propargylglycine and 1mM *S*-adenosylmethionine. Propargylglycine is an inhibitor of γ-cystathionase, which cleaves cystathionine to α-ketobutyrate and cysteine. Although very little cysteine was detected during previous assays, propargylglycine was added as a cautionary measure. *S*-adenosylmethionine is an allosteric activator of CBS and enzyme activity shows a threefold increase in response to an increase in SAM concentration from 0 to 100μM (Kluijtmans et al, 1996).

CBS activity in PVD patients

The standardised enzyme assay was subsequently used to investigate a group of hyperhomocysteinaemic patients suffering from peripheral vascular disease. The group consisted of non-diabetic, non-smokers to whom no known clinical reason for their condition could be ascribed. CBS activity was assayed in lysates of fibroblast cell lines obtained from the PVD group as well as a group of age and sex matched controls (mainly parents of patients who tested negative for suspected mitochondrial disorders). Three known homozygotes for CBS deficiency and three obligate heterozygotes were included in the experiment. The assays were all performed with lysates prepared on the same day and processed in a random order. All cultures had been in a highly confluent state for at least a week before the assay was performed.

Assays were performed under the conditions established to produce maximal activity as well as under conditions of sub-maximal activity, with substrate concentrations close to their measured K_m's to determine which would best discriminate between the control and heterozygote groups. Assaying under conditions with substrate concentrations at or below K_m, should reveal any mutations affecting the affinity of the enzyme for its substrate. The sub-maximal conditions where 10mM homocysteine and 5mM serine. The values obtained for groups were compared using a two-tailed Students t-test.

Figure 9 (top) shows the enzyme activity obtained under conditions of maximal activity for the control and PVD patient groups as well as the homozygotes and obligate heterozygotes for CBS deficiency. The average enzyme activity for the control group was 46.6 ± 17.0 U/mg and the 95% confidence interval between 12.6 and 80.6 U/mg under conditions of maximal activity. The enzyme activity measured in the heterozygote group was 32.6 ± 15.3 U/mg, which was not significantly different from the control group. Surprisingly, the one homozygous cell line that was assayed under conditions of maximal activity showed activity at 14.2 U/mg, which can most likely be ascribed to stimulation of residual enzyme activity by pyridoxal 5-phosphate and *S*-adenosyl methionine.

In the PVD group, the enzyme activity ranged between 13.5 and 64.0 U/mg. The average enzyme activity was 36.6 ± 16.2U/mg, which does not differ significantly from the activity in the control group. None of the cell lines from the PVD group showed enzyme activity outside of the 95% confidence interval of the control group. The only cell line that showed CBS activity close to the lower 95th percentile was F1000 at 13.5 U/mg.

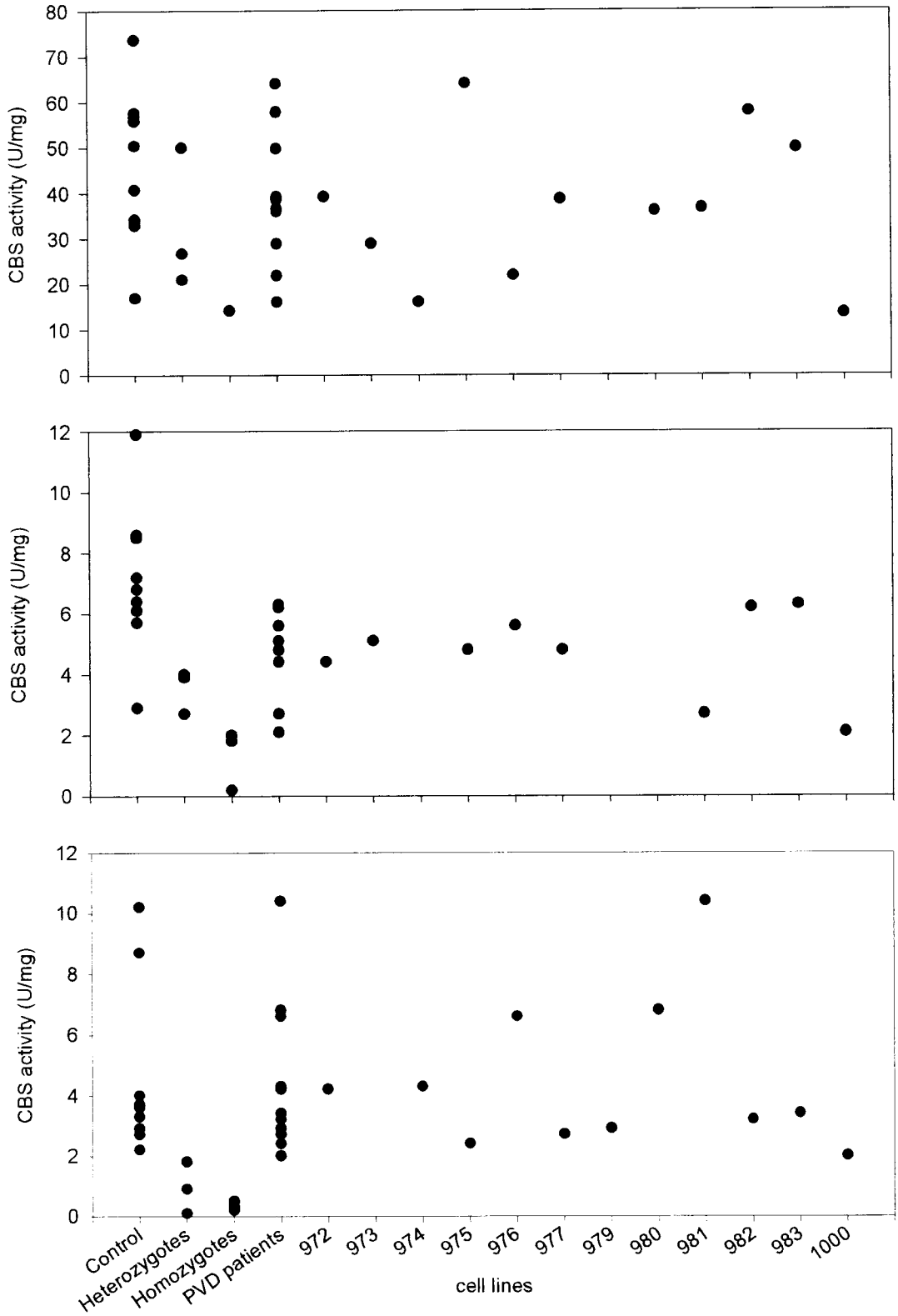


Figure 9. CBS activity measured in the PVD group, an age and sex matched control group, three known homozygotes and three obligate heterozygotes for CBS deficiency. The activity of each cell line in the PVD group (972 to 1000) is also shown individually. The assay under conditions of maximal activity is shown in the top figure, under conditions of sub-maximal activity in the middle figure, and on a second occasion under conditions of sub-maximal activity in the bottom figure.

Figure 9 (middle) shows the enzyme activity obtained on the identical lysates using conditions of sub-maximal activity. The enzyme activity measured under conditions of sub-maximal activity appeared to better discriminate between the control and heterozygote groups compared to the conditions of maximal activity. The enzyme activity measured in the control group under conditions of sub-maximal activity was 7.1 ± 2.5 U/mg and the 95% confidence interval between 2.1 and 12.1 U/mg which corresponds with previously published data using similar assay conditions (Fowler, 1978 and Kluijtmans, 1996).

The enzyme activity measured in the group of obligate heterozygotes was 3.5 ± 0.7 U/mg, which is significantly different from the activity measured in the control group ($P<0.01$). There is however some overlap between the two groups as has been reported by McGill et al (1990). The enzyme activity measured in the patients homozygous for CBS deficiency showed reduced activity at 1.8, 2.0 and 0.2 U/mg respectively. The enzyme activity in the PVD group ranged between 2.1 and 6.3, all of which are within the 95% confidence interval for the control group. The average enzyme activity of the PVD group was 4.7 ± 1.4 U/mg, which is significantly different from that of the control group ($P<0.05$). Once again, as with the assay under conditions of maximal activity F1000 was the only cell line to show activity very close to the lower 95th percentile of the control range (2.1 U/mg). Patients 974, 979 and 980 could not be assayed under these conditions, as insufficient material was isolated from their cultured cells.

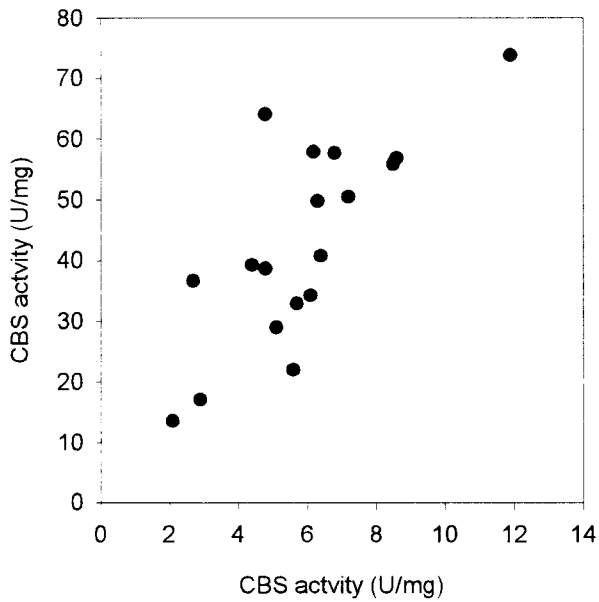


Figure 10. Scatter plot of CBS activity measured on identical lysates under conditions of maximal activity (y-axis) versus sub-maximal activiy (x-axis). The correlation coefficient between the two sets of data is 0.76.

The enzyme activity measured under conditions of sub-maximal activity corresponded well with the activity obtained under conditions of maximal activity, with a correlation coefficient of 0.76 between the two sets of data. The scatter plot of enzyme activity under conditions of maximal activity versus enzyme activity under conditions of sub-maximal activity is illustrated in Figure 10.

On average the enzyme activity was about 6 times higher using the conditions of maximal enzyme activity. This increase in activity is most likely due to an estimated threefold increase in enzyme activity when adding *S*-adenosylmethionine to 1mM, as reported by Kluijtmans et al (1996), as well as an estimated 2.5 fold increase in activity when increasing serine from 5 to 40mM, using in the values obtained from the serine titration. The addition of 2mM pyridoxal 5-phosphate and the increase in homocysteine from 10 to 20mM may also make slight contributions to the increase in activity.

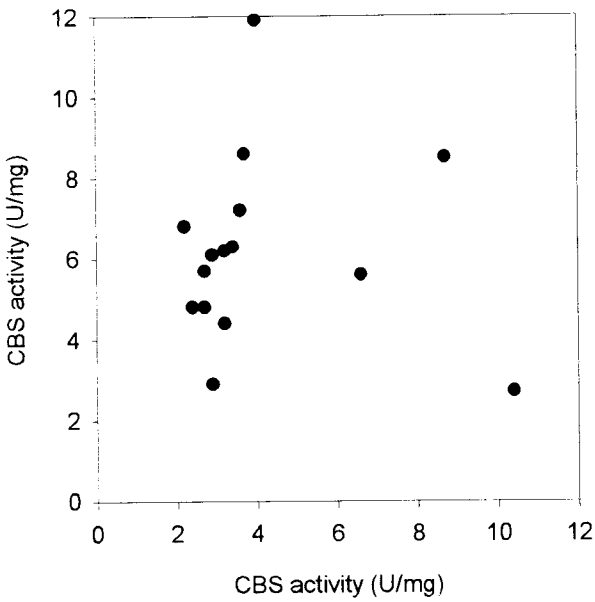


Figure 11. Correlation between CBS activity measured in lysates of fibroblast cultures of the control and PVD groups under conditions of sub-maximal activity in the first (y-axis) and second (x-axis) experiment.

The whole experiment was repeated under conditions of sub-maximal activity to investigate the reproducibility of the assay, the result of which can be seen in Figure 9 (bottom). Cell cultures were seeded again after the first experiment and allowed to grow for two weeks to ensure that all cultures had reached confluence. Cultures were fed twice a week and two days before the assay. The control group displayed an average enzyme activity of 4.3 ± 2.1 U/mg. These values were as a group significantly lower than the values obtained for the control group in the previous experiment ($P<0.01$). Both the homozygote and heterozygote groups also showed lower activity than in the previous experiment. The heterozygote group displayed an average enzyme activity of 0.9 ± 0.8 U/mg, while all the homozygotes showed activity below 1 U/mg. The activity measured in the PVD group ranged between 2.0 and 10.4

U/mg with an average of 4.3 ± 2.4 U/mg. The enzyme activity measured in the PVD group was not significantly different from that of the control group (P>0.9), which is contradictory to the first experiment. The average enzyme activity measured for the PVD group was also not significantly different from that of the first experiment. The correlation between the values obtained for the two experiments was low (correlation coefficient <0.5) and the scatter plot is shown in Figure 11.

The above mentioned data illustrates that regardless of the precautions taken the individual measurements still show great variability. The only constant effect was the average differences between the control, heterozygous and homozygous patient groups. Thus, the only way to currently identify a patient as a likely candidate for heterozygosity for CBS deficiency is to show that the enzyme activity released from cultured fibroblasts is reproducibly low when compared to a panel of control cell lines on a number of occasions.

The only patient in the PVD group that met these criteria was patient 1000. The enzyme activity released from this cell line was low compared to the control range in two experiments (2.2 and 2.0 U/mg), under conditions of maximal activity in the same experiment (13.5 U/mg). The enzyme activity was found to be low in another experiment using conditions of maximal activity (10.4 U/mg). Patient 960, suffering from homocystinuria, consistently showed virtually no detectable CBS activity. The patient's mother (960) showed CBS activity in the heterozygote range (5.1 and 2.7 U/mg) that was lower than a control cell line (959) assayed in parallel (14.8 and 14.2 U/mg). The values obtained for the mother was however in the range that overlaps with the established normal range and viewed in isolation they would not have suggested heterozygosity for CBS deficiency.

The association of CBS activity in lysates of fibroblast cultures with fasting and post methionine load plasma homocysteine levels was also investigated. The correlation between plasma homocysteine and CBS activity is graphically illustrated in Figure 12 as scatter plots of fasting plasma homocysteine, post methionine load plasma homocysteine and change in plasma homocysteine versus CBS activity measured in the two above-mentioned experiments under both conditions of maximal and sub-maximal activity.

In the first experiment, both using conditions of maximal and sub-maximal activity there is a slight negative correlation between both the fasting and post methionine load plasma homocysteine levels measured in the patients and the CBS activity measured in lysates of the cultured fibroblasts. This finding could not, however, be reproduced in the repeat experiment as no clear correlation could be observed between any of the homocysteine measurements in plasma and CBS activity. There was also no clear correlation between the change in homocysteine level between the fasting and post methionine load state and enzyme activity in any of the experiments.

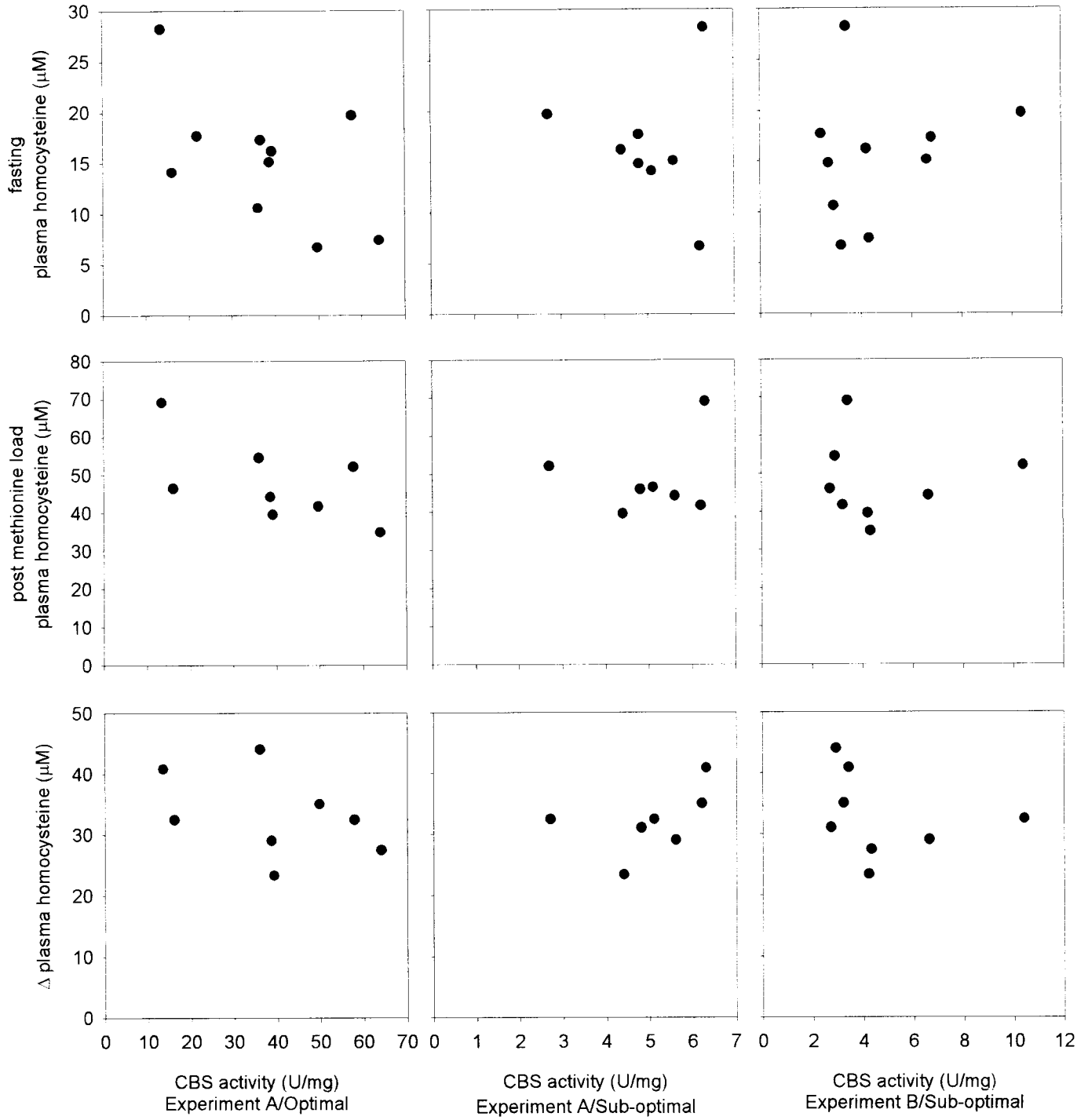


Figure 12. Correlation between fasting plasma homocysteine (top row), post methionine load plasma homocysteine (middle row) and change in plasma homocysteine (bottom row) AND CBS activity measured under conditions of maximal activity (first column) and conditions of sub-maximal activity (middle column) in the first experiment and second experiment (last column).

Molecular Results

The aim of the molecular techniques applied during this study was to identify the exact genetic mutations in the CBS gene leading to the production of dysfunctional enzyme and decreased activity. Patient 1000 as well as patient 960 and her mother, 961 were investigated.

mRNA was extracted from cultured lymphoblasts and fibroblasts using the acid guanidinium thiocyanate-phenol-chloroform method of Chomczynski and Sacchi (1987). Diethyl pyrocarbonate treated water and RNase free pipette tips were used in these procedures to prevent the degradation of the isolated message by contaminating RNases. The quality of the preparations was assessed by measuring the ratio of the absorbance at 260nm to 280nm. The $A_{260/280}$ ratio in all preparations exceeded 1.7. cDNA was reverse transcribed from the isolated mRNA using an oligo dT primer and AMV reverse transcriptase.

cDNA was amplified with the polymerase chain reaction (PCR) using *Thermus aquaticus* DNA polymerase (Taq). PCR products were size separated by agarose gel electrophoresis and visualized by ethidium bromide staining and UV detection. All PCR's were optimized by initially performing the reactions at 2mM $MgCl_2$ and at an annealing temperature 2°C below the theoretical melting temperature of the primer with the lowest melting point. If no product was observed, the annealing temperature was dropped by another two degrees in a stepwise fashion until a reasonable amount of product was formed. If many non-specific products were observed the annealing temperature was similarly raised in 2°C steps until a balance was achieved between yield and specific product. Once the optimum temperature had been established a $MgCl_2$ titration was performed between 1 and 4mM $MgCl_2$ in increments of 0.5mM. Again, the $MgCl_2$ concentration yielding the most product and highest specificity was subsequently used. An extension time in excess of 1 minute per thousand base pairs amplified was generally used.

For the purpose of direct sequencing the CBS cDNA was amplified in two overlapping sections of about 1000bp each. A semi-nested approach using three primers in two rounds of amplification was followed in both cases. The 5' section was amplified using the primers CBS f-107 and CBS r1393 for 40 cycles in the initial round of amplification. The PCR products from the initial amplification were subjected to a second round of amplification using primers CBS f-57 and CBS r1393 for 30 cycles. The temperature profile comprised denaturing at 94°C for 45s, annealing at 50°C for 45s and extension at 72°C. Extension time was increased in a stepwise manner and was 120s during the first thirteen cycles, 150s during the middle thirteen cycles and 180s during the last thirteen cycles in the first round of amplification. During the second round of amplification the extension time was increased in a similar fashion every ten cycles. The 3' section of the CBS cDNA was amplified in a similar

fashion using the primers CBS f1106, r1955 and r1928 and an annealing temperature of 54°C. In both cases the optimal $MgCl_2$ concentration was established to be 2mM.

PCR products were subsequently purified by resolving the products on an agarose gel, physically cutting out the desired band and extracting the DNA from the gel slice using a Qiaex II gel extraction kit from Qiagen. Cleaned PCR products were subsequently directly sequenced at the Core Facility at UCT using internal primers and dye labeled dideoxy terminators. A Thermosequenase cycle sequencing kit (Amersham Life Science) was used with an ABI 373 DNA sequencer (Perkin Elmer). Direct sequencing of PCR products, however, proved to be an inappropriate method for searching for different mutations on two alleles over 2000 base pairs. Heterozygous mutations show up as two peaks or bands, one for the normal base and one for the altered base. Due to background signals quite a number of bases show two signals, which makes it virtually impossible to identify the true mutations.

Cloning of PCR products was utilized as a solution to this problem. Each clone represents only one allele and any changes show up distinctly upon sequencing. A 1985bp PCR was set up spanning the entire coding region of the CBS cDNA using a nested approach. The primers CBS f-107 and CBS r1955 were used for 40 cycles in the initial round of amplification. The PCR products from the initial amplification were subsequently further amplified using CBS f-57 and CBS r1928 for 30 cycles. The temperature profile comprised denaturing at 94°C for 45s, annealing at 54°C for 45s and extension at 72°C. Extension time was increased in a stepwise manner and was 120s during the first thirteen cycles, 180s during the middle thirteen cycles and 240s during the last thirteen cycles in the first round of amplification. During the second round of amplification the extension time was increased in a similar fashion every ten cycles. Figure 13 shows the $MgCl_2$ titration for the above mentioned PCR. The optimal $MgCl_2$ concentration for this PCR is 1.5mM, yielding the greatest amount of product and only one specific band with an apparent size of ~2000bp. The products of the first and second rounds were loaded successively. No product is visible after the first round of amplification illustrating the need for two rounds of PCR to amplify the low copy number CBS mRNA.

Cleaned PCR products were ligated into the plasmid vector pGEM-T, which contains an Ampicillin resistance gene and the *lacZ* gene in the multiple cloning site allowing for blue/white selection of insert-containing vector. pGEM-T also carries single 5' thymidine overhangs, which are complementary to single 3' adenosine overhangs in the PCR products created by *Taq* polymerase's terminal transferase activity. This feature facilitates the direct cloning of PCR products into pGEM-T. PCR product was added in a molar ratio of 5:2 to the plasmid as recommended, and the manufacturer's protocol followed exactly.

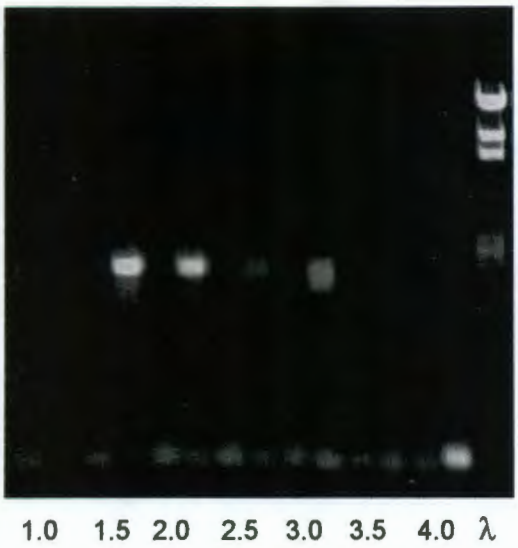


Figure 13. $MgCl_2$ titration of a 1985bp PCR amplifying the entire coding region of the CBS cDNA. The $MgCl_2$ concentration of each reaction is given below each lane in mM. The initial and nested amplifications for each reaction were loaded in successive lanes. The lane marked λ represents λ-DNA digested with *Hind*III

Competent XL1 Blue *E.coli* cells were produced by $RbCl_2$ treatment and transformed with the ligated vector using a 30 second heat shock at 37°C. Transformed bacterial cultures were plated onto LB agar plates containing Ampicillin and coated with Xgal and IPTG and grown overnight at 37°C. White colonies, carrying insert-containing vector, as well as a few of blue colonies were streaked onto fresh Ampicillin-containing LB agar plates and grown overnight at 37°C. The transformation efficiency was calculated by transforming the competent cells with 1ng of pUC18 plasmid, which is of similar size as pGEM-T. The transformation efficiency was reproducibly found to be 10^7 colony-forming units per microgram of DNA.

Single colonies were grown overnight at 37°C in LB broth containing Ampicillin. Plasmid DNA was prepared using the alkaline lysis miniprep method of Birnboin and Doly (1979) or a commercial Wizard Plus SV miniprep kit (Promega). Bacterial cells were sedimented by centrifugation, resuspended in buffer, lysed and proteins and nucleic acids denatured by adding SDS and NaOH. Potassium acetate was added to neutralize the mixture causing plasmid DNA to re-anneal rapidly and SDS, bacterial proteins and chromosomal DNA to precipitate. Precipitated material was subsequently removed by centrifugation. Hereafter different means of purifying the plasmid DNA were employed by the two methods. A spin column, containing a porous silica gel matrix to bind DNA at acidic pH and filter any precipitated contaminants was used in the commercial kit. After two washes, DNA was eluted at neutral pH with sterile distilled water. The method of Birnboin and Doly used phenol/chloroform/isoamyl alcohol and chloroform extractions to remove contaminants, where after DNA was washed by precipitating with ethanol. Precipitation of the plasmid DNA with polyethylene glycol at a high salt concentration was added as a further purification step. PEG was dissolved and plasmid DNA precipitated in 70% ethanol and DNA dissolved in sterile distilled water after drying.

Plasmids were purified from white colonies, which should contain insert-containing vector as well as blue colonies, which contain self-ligated vector. The plasmids isolated from the blue

colonies are used as a size marker, to show that recombinant plasmid vectors of higher molecular weight had indeed been isolated from the white colonies. Figure 14 shows the purified plasmids resolved on a 1% agarose gel visualized with ethidium bromide. The first lane contains a DNA size marker and the last, plasmid isolated from a blue colony, which contains no insert. As is clear from the sizes of the bands in lanes A to D, most plasmids isolated from white colonies are of higher molecular weight and therefore contain an insert. Some of the white colonies however, yielded plasmids of the same apparent size as the self-ligated vector (lane E) and contained no insert. The higher molecular weight plasmids all appear to be of the same size, and should therefore all contain either one of the alleles as an insert.

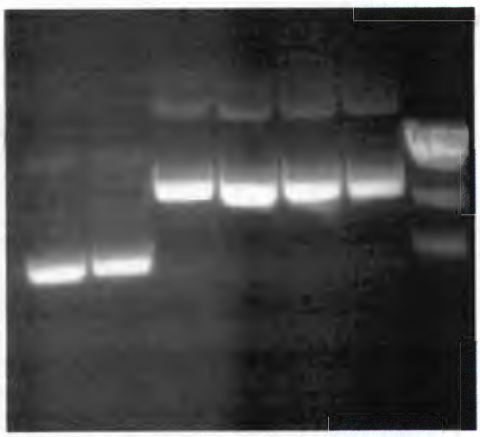


Figure 14. Plasmid minipreps separated on a 1% agarose gel and visualized with ethidium bromide. The right hand lane represents a linear size marker (λ DNA digested with *Dra*I). The lanes A to E represent plasmids purified from white colonies. The left hand lane (blue) represents plasmid purified from a blue colony, which contains no insert. The numbers on the right give the sizes of the standards in base pairs.

Manual sequencing was employed to confirm the identity as well as the orientation of the insert. A Sequenase DNA sequencing kit (Amersham Life Science), which is based on the Sanger dideoxy nucleotide sequencing method was used with a commercially obtained M13 – 40 forward sequencing primer. [α-^{35}S]-dATP was used as a label to visualize the reaction products using autoradiography. Plasmid DNA was denatured under basic conditions with NaOH and single stranded molecules selectively renatured upon neutralization with ice cold sodium acetate. DNA was sedimented with 100% ethanol, washed with 70% ethanol and dissolved in sterile distilled water. The primer was annealed at 37°C for 30 minutes and extension allowed to occur for 3 minutes at ambient temperature. Extension was terminated by adding the respective dideoxy nucleotides. The reaction products were separated on a 6% polyacrylamide gel and exposed to autoradiography film for 72h. Figure 15 shows an example of a portion of a typical sequencing autoradiograph. Both the upstream plasmid sequence as well as the sequence of the insert, starting at the primer can be read using the M13 –40 forward sequencing primer.

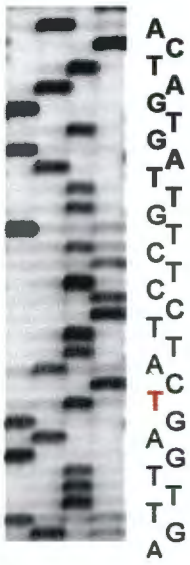


Figure 15. Autoradiograph of a sequencing reaction resolved using polyacrylamide gel electrophoresis. This autoradiograph shows the sequence of a clone of the CBS cDNA obtained from patient 960. The clone was sequenced with the M13 –40 forward sequencing primer that appears upstream from the multiple cloning site. The upstream plasmid sequence is annotated in bold lettering while the insert sequence starting at the beginning of the primer CBS f-56 annotated in normal lettering. The red T represents an A to T change that is likely due to *Taq* polymerase error as it was not found in any of the other sequenced clones.

Plasmids containing the correct insert were sequenced on an automated sequencer to obtain longer sequences. This was performed at the Department of Biochemistry and Microbiology at UCT on a ALFexpress DNA automated sequencer, using a ThermoSequenase fluorescent labeled primer cycle sequencing kit. The M13 –40 forward and reverse sequencing primer labeled with a fluorescent dye was used in the reactions. Sequencing of the vector constructs with the M13 sequencing primers covered 750bp in the forward and 750bp in the reverse direction, leaving the middle ~500bp unsequenced. The middle section of the CBS cDNA was sequenced at the Core Facility at UCT using the internal primers CBS f560, f769 and f1106.

Patient 960 was identified as a compound heterozygote, carrying a different mutation on each allele. The first mutation was confirmed as a duplication of the 7 bases between position 1553 and 1559. The 7 base pair insert leads to a frameshift and premature termination of the protein, affecting the last 32 amino acids of the 551 amino acid CBS monomer. The sequencing profile for the mutant allele as well as the normal sequence is illustrated in Figure 16. Figure 17 shows the translated sequence for the last 41 amino acids for the normal CBS sequence compared to the mutant sequence. This mutation was found to novel and deposited at the CBS Database (http://www.uchsc.edu/sm/cbs/cbsdata/cbsmain.htm). The second mutation was confirmed in three clones as well as the patient's cDNA as the common G919A transition leading to a glycine to serine substitution at amino acid 307. The sequencing profiles of both the patient cDNA as well as a normal control are shown in Figure 18. These were sequenced with an internal primer, CBS f769. This mutation creates an *Alu*I site and was confirmed in other clones and in the patient cDNA by restriction digest of a 443bp PCR product of primers CBS f769 and r1212. The normal PCR product is digested into four fragments of 31, 75, 124 and 213bp, while the 213bp fragment is digested to a 44 and 169bp fragment in the PCR product of the mutant allele. The restriction digest of the PCR products is illustrated in Figure 19. Both the normal and mutant sequence at position

919 are present in the patient, whose restriction digest products shows both the 169 and 213bp bands, while only the normal sequence is present in the mother, who's 213bp PCR fragment remains undigested.

Cloned CBS cDNA from patient 1000 was sequenced to determine the mutation/s leading to decreased enzyme activity and mild hyperhomocysteinaemia in the patient. An A to G transition was identified at nucleotide 695 in two clones out of seven sequenced clones. No other mutations were consistently found in any of the remaining clones. This mutation leads to the substitution of a histidine for an arginine at amino acid 232 (H232R). The sequencing profiles for the normal and mutant alleles are shown in Figure 20.

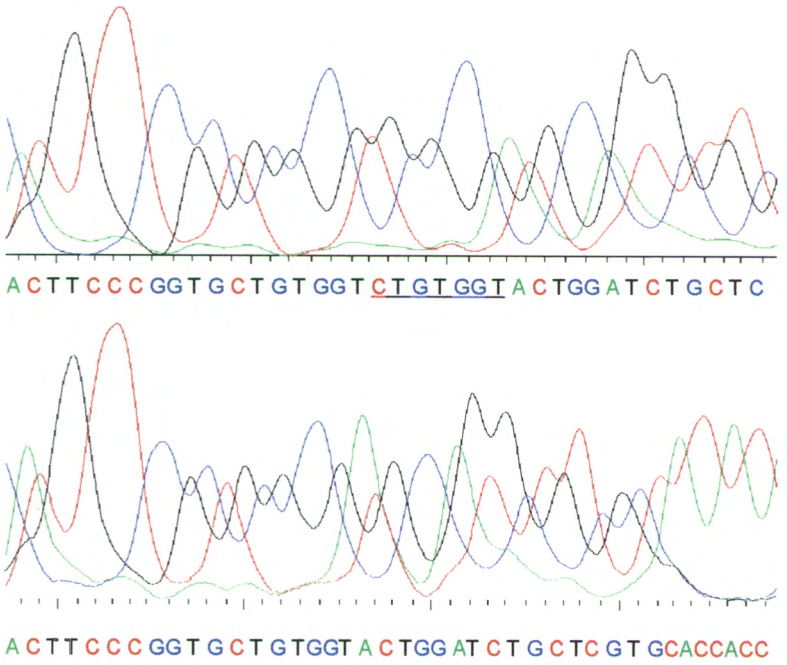


Figure 16. Sequence profile of the complementary strand of a mutant allele of patient 960 showing the 7bp duplication (top) and the normal allele (bottom). The duplicated bases are underlined.

```
 V  V  H  E  Q  I  Q  Y  H  S  T  G  K  S  S  Q  R  Q  M  V
GTGGTGCACGAGCAGATCCAGTACCACAGCACCGGGAAGTCCAGTCAGCGGCAGATGGTG
 F  G  V  V  T  A  I  D  L  L  N  F  V  A  A  Q  E  R  D  Q
TTCGGGGTGGTCACCGCCATTGACTTGCTGAACTTCGTGGCCGCCCAGGAGCGGGACCAG
 K  *
AAGTGA


 V  V  H  E  Q  I  Q  Y  H  R  P  Q  H  R  E  V  Q  S  A  A
GTGGTGCACGAGCAGATCCAGTACCACAGACCACAGCACCGGGAAGTCCAGTCAGCGGCA
 D  G  V  R  G  G  H  R  H  *
GATGGTGTTCGGGGTGGTCACCGCCATTGACTTGCTGAACTTCGTGGCCGCCCAGGAGCG

GGACCAGAAGTGA
```

Figure 17. Translated sequence for the last 41 amino acids of the normal CBS sequence compared to the insert containing mutant CBS allele in patient 960. The normal sequence is shown on top while the mutant sequence is shown at the bottom. The duplicated bases are underlined and the amino acid changes in bold lettering. The stop codon for each reading frame is shown as a star.

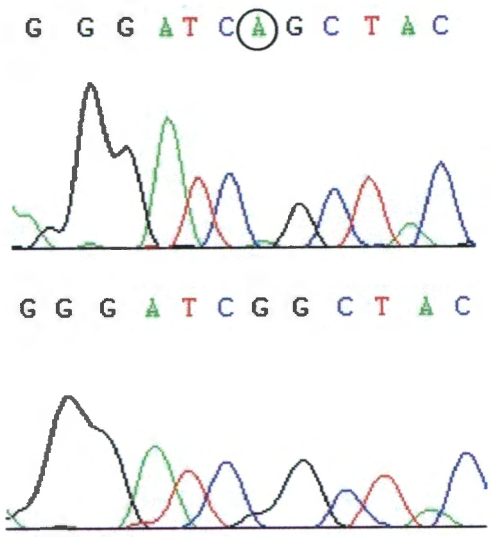


Figure 18. Sequencing profiles of both the patient's cDNA as well as a normal control. This sequence shows the G919A transition that leads to a glycine to serine substitution at amino acid 307. The changed base is circled.

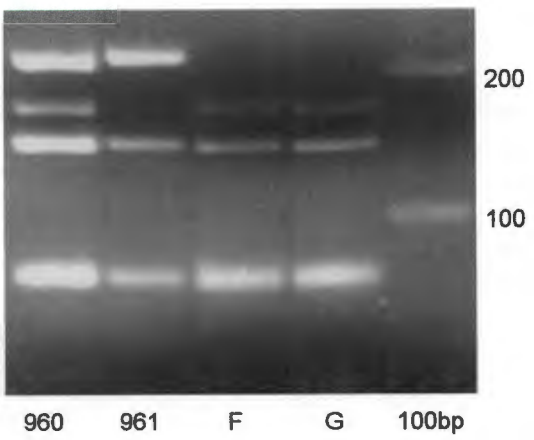
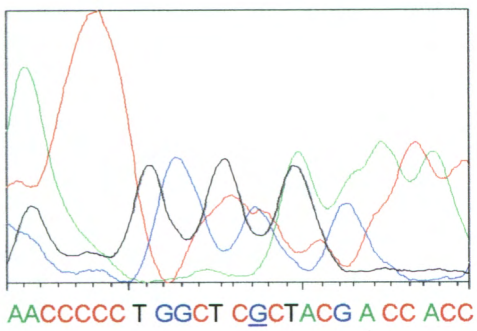


Figure 19. *Alu*I digest of a 443bp PCR product from cDNA of patient 960, his mother (961), and two clones of the patients' cDNA (F and G) containing the G919A transition. The PCR product is digested to four fragments, 31, 75, 124 and 213bp in length in the normal sequence and the 213bp fragment is further digested to two fragments, 44 and 169bp in length in the mutant. The mother shows no 169bp band while the patient shows both alleles (213 and 169bp). The right hand lane shows a 100bp ladder.



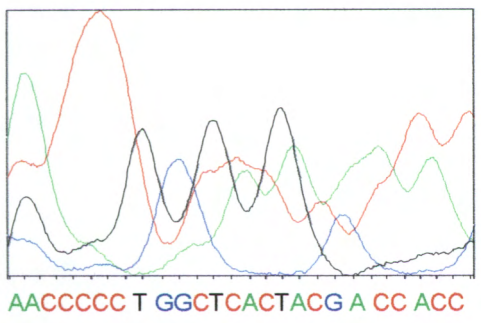


Figure 20. Sequencing profile of cloned CBS cDNA of patient 1000. The top profile shows the sequence of the mutant allele, with the A to G transition at nucleotide 695 underlined. The bottom sequence shows the normal allele of the same patient. This transition leads to a histidine to an arginine substitution at amino acid 232 (H232R).

Dual metabolic labeling

The metabolism of methionine in cultured fibroblasts was investigated using two radiolabeled isotopes of methionine. L-[^{35}S]-methionine was used to follow methionine through the transsulfuration and remethylation pathways, while L-[methyl-^{3}H]-methionine was used as a marker for methyl transfer reactions. Figure 21 shows the metabolism of methionine with the metabolites that the ^{35}S and tritium labels can be incorporated into in yellow and blue respectively. The ^{35}S radiolabel can be incorporated into protein directly as methionine or after being metabolized through the transsulfuration pathway, as cysteine. The tritium radiolabel, however is on the methyl group of methionine, and is transferred to a methylated acceptor, mainly nucleic acid and protein. Changes in the flux through the transsulfuration and remethylation pathways, such as caused by enzyme deficiencies, could therefore affect the ratio of tritium to ^{35}S incorporated into acid precipitable material. Confluent fibroblast cultures were incubated in pre-warmed buffer or tissue culture media containing the isotopes. At various time intervals culture medium was removed, the cultures washed with saline and protein and DNA precipitated from the cultures with 5% trichloracetic acid. The precipitated material dissolved in 0.5M NaOH. The culture medium, as well as the acid soluble and acid precipitable fractions obtained from the cultures at each time point was counted in a scintillation counter. A 0 to 200keV window was used to detect tritium (which includes a defined contribution from ^{35}S) and a 400 to 600keV window was used to detect only ^{35}S. The amount of each isotope was calculated for the medium, the TCA soluble fraction containing all the small molecular components and the TCA precipitated macromolecules.

The initial experiments focussed on the effects of different culture media on the uptake and incorporation of the labels into acid precipitable material. The first medium that was used was a balanced salt solution (Medium Z) containing 1g/L glucose as carbon source. A control cell line was followed over a period of 2 hours and the results of this experiment are shown in Figure 22. The rapid entry of both labels into the cytoplasmic compartment from the medium is visible after the first 15 minutes of incubation. From fifteen minutes on, the amount of both labels remains relatively constant within the acid soluble fraction as well as the medium at about 20% and 80% respectively. After 120 minutes there appears to be a slight decrease in the amount of both labels in the acid soluble cytosolic components matched by an equal increase in the amount of both labels in the medium. The incorporation of both labels into acid precipitable material appears to increase in a linear fashion from the start of the experiment up to two hours suggesting a more or less constant rate of protein synthesis. The amount of label incorporated into acid precipitable material only reaches about 1% of the total amount of label added. The ratio of ^{3}H/^{35}S stayed constant at ~1 for all three compartments over the two hour period.

Methionine-free Eagles Minimal Essential Medium (MEM) was subsequently tested to ascertain the effect that a more complete medium would have on labeling. The medium was also supplemented with 1mg/L methionine to test whether the absence or presence of methionine has a profound effect on the metabolism and labeling of the cultured cells. Virtually identical results were obtained in the presence and absence of the added methionine and it appeared that the dilution of the radiolabeled methionine with unlabeled methionine was not a significant problem as there was only a 50% reduction in the amount of radiolabel incorporated into acid precipitable material. The results for the methionine-free MEM supplemented with 1mg/L methionine are shown in Figure 23. The overall trend is similar to the first experiment with two important exceptions. Firstly, the amount of label that enters the acid soluble fraction is only about 0.5% of the total amount of label, in contrast to the 20% in the first experiment. Both these findings were reproducible. Interestingly, the incorporation of both labels into acid precipitable material was still linear and reached about 1% of the total amount of label as in the previous experiment. Secondly, the ratio of $^{3}H/^{35}S$ in the acid soluble fraction increases markedly over the two hour time course of the experiment. This was not observed in the first experiment where the ratio of the two labels stayed constant for all three fractions. The ratio of $^{3}H/^{35}S$ in the medium and acid precipitable fractions remained completely constant over the two hour time period.

The dual label assay was then applied to investigate known homozygotes, heterozygotes and control lines in an attempt to find possible differences in the ratio of the two labels in any of the fractions. The homozygous cell lines were obtained from two siblings (patients 488 and 489) who were compound heterozygotes for CBS deficiency with the same mutant CBS genotype that completely abolishes enzyme activity. The heterozygotes were their parents (patients 486 and 487) who each carried one of the two compound mutations present in their offspring. The general trend of the results obtained for each of the cell lines was almost identical to the previous experiment illustrated in Figure 23. There is an initial increase of label in the acid soluble pool, which flattens of after 15 minutes. The incorporation of both labels into acid precipitable material is linear over time and the ratios of $^{3}H/^{35}S$ in the medium and the acid precipitable fraction stayed constant for the duration of the assay. As in the second experiment there is a marked increase in the ratio of $^{3}H/^{35}S$ in the acid soluble fraction over the time course of the experiment. The ratio of $^{3}H/^{35}S$ in the acid soluble fraction is illustrated in Figure 24 for each of the cell lines. It is clear that there is no correlation between the rate of change in the ratio of $^{3}H/^{35}S$ in the acid soluble fraction and the zygosity of the cell line for CBS deficiency and that the change in ratio of $^{3}H/^{35}S$ in the acid soluble fraction over time can therefore not be used as an indicator for CBS deficiency. These investigations, however present only pilot studies on the use of dual metabolic labels in the investigation of methionine metabolism and much further work is needed before a definite indication of the usefulness of this technique in this regard can be ascertained.

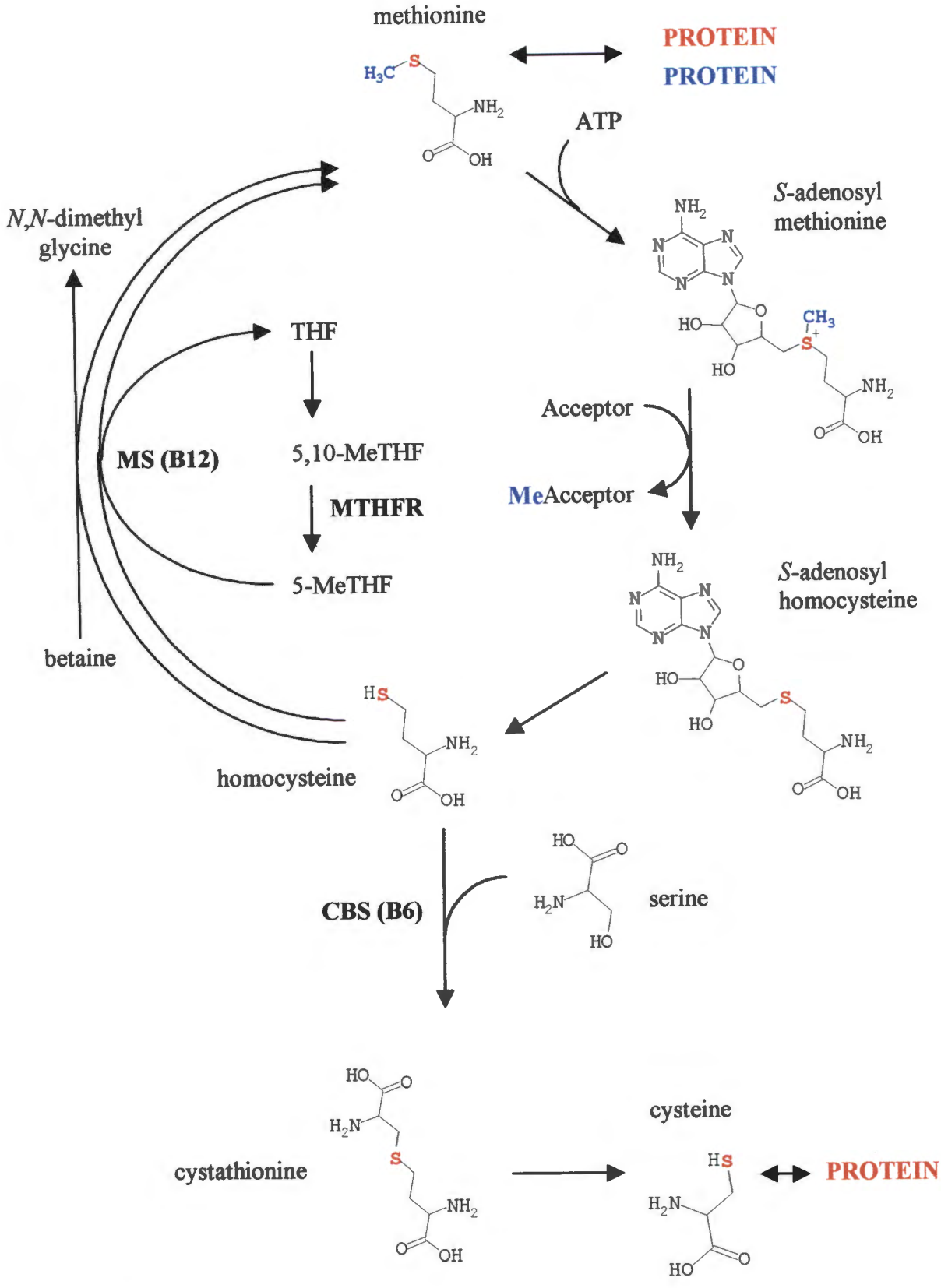


Figure 21. Summary of methionine metabolism, illustrating into which metabolites the ^{35}S (orange) and ^{3}H (blue) are converted.

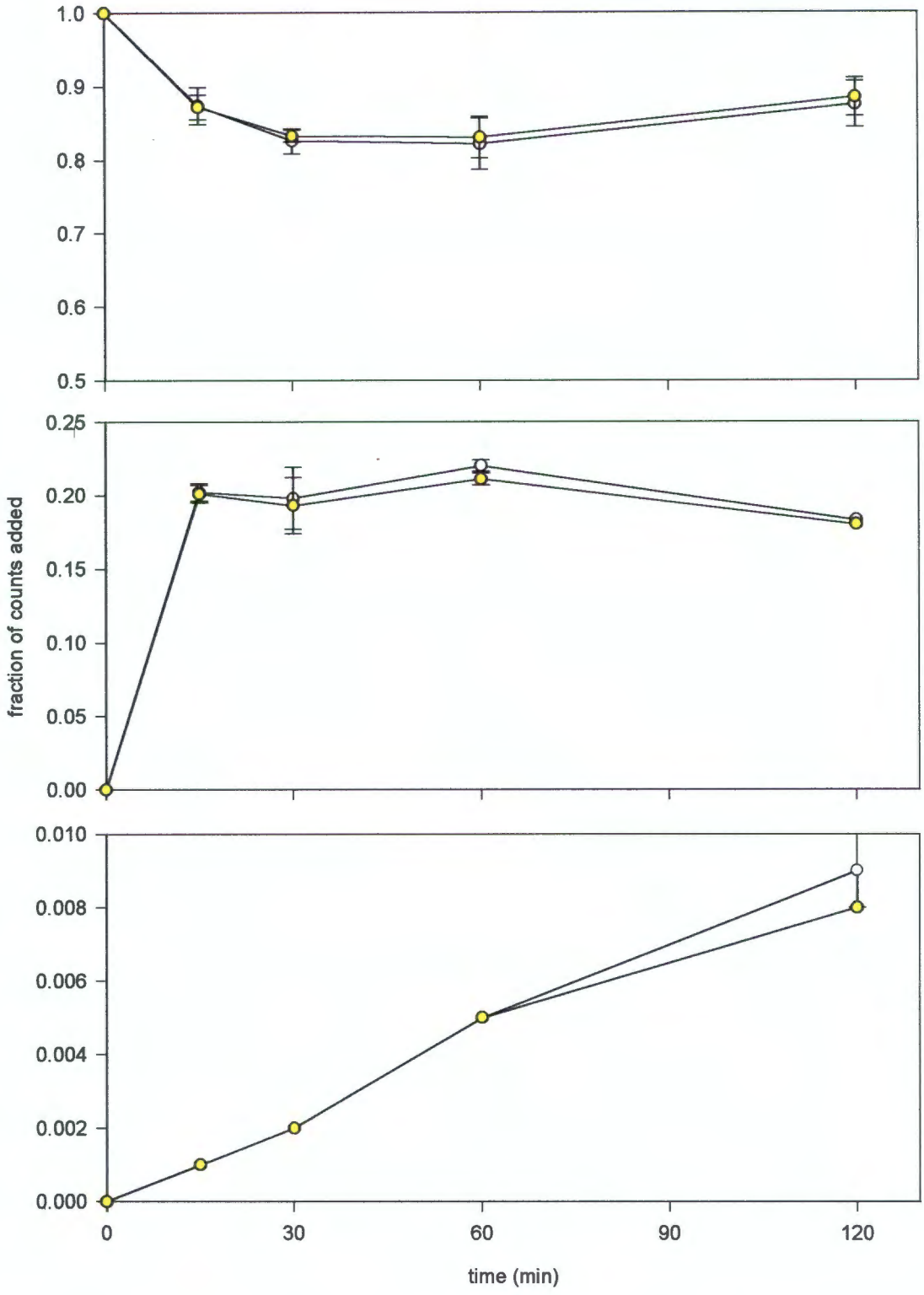


Figure 22. Dual label of a control cell line in Medium Z containing 1mg/L glucose. The top figure represents the medium, the middle figure the acid soluble pool and the bottom figure the acid precipitable material. The yellow circles represent ^{35}S, while the white circles represent ^{3}H.

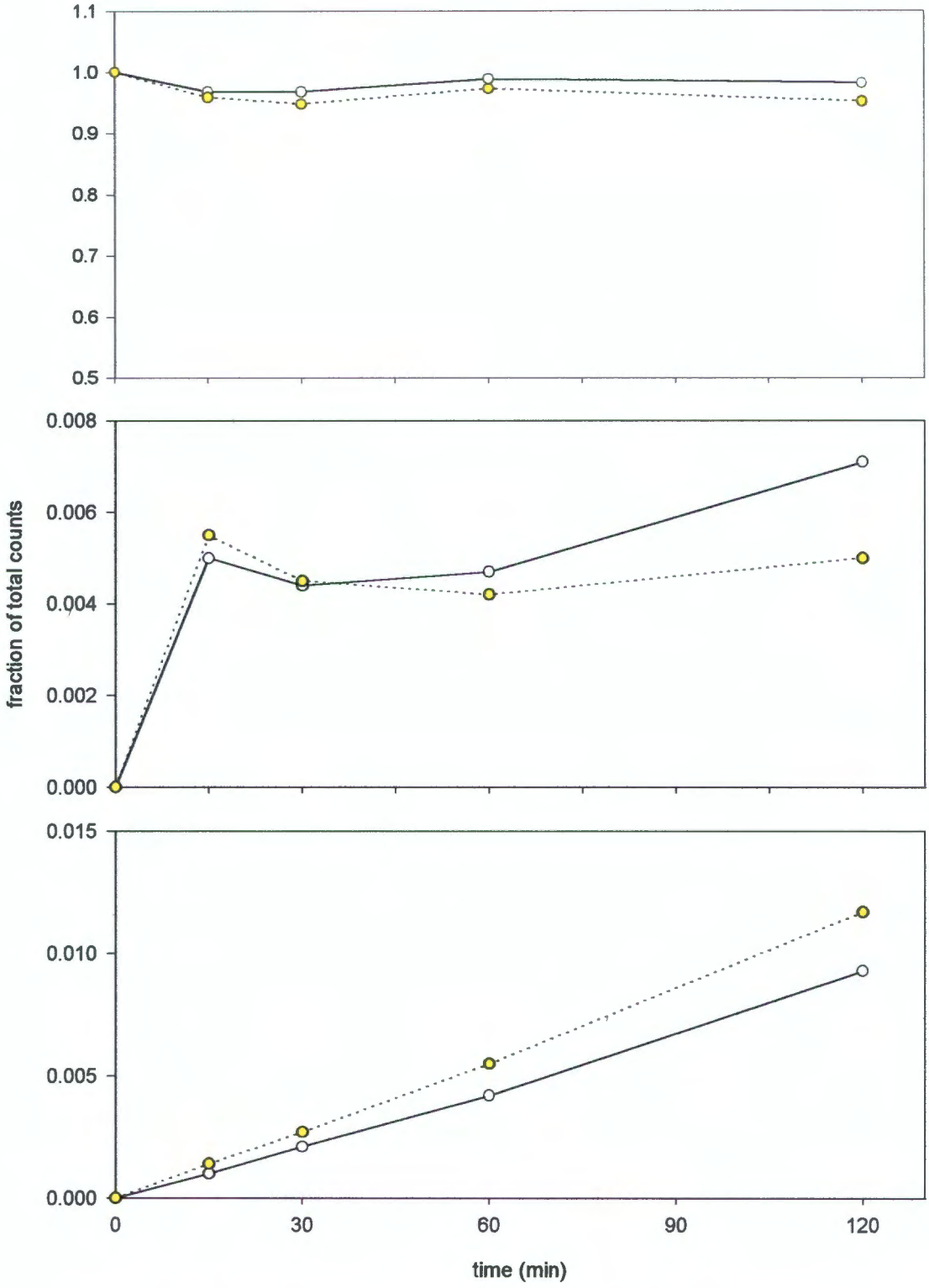


Figure 23. Dual label of a control cell line in methionine free MEM supplemented with 1mg/l methionine. The top figure represents the medium, the middle figure the acid soluble pool and the bottom figure the acid precipitable material. The yellow circles represent ^{35}S, while the white circles represent ^{3}H.

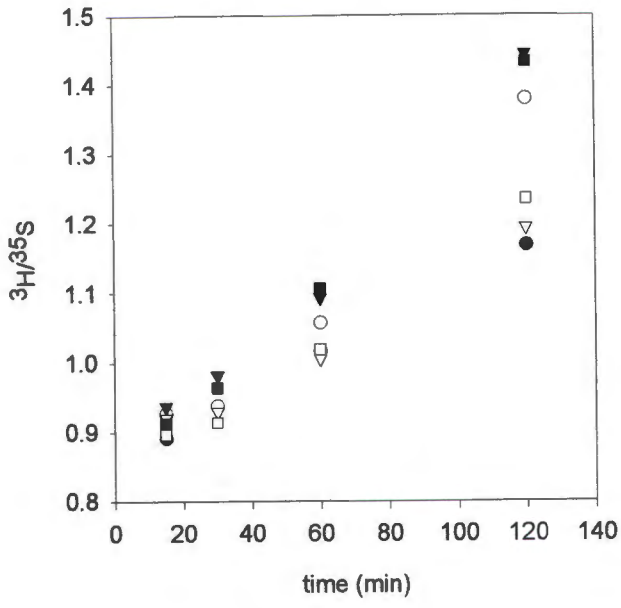


Figure 24. Ratio of $^3H/^{35}S$ in the acid soluble pool over time. The circles represent two compound heterozygotes siblings for CBS deficiency with the same genotype, the triangles their heterozygote parents and the squares two control cell lines. No difference in the change in ratio is observed between the three groups.

DISCUSSION

Hyperhomocysteinaemia is an established independent risk factor for the development of vascular disease. In a study of over 600 patients with homocystinuria due to CBS deficiency, 51% of the 158 thromboembolic events that occurred were in the peripheral veins (Mudd et al, 1985). Two separate studies (Boers et al, 1985 and Clarke et al, 1991) also found mild hyperhomocysteinaemia in 28% of subjects suffering from peripheral vascular disease. Boers also measured decreased CBS activity in all the hyperhomocysteinaemic subjects studied. Den Heijer et al (1996) recently found a greatly increased probability of the occurrence of deep-vein thrombosis in subjects with mild hyperhomocysteinaemia. Our study focussed on the incidence of CBS deficiency in subjects with peripheral vascular disease that had none of the conventional risk factors for the development of vascular disease and were selected for having hyperhomocysteinaemia. The study group of 12 young patients was of varied ethnic and racial origin. Although the hyperhomocysteinaemia may have contributed to the vascular disease in these patients, it is important to note that other factors, such as genetic defects in Factor V could also have contributed to the development of vascular disease in this group of patients (Mandel et al, 1996).

Plasma homocysteine levels

Fasting plasma homocysteine measurements revealed that 6 of the 12 PVD patients suffered from mild hyperhomocysteinaemia (Table 1). The measurement of plasma homocysteine levels after an oral methionine load was developed to identify heterozygotes for CBS deficiency (Fowler et al, 1971) and clinical studies have shown that the methionine loading test can identify subjects that are missed by measuring only fasting plasma homocysteine (Graham et al, 1997). Two of the subjects with mildly elevated fasting plasma homocysteine were not available for methionine loading. Three of the four remaining subjects with mild fasting hyperhomocysteinaemia also showed abnormally elevated plasma homocysteine levels after methionine load, as ascertained by the absolute value as well as the increase in plasma homocysteine. All six PVD patients who had normal fasting plasma homocysteine levels displayed hyperhomocysteinaemia after methionine load as measured by the absolute value and/or the increase from the fasting level. Thus nine out of ten subjects that had the methionine loading test done showed hyperhomocysteinaemia, indicating that defects in the transsulfuration pathway, such as heterozygosity for CBS deficiency might play an important role in this population.

Standardization of the CBS assay

The initial stumbling block in this project was that the CBS assay had been found to yield unreproducible results from one assay to the next in previous studies done in our laboratory.

The assay needed to be standardized and the sources of variation identified and eliminated before it could be applied to the study population.

Digitonin treatment was investigated as a mild method of cell lysis that would preferentially release cytoplasmic components and leave organelles intact. This method of lysis would leave lysozomes intact, which contain proteolytic enzymes that might cause variations in enzyme activity. Using citrate synthase as a mitochondrial marker and lactate dehydrogenase as a cytoplasmic marker it was found that Digitonin treatment releases about 25% less of the cytoplasmic components compared to freeze/thawing and sonication (Figure 2). Digitonin treatment however released five and ten times less citrate synthase activity than freeze/thawing and sonication respectively. Digitonin treatment reproducibly released both protein and CBS activity from trypsinized fibroblast suspensions and left a constant amount of CBS activity associated with the cellular debris (Table 3).

Separation of reaction products by TLC was compared to separation by amino acid analyzer (Table 2). The enzyme activity obtained with separation by TLC was consistently about 10% less than the enzyme activity obtained with separation by amino acid analyzer. The difference between the two methods was acceptable in light of the saving in hands-on time. In both cases virtually no radioactive cysteine could be measured.

The extent of the variation in enzyme activity over time was investigated by seeding three separate tissue culture flasks with an identical starting culture and assaying each of the three flasks in triplicate, every week for a period of five weeks (Figure 4). The results of this experiment showed a fourfold difference between the highest and lowest measured values between experiments. The variation between replicates of the same culture and between the separate cultures was very similar (~10%) and marginal compared to the variation between experiments. The variation between experiments did not correlate with the amount of protein released from the cells and showed a stronger association with the absolute amount of enzyme activity released from the cells. Importantly, the enzyme activity released from the cultures varied in tandem. Whatever factor/s caused the variation therefore affected all the cultures to the same degree.

Various factors that could possibly contribute to the observed variation were investigated. The release of CBS activity by Digitonin had been found to be reproducible (Table 3) and the variation in the enzyme activity between different Digitonin lysates of identical cell suspensions was found to be the same as the variation between replicate of identical lysates (Table 5). The variation between different substrate solutions was also found to be comparable to the variation between replicate of identical lysates (Table 2). The Biorad protein assay was found to be very reproducible and to make only a minor contribution to the overall variation. Contaminating protein from the fetal bovine serum in the tissue culture

medium contributed little to the protein measurements, as washing the cell pellet with PBS had no effect on the enzyme activity (Table 4).

The reproducibility of the assay procedure itself was investigated by freezing away identical cell pellets and lysates of thereof and assaying these over a period of two weeks (Figure 5). The culture from which the cell pellets and lysates were obtained was maintained and also assayed. The results indicated that the assay was reproducible. The assays on the frozen lysates and fresh lysate of the frozen cell pellets did not vary markedly. The frozen cell pellets reproducibly showed ~10% more activity than the lysates indicating that the freezing and thawing of the cell pellets released additional enzyme. The assays on the lysates of the original cultures that were maintained over the time of the experiment showed the same fourfold difference between the highest and lowest value as was found initially, suggesting that factors during the culture of the cells made the greatest contribution towards the variation.

The density of the cultures seemed a likely source of variations in the levels of expression of the enzyme. All the previous experiments had been performed on cultures that had just reached confluence or that were sub-confluent. Equal amounts of a cell suspension were seeded into eight tissue culture flasks and the cultures were allowed to reach a highly confluent state. Two flasks where trypsinized, lysed and assayed each week for four weeks (Figure 6). The results from this experiment showed that once the cultures had reached confluence, the amount of measurable enzyme activity remained stable and all subsequent experiments were performed on highly confluent cultures.

The kinetic properties of the enzyme in the assay were also investigated to establish the conditions under which maximal activity could be obtained. The K_m for serine in the assay was determined to be 10.2mM using a Michaelis-Menten fit and 7.2mM using a reciprocal Lineweaver-Burk plot (Figure 7). These values correspond well with the published value of 4mM obtained for the enzyme purified from human liver. The assay showed substrate inhibition with respect to homocysteine (Figure 8). Product inhibition was excluded by assaying in the presence of increasing cystathionine, which had no effect on CBS activity. Maximal enzyme activity was obtained at 20mM homocysteine and the K_m calculated as 8.4mM by fitting a curve to the data using an equation describing substrate inhibition. Fleisher et al (1973) reported similar data but did not comment further. A possibility that cannot be excluded and that was not tested was that other substances in the homocysteine preparation caused the observed inhibition and not the homocysteine itself. Maximal enzyme activity was achieved at 0.25mM pyridoxal 5-phosphate, as there was no change in activity between 0.25mM and 2mM PLP.

The conditions under which maximal activity was obtained were defined as 20mM homocysteine, 40mM serine and 2mM PLP. Propargylglycine, an inhibitor of γ-cystathionase was added to 5mM and *S*-adenosylmethionine and allosteric activator of CBS was added to 1mM. The addition of 100μM SAM has been shown to stimulate CBS activity threefold (Kluijtmans et al, 1996). The conditions of sub-maximal activity were adapted from Fowler et al (1978) and were defined as 10mM homocysteine and 5mM serine.

CBS activity in the PVD group

The standardized enzyme assay was subsequently applied to the PVD patient population. A control group consisting of age and sex matched subjects with no indication of disorders of methionine metabolism was assayed as well as known homozygotes for CBS deficiency and their obligate heterozygote parents. The enzyme assay showed the best discrimination between the heterozygote and control subjects under conditions of sub-maximal enzyme activity (Figure 9, top & middle). Under conditions of maximal activity, the residual activity in the heterozygote patients was stimulated to such a degree that all the values obtained for this group fell within the control range, making these conditions unsuitable for distinguishing between heterozygotes for CBS deficiency and normal subjects. Under these conditions, no significant difference was observed between the enzyme activity measured in the PVD group and the control subjects.

Under conditions of sub-maximal activity the overlap between the control and obligate heterozygotes was substantially less and the difference in enzyme activity between the two groups was significant ($P<0.01$). The 95% confidence interval for the control group was between 2.1 and 12.1U/mg, which agrees well with previously published normal ranges established using similar conditions (Fowler et al, 1978 and Kluijtmans et al, 1996). Most importantly, the enzyme activity measured in the PVD patient population was significantly different from the control group ($P<0.05$). The values obtained under conditions of maximal activity correlated well with the values obtained under conditions of sub-maximal activity, with a correlation coefficient of 0.76 (Figure 10).

On average the enzyme activity was about 6 times higher using the conditions of maximal enzyme activity. This increase in activity is most likely due to an estimated threefold increase in enzyme activity when adding *S*-adenosylmethionine to 1mM, as reported by Kluijtmans et al (1996), as well as an estimated 2.5 fold increase in activity when increasing serine from 5 to 40mM, using the values obtained from the serine titration. The addition of 2mM pyridoxal 5-phosphate and the increase in homocysteine from 10 to 20mM also make lesser contributions to the increase in activity.

Repetition of the experiment under conditions of sub-maximal activity (Figure 9, bottom) however, revealed that CBS activity measured in lysates of fibroblast cultures was not reproducible. The correlation coefficient between the CBS activity measured on the two occasions was <0.5 for both the control and PVD groups (Figure 11), even though the CBS activity in the PVD group was not significantly different (P>0.75) between the two experiments and the range of values nearly identical. The CBS activity in the control group was however significantly lower in the second experiment compared to the first. Most importantly though, CBS activity was not significantly different between the control and PVD groups in contrast to the first experiment.

Therefore, the assay still yields unreproducible results from one assay to the next in spite of all the efforts to standardize it. It would seem that the individual values vary, but the overall trend remains the same, as CBS activity measured in the obligate heterozygote group was still significantly lower than the controls, and homozygotes showed very little activity. The most appropriate way of identifying heterozygotes under these circumstances is to assay for CBS activity in patients and a group of controls on more than one occasion. If the measured enzyme activity is consistently low compared to the control group, it is highly likely that the patient is a heterozygote for CBS deficiency.

Only one patient in the PVD group, patient 1000 truly fulfilled these criteria. Low CBS activity compared to controls was measured in lysates of fibroblasts of this patient on three separate occasion under both conditions of maximal and sub-maximal activity, and this patient consistently showed the lowest CBS activity in the PVD group. It is not surprising to find that patient 1000 consistently showed significantly reduced CBS activity as the patient had the second highest fasting and post methionine-load plasma homocysteine levels in the group of PVD patients. Intriguingly patient 983, who had the highest fasting and post methionine-load plasma homocysteine levels in the group, showed highest CBS activity (6.3U/mg) in the first experiment. The second CBS assay on this patient was much lower (3.3U/mg). Most patients typically showed moderate CBS activity in one or both of the assays. The fact that the CBS activity measured in fibroblasts is not reproducible made it nearly impossible to truly state whether or not some of the subjects are not in fact heterozygous for CBS deficiency. An asymptomatic obligate heterozygote mother (959) of a homocystinuric patient (960) showed variable CBS activity (5.1 and 2.7U/mg) very similar to what was found for patient 983. This patient would have been excluded under the criteria applied to the study group and most of the PVD patients can therefore not with certainty be excluded as possible heterozygotes for CBS deficiency. It is important to note that in a similar study by Boers et al (1985), reduced CBS activity compared to controls was measured in all seven of the hyperhomocysteinaemic patients with peripheral vascular disease. It is possible that the different findings in this study and the study by Boers et al are the result of the strict criteria applied to the patient

population, or that differences are the result of varying incidence of CBS deficiency in two populations of different ethnic origin.

The possibility remains that defects in enzymes other than CBS are the cause of the hyperhomocysteinaemia in these patients. The thermolabile variant of MTHFR causes mild hyperhomocysteinaemia and has been shown to occur with a frequency of ~10% in the Caucasian population (Rosenblatt, 1995), but could not be associated with vascular disease (Brattstrom et al, 1998). Defects in methionine synthase or other disorders of cobalamin metabolism are other possible causes of hyperhomocysteinaemia. These enzyme defects were not assayed for in any way. Nutritional deficiencies of B6, B12 or folate could also have made a contribution to the observed hyperhomocysteinaemia (Robinson et al, 1998), but would not have been reflected in the CBS assay, as it is performed on material isolated from cultured cells, grown in medium that is supplemented with vitamin cofactors. Only genetic defects in the metabolism of the vitamin cofactors would have yielded reduced enzyme activity. It is also possible that some patients may have reduced CBS activity *in vivo*, which is not detected using material obtained from cultured cells, as culture conditions not represent *in vivo* conditions accurately.

The exact source of the variation still remains unclear. Most factors that might influence the activity of the enzyme in the assay procedure itself were investigated and found to have a minor effect. The assay procedure itself was also found to be reproducible both on the same occasion and on separate days. The only aspect that seemed likely to influence the activity to a large extent was the tissue culture conditions. It was initially thought that the confluence of the cultures was the most influential factor on the CBS activity. Experimental evidence also showed that after cultures had reached confluence that the activity remains stable (Figure 6). Even though highly confluent cultures were used in the investigation of the PVD group, the CBS activity was still not reproducible (Figure 11).

It is possible that factors beyond experimental control are responsible for the large inter-experimental variation, and as CBS is mainly expressed in liver (Mudd et al, 1995), the expression of CBS is possibly not as tightly controlled in extra hepatic tissues. CBS activity is detectable in fibroblast cells grown from skin, but not in homogenized skin (Uhlendorf et al, 1968), and is much less in fibroblasts than in homogenized liver. Liver biopsy material would also reflect the *in vivo* status of the patients much more accurately than fibroblasts grown in culture. Fibroblasts however, have to substitute as a less optimal starting material, as liver biopsies are difficult and risky to obtain. Whatever the source of the variation, it makes it very difficult to identify heterozygotes for CBS deficiency, especially when the overlap in the ranges of activities measured in controls and heterozygotes is taken into account.

The association of CBS activity measured in lysates of cultured fibroblasts with the fasting and post methionine load plasma homocysteine levels was also investigated. Scatter plots of fasting and post methionine load plasma homocysteine as well as the change in plasma homocysteine versus CBS activity measured under conditions of maximal and sub-maximal activity are illustrated in Figure 12. A slight negative correlation is noted between the fasting and post methionine-load homocysteine levels and CBS activity under both conditions of maximal activity as well as conditions of sub-maximal activity in the first experiment. This type of correlation is to be expected as subjects with higher CBS activities will process homocysteine more rapidly and reduce its concentration in plasma. No correlation between plasma homocysteine levels and CBS activity is apparent in the second experiment. The reason for the weak correlation between plasma homocysteine levels and CBS activity might be related to the fact that plasma homocysteine levels, measured *in vivo*, is the product of many genetic loci, while CBS activity, measured *in vitro* under controlled tissue culture and assay conditions, is only the product of one genetic locus. Dawson et al (1996) has provided evidence that even in family members with an identical genotype for defects in the CBS coding sequence, the phenotype for plasma homocysteine levels might be different.

Molecular investigations

The aim of the molecular techniques applied in this research was to identify the exact genetic mutations in the CBS coding sequence causing reduced enzyme activity and ultimately hyperhomocysteinaemia. The subjects that were investigated were patient 1000, a likely candidate for CBS deficiency based on plasma homocysteine measurements and CBS activity in cultured fibroblasts, patient 960, suffering from severe homocystinuria and her asymptomatic mother, patient 961.

The initial approach was to directly sequence PCR amplified cDNA that was reverse transcribed from mRNA, isolated from cultured fibroblasts of the patients. Two rounds of PCR using nested primers was necessary to obtain a sufficient quantity of PCR product due to the fact that the CBS gene is expressed at low levels in fibroblast cells (Figure 13). Nested PCR greatly enhances the specificity of PCR as two different sets of primers are used in the successive amplifications. PCR products were gel purified using commercial kits and sequenced using internal primers and dye labeled terminators. This approach proved to be unsuccessful as *Taq* polymerase could introduce single base changes in the PCR product due to a lack of proofreading activity. Sequencing of PCR products also screens both alleles simultaneously, causing point mutations to show up as double peaks or bands. By the nature of the sequencing reaction, numerous double peaks or bands are present, making it virtually impossible to distinguish the true mutations.

These difficulties were overcome by cloning the PCR amplified CBS cDNA. This allows for the sequencing of one allele at a time. The entire coding sequence of the CBS cDNA was amplified and ligated into pGEM-T and used to transform $RbCl_2$ competent *E.coli* (Figure 14). The pGEM-T vector was chosen as it allows for the convenient direct ligation of PCR product into the vector. It contains single overhanging 5' thymidines which are complementary to single 3' adenines on the PCR product created by *Taq* polymerase's terminal transferase activity. $RbCl_2$ treatment was chosen as a method for producing competent *E.coli* as it yields very reproducible transformation although at a lower efficiency than electroporation. High transformation efficiency is not a prerequisite when cloning PCR amplified DNA, as a high number of copies of the gene are present. Also, when screening for mutations, only two alleles need to be found. Twenty insert-containing clones are usually more than sufficient to find more than one copy of the mutant allele. The whole system once standardized worked very effectively.

Sequencing of the clones was performed using fluorescently labeled M13 –40 forward and reverse sequencing primers. This type of approach usually allowed 750bp to be read in the forward and reverse directions, leaving the middle 500bp of each clone unsequenced. These bases were sequenced using internal primers and fluorescently labeled terminators.

Patient 960, an adolescent female who suffered from severe homocystinuria was confirmed to be a compound heterozygote for CBS deficiency. One allele carried the commonly occurring G to A transition at nucleotide 919 resulting in the substitution of glycine for serine at amino acid 307 (G307S). Sequencing confirmed this mutation to be present in one clone as well as the cDNA of the patient (Figure 18). This is the most common mutation in the CBS coding sequence in populations of Celtic origin and is associated with B6 non-responsiveness *in vivo* and complete loss of enzyme activity (Hu et al, 1993). This mutation creates an *AluI* restriction enzyme digest site, which was used to confirm the presence of the mutation in three clones as well as the patient's cDNA (Figure 19). The other allele carried a duplication of the 7 bases between nucleotide 1553 and 1559. This 7 base pair insert results in a reading frame shift, altering the amino acid sequence from position 519, as well as the premature introduction of a stop codon, truncating the protein by 12 amino acids. This drastic change in primary amino acid sequence most likely has a severe impact on the protein structure and function as virtually no enzyme activity could be detected in this patient. This sequence change could also negatively impact on the ability of the protein to form active tetramer. The last 137 amino acids have been illustrated to be important for tetramer formation by trypsin cleavage of the enzyme (Kery et al, 1998). The severe clinical condition of the patient is substantiated by the profound effect that both mutations have on the enzyme activity.

Sequencing revealed that the patient inherited the allele containing the 7bp duplication from her mother. Restriction digest analysis also revealed that the mother did not carry the $G_{919}A$

transition (Figure 19). The mother was completely asymptomatic, had moderate CBS activity in lysates of fibroblast cultures and her plasma homocysteine status is unknown. The moderate activity is probably sufficient to maintain normal plasma homocysteine concentrations and avert the development of disease. The fact that this mutation probably directly impairs the ability of the mutant protein to form tetramer most likely mitigates the dominant negative interaction between mutant and normal subunits, since only tetramer composed of normal subunits will be formed to an appreciable degree. This could possibly explain the presence of a moderate amount of residual enzyme activity in the mother.

Patient 1000 showed definite fasting and post methionine load hyperhomocysteinaemia as well as markedly reduced CBS activity compared to controls on a number of occasions and was therefore a likely heterozygote for deficiency of this enzyme. The same sequencing approach as outlined above was used. An A to G transition was identified at nucleotide 695 in two clones out of seven sequenced clones. No other mutations were consistently found in any of the remaining clones. This mutation leads to the substitution of a histidine for an arginine at amino acid 232 (H232R). The sequencing profile for the normal and the mutant alleles are illustrated in Figure 20. The arginine to histidine substitution, although not changing the polarity of the amino acid side chain, does substitute a long aliphatic chain for a bulky cyclic group. This moderate character change in the amino acid side chain is consistent with the preservation of a fair amount of enzyme activity in this patient.

Hyperhomocysteinaemia combined with reduced CBS activity compared to controls does not in all cases seem to indicate genetic defects in the CBS coding sequence. Kozich et al (1995) measured the activity of the gene products of single CBS alleles purified form an *E.coli* expression system, obtained from patients with occlusive arterial disease, hyperhomocysteinaemia and reduced CBS activity in fibroblasts. They could not find reduced activity in seven of the eight individual alleles from four patients, and direct sequencing of both alleles of one of the patients yielded no pathogenic mutations. Mutations in the untranslated or promoter regions of the CBS gene, which were not sequenced could have contributed to the reduced enzyme activity. Another possibility is that the criteria applied are not strict enough for the correct identification of heterozygotes for CBS deficiency.

Dual metabolic labelling

The metabolism of methionine in cultured fibroblasts was investigated using two radiolabeled isotopes of methionine. L-[^{35}S]-methionine was used to follow methionine through the transsulfuration and remethylation pathways, while L-[methyl-^{3}H]-methionine was used as a marker for methyl transfer reactions. Figure 21 shows the metabolism of methionine with the metabolites that the ^{35}S (yellow) and tritium (blue) labels can be incorporated into. The ^{35}S radiolabel can be incorporated into protein directly as methionine or after being metabolized

through the transsulfuration pathway, as cysteine. The tritium radiolabel, is on the methyl group of methionine and is transferred to a methylated acceptor, including nucleic acid and protein. It can also be incorporated directly into protein as methionine. Changes in the flux through the transsulfuration and remethylation pathways, such as caused by enzyme deficiencies, could therefore affect the ratio of tritium to ^{35}S as well as the absolute amount of each label incorporated into acid precipitable macromolecules as well as acid soluble small molecular components.

Confluent fibroblast cultures were incubated in pre-warmed buffer or tissue culture media containing the isotopes. At various time intervals culture medium was removed, protein and DNA precipitated with 5% trichloracetic acid and the precipitated material released with 0.5M NaOH. The medium, acid soluble pool and acid precipitable pool at each time point were counted in a scintillation counter. A 0 to 200keV window was used to detect tritium (which includes a defined contribution from ^{35}S) and a 400 to 600keV window was used to detect only ^{35}S. The amount of each isotope was calculated for the medium, the TCA soluble fraction containing all the small molecular components and the TCA precipitated macromolecules.

Initially, experiments were performed to investigate the effects of different types of tissue culture media and buffers on the uptake and incorporation of the two labels into acid precipitable material (Figure 22 and 23). Essentially, two types of media were investigated, Medium Z, a balanced salt solution containing 1g/L glucose as a carbon source as well as methionine-free Eagles minimal essential medium (MEM). The methionine-free MEM was supplemented with 1mg/L unlabeled methionine, to avoid its depletion in the medium during the course of the assay as the labels make a very small quantitative contribution to the amount of methionine present in molar terms. Overall, the addition of 1mg/L unlabeled methionine had little effect on the overall uptake and incorporation of either label, and diluted the radiolabeled methionine fifteen-fold less than normal MEM, which contains 15mg/L methionine.

The overall trend in the uptake and incorporation of both labels was very similar whether the incubation was done in Medium Z or methionine-free MEM supplemented with 1mg/L methionine. In the first fifteen minutes there is a sharp increase in the amount of both labels in the acid soluble fraction (Figure 22 and 23, middle panels), accompanied by a similar reduction in both labels in the medium (top panels), illustrating the uptake of the labels into the cells. The amount of both labels subsequently stabilizes in the acid soluble fraction and the medium after fifteen minutes. It is possible that influx simply ceases, although it is difficult to provide a reason therefore, or that a steady state between influx and efflux of both labels is achieved which is more likely. Influx and efflux of the labels could occur as methionine, or the methionine could be metabolized to different compounds, which could then be exported from

the cells. In contrast to this, a linear increase in the amount of both labels incorporated into acid precipitable macromolecules is apparent (bottom panels).

Two important differences where observed. Firstly, the fraction of both labels that entered the acid soluble pool from the medium was about 30 times higher when Medium Z was used (0.20) compared to when methionine free MEM supplemented with 1mg/L methionine was used (0.006). Secondly, the ratio of $^{3}H/^{35}S$ in the acid soluble pool steadily increased when the cells where incubated in methionine free MEM supplemented with 1mg/L methionine, while the amounts of tritium and ^{35}S remained almost identical when the cells where incubated in Medium Z. Most importantly, the fraction of the labels that was incorporated into acid precipitable material was almost identical in both media, regardless of the fact that about thirty times more label was present in the acid soluble pool when the cells were incubated in Medium Z. Methionine-free MEM supplemented with 1mg/L methionine was chosen for subsequent investigations as it is a more complete medium which will be less likely to lead to deficiencies that could alter the normal metabolic flux in the cultured cells.

The dual label assay was subsequently applied to known homozygotes and heterozygotes for CBS deficiency. Fibroblasts from two homocystinuric siblings with identical CBS genotypes (patients 488 and 489) and their obligate heterozygote parents (patients 486 and 487) as well as two control cell lines were compared. Overall, the results of this experiment looked almost identical to the first control experiment with methionine-free MEM supplemented with 1mg/L methionine. The ratio of $^{3}H/^{35}S$ in the medium and acid precipitable pool remained constant over the time course of the experiment and was not different between the homozygotes, heterozygotes and controls. The ratio of $^{3}H/^{35}S$ in the acid soluble pool (Figure 24) showed the same increase over time, but was also not different between the three groups. Neither the ratio of $^{3}H/^{35}S$ in the acid precipitable pool or the acid soluble pool is therefore useful as a marker for the zygosity of the cell line for CBS deficiency, under these conditions.

These investigations, however present only pilot studies on the use of dual metabolic labels in the investigation of methionine metabolism and much further work is needed before a definite indication of the usefulness of this technique in this regard can be ascertained. The metabolites in each pool can be separated chromatographically to gain further insight into the identity of the metabolites carrying the labels. The acid precipitable macromolecules can be hydrolyzed and separated and perhaps other ratios such as the ratio of the amount of ^{35}S incorporated as methionine to the amount of ^{35}S incorporated as cysteine can be used as markers of the amount of flux through the transsulfuration pathway relative to the remethylation pathway.

Conclusions

This study explored the incidence of CBS deficiency in a group of twelve young patients of varied ethnic origin, with peripheral vascular disease that could not be ascribed to any of the conventional risk factors for the development of vascular disease and who were selected for having hyperhomocysteinaemia.

The CBS assay proved to be highly variable despite all efforts to standardize the assay. The source of the variation could not be pinpointed, but seems to be related to varying physiology of the cell cultures over time, as the assay is reproducible when replicates of the same lysates are analyzed, even on different days. All other factors tested, such as varying substrate solutions, the protein assay, or the lysis of the cells appeared to contribute little to the very wide variation in CBS activity over time. Conditions under which maximal CBS activity could be obtained were defined, but proved to be less effective in distinguishing heterozygotes from normal subjects, compared to conditions of sub-maximal activity, with substrate concentrations below their K_m for the enzyme.

Only one out of the twelve patients assayed could truly be identified as a heterozygote for CBS deficiency (patient 1000) by repeatedly having low enzyme activity compared to controls. Under the strict criteria applied to the PVD population, however some of the heterozygotes for CBS deficiency could have been missed, as the obligate heterozygote mother (patient 961) of a homocystinuric patient (patient 960) showed CBS activity very similar to subjects in the PVD group. The hyperhomocysteinaemia measured in the other patients could also be due to defects in methionine synthase or MTHFR, or nutritional deficiencies of B6, B12 or folate which would not have been detected by the CBS assay.

Selected patients were sequenced to determine the exact genetic mutations causing the reduced enzyme activity. An A to G transition at nucleotide 695 leading to histidine to arginine substitution at amino acid 232 was found in one allele of patient 1000. A young homocystinuric female (patient 960) was confirmed to be compound heterozygote for CBS deficiency, with the common Celtic $G_{919}A$ transition on the one allele and a duplication of the 7 bases between position 1553 and 1559 on the other allele, which was of maternal origin.

Dual metabolic labeling of cultured fibroblasts with L-[methyl-^{3}H]-methionine and L-[^{35}S]-methionine was also investigated as a possible method of identifying heterozygotes for CBS deficiency more effectively. The ratio of $^3H/^{35}S$ was however not different in either the medium, the acid soluble pool or acid precipitable pool between controls, heterozygotes and homozygotes for CBS deficiency. These studies represent merely pilot experiments, and much work can still be done to investigate methionine metabolism by development of this approach.

In conclusion, the very wide between-experimental variation observed in the CBS assay was investigated and is most likely the result of changes in enzyme activity during cell culture. This variation suggests that reduced enzyme activity must be measured on several occasions compared to a control group, before a patient can be identified as a heterozygote for CBS deficiency. By applying the assay to a group of twelve PVD patients with hyperhomocysteinaemia, only one patient was shown to have CBS deficiency, which was confirmed on a genetic level.

BIBLIOGRAPHY

Abbott, M.H., S.E. Folstein, H. Abbey and R.E. Pyeritz. 1987. Psychiatric manifestations of homocystinuria due to cystathionine beta-synthase deficiency: prevalence, natural history, and relationship to neurologic impairment and vitamin B6-responsiveness. Am J Med Genet, 4:959-969.

Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith and K. Struhl. 1995. Current Protocols in Molecular Biology. John Wiley and Sons, Inc. New York.

Bao, L., C. Vlcek, V. Paces and J.P. Kraus. 1997. Identification and tissue distribution of human cystathionine beta-synthase mRNA isoforms. Arch Biochem Biophys. 350:95-103.

Barber, G.W. and G.L. Spaeth. 1967. Pyridoxine therapy in homocystinuria. Lancet 1:337.

Baumgarten, I.M. 1984. A comparison of metabolic pathway dynamics in man and other animals. Masters Diploma Thesis, Cape Technikon.

Baumgarten, I.M. and E.H. Harley. 1995. Uptake of purine substrates shows major physiological variations in mammalian skin fibroblasts. Comp Biochem Physiol. 110: 37-46.

Birnboim, H.C. and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucl. Acids Res. 7: 1513-1523.

Boers, G.H., A.G. Smals, F.J. Trijbels, B. Fowler, J.A. Bakkeren, H.C. Schoonderwaldt, W.J. Kleijer and P.W. Kloppenborg. 1985. Heterozygosity for homocystinuria in premature peripheral and cerebral occlusive arterial disease. N Engl J Med. 313:709-715.

Boushey, C.J., S.A. Beresford, G.S. Omenn and A.G. Motulsky. 1995. A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. Probable benefits of increasing folic acid intake. JAMA. 274:1049-1057.

Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254

Brattstrom, L., B. Israelsson, F. Lindegarde and B Hultberg. 1988. Higher total plasma homocysteine in vitamin B12 deficiency than in heterozygosity for homocystinuria due to cystathionine beta-synthase deficiency. Metabolism. 37:175-178.

Brattstrom, L., B. Israelsson, L. Tengborn and B Hultberg. 1989. Homocysteine, factor VII and antithrombin III in subjects with different gene dosage for cystathionine beta-synthase. J Inherit Metab Dis. 12:475-482.

Brattstrom, L., A. Lindgren, B. Israelsson, A. Andersson and B. Hultberg. 1994. Homocysteine and cysteine: determinants of plasma levels in middle-aged and elderly subjects. J Intern Med. 236:633-641.

Brattstrom, L.A., D.E. Wilcken, J. Ohrvik and L. Brudin. 1998. Common methylenetetrahydrofolate reductase gene mutation leads to hyperhomocysteinemia but not vascular disease: the result of a meta-analysis. Circulation. 98:2520-2526.

Celermajer, D.S., K. Sorenson, M. Ryalls, J. Robinson, O. Thomas, J.V. Leonard and J.E. Deanfield. 1993. Impaired endothelial function occurs in the systemic arteries of children with homozygous homocystinuria but not in their heterozygous parents. J Am Coll Cardiol. 22:854-858.

Chassé, J.F. E. Paly, D. Pairs, V. Paul, P.M. Sinet, P. Kamoun and J. London. 1995. Biochem Biophys Res Commun. 211: 826-832.

Chen, J.R. 1977. In situ detection of mycoplasma contamination in cell cultures by fluorescent Hoechst 33258 stain. Experimental Cell Research. 104: 255-256.

Chiang, P.K., R.K. Gordon, J. Tal, G.C. Zeng, B.P. Doctor, K. Pardharadhi and P.P. McCann. 1996. S-adenosylmethionine and methylation. FASEB J. 10: 471-480.

Chomczynski, P. and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162: 156-159.

Clarke, R., L. Daly, K. Robinson, E. Naughton, S. Cahalane, B. Fowler and I. Graham. 1991. Hyperhomocysteinemia: an independent risk facto for vascular disease. N Engl J Med. 324: 1149-1155.

Dawson, P.A., D.A.E. Cochran, B.T. Emmerson, J.P. Kraus, N.P.B. Dudman and R.B. Gordon. 1996. Variable hyperhomocysteinaemia phenotype in heterozygotes for the Gly307Ser mutation in cystathionine β-synthase. Aust NZ J Med. 26:180-185.

Den Heijer, M., T.D. Koster, H.J. Blom, G.M.J. Bos, E. Briët, P.H. Rietsma, J.P. Vandenbroucke and F.R. Rosendaal. 1996. Hyperhomocysteinemia as a risk factor for deep-vein thrombosis. N Engl j Med. 334:759-762.

Dietz H.C., J.M. Saraiva, R.E. Pyeritz, G.R. Cutting and C.A. Francomano. 1992. Clustering of fibrillin (FBN1) missense mutations in Marfan syndrome patients at cysteine residues in EGF-like domains. Hum Mutat. 1:366-374.

Di Minno, G., G. Davi, M. Margaglione, F. Cirillo, E. Grandone, G. Ciabattoni, I. Catalano, P. Strisciuglio, G. Andria, C. Patrono, et al. 1993. Abnormally high thromboxane biosynthesis in homozygous homocystinuria. Evidence for platelet involvement and probucol-sensitive mechanism. J. Clin. Invest. 92:1400-1406.

Dudman, N.P., C. Hicks, J. Wang and D.E. Wilcken. 1991. Human arterial endothelial cell detachment in vitro: its promotion by homocysteine and cysteine. Atherosclerosis. 91:77-83.

Finkelstein, J.D., and J.J. Martin. 1984. Methionine metabolism in mammals. Distribution of homocysteine between competing pathways. J.B.C. 259: 9508-9513.

Finkelstein, J.D., and J.J. Martin. 1986. Methionine metabolism in mammals. Adaption to methionine excess.. J.B.C. 261: 1582-1587.

Finkelstein, J.D. 1990. Methionine metabolism in mamals. J Nutr Biochem. 1:228-237.

Fleisher, L.D., H.H. Tallan, N.G. Beratis, K. Hirschhorn and G.E. Gaull. 1973. Cystathionine beta-synthase deficiency: heterozygote detection using skin fibroblasts. Biochem. Biophys. Res. Com. 55: 38-44.

Fowler, B., I.B. Sardharwalla and A.J. Robbins. 1971. The detection of heterozygotes for homocystinuria by oral loading with L-methionine. Biochem J. 122:23-24.

Fowler, B., Kraus, J., Packman, S. and Rosenberg, L.E. 1978. Homocystinuria: Evidence for three distinct subclasses of cystathionine β-synthase mutants in cultured fibroblasts. J. Clin. Inv. 61: 645-653.

Fowler, B. Disorders of homocysteine metabolism. 1997. J Inher Metab Dis. 20:270-285.

Franco, R.F., J. Elion, J. Lavinha, R. Krihnamoorthy, M.H. Tavella and M.A. Zago. 1998. Heterogeneous ethnic distribution of the 844ins68 in the cystathionine beta-synthase gene. Hum Hered. 48:338-342.

Freshney, R.I. 1994. Culture of animal cells: a manual of basic technique. John Wiley and Sons, Inc. New York.

Gallagher, P.M., P. Ward, S. Tan, E. Naughten, J.P. Kraus, G.C. Sellar, D.J. McConnell, I. Graham and A.S. Whitehead. 1995. High frequency (71%) of cystathionine beta-synthase mutation G307S in Irish homocystinuria patients. Hum. Mutat. 6:177-180

Gerritsen, T., J.G. Vaughn and H.A. Waisman. 1962. The identification of homocystine in the urine. Biochem Biophys Res Commun. 9: 493-496.

Giannini, M.J., M. Coleman and I. Innerfield. 1975. Letter: Antithrombin activity in homocystinuria. Lancet 1:1094

Gibson, J.B., N.A.J. Carson and D.W. Neil. 1964. Pathalogical findings in homocystinuria. J Clin Path. 17:427-437.

Goldstein, J.L., B.K. Campbell and S.M. Gartler. 1972. Cystathionine synthase activity in lymphocytes: induction by phytohemagglutinin. J Clin Invest. 51:1034-1037.

Goyette, P. J.S. Sumner, R. Milos, et al. 1994. Human methylenetetrahydrofolate reductase: isolation of cDNA, mapping and mutation identification. Nat Genet. 7:1955-200.

Graeber, J.E., J.H. Sltt, R.E. Ulane, J.D. Schulman and M.J. Stuart. 1982. Effect of homocystine on platelet and vascular arachidonic acid metabolism. Pediatr Res. 16:490-493.

Graham, I.M., L.E. Daly, H.M. Refsum, et al. 1997. Plasma homocysteine as a risk factor for vascular disease. The European Concerted Action Project. JAMA. 277:1775-1781.

Harker, L.A., S.J. Slichter, C.R. Scott and R. Ross. 1974. Homocystinemia. Vascular injury and arterial thromobosis. N Engl L Med. 291:538-54.

Harker, L.A., R. Ross, S.J. Slichter and C.R. Scott. 1976. Homocystine-induced arteriosclerosis. The role of endothelial cell injury and platelet response in its genesis. J Clin Invest. 58:731-741.

Hayashi, T., G. Honda and K. Suzuki. 1992. An atherogenic stimulus homocysteine inhibits cofactor activity of thrombomodulin and enhances thrombomodulin expression in human umbilical vein endothelial cells. Blood. 79:2930-2936.

Hu, F.L, Z. Gu, V. Kozich, J.P. Kraus, V. Ramesh and V.E. Shih. 1993. Molecular basis of cystathionine beta-synthase deficiency in pyridoxine responsive and nonresponsive homocystinuria. Hum Mol Genet. 2:1857-1860.

Kang A.H. and R.L. Trelstad 1973. A collagen defect in homocystinuria. J Clin Invest. 10:2571-2578

Kang S.S., P.W.K. Wong and M. Norusis. 1987. Homocysteinemia due to folate deficiency. Metabolism. 36:458-462.

Kang S.S., J. Zhou, P.W.K. Wong, J. Kowalisyn and G. Strokosch. 1988. Intermediate homocysteinemia: a thermolabile variant of methylenetetrahydrofolate reductase. Am J Hum Genet. 43:414-421.

Kang S.S., P.W.K. Wong and M.R. Malinow. 1992. Hyperhomocyst(e)inemia as a risk factor for occlusive vascular disease. Annu Rev Nutr. 12:279-298.

Kery, V., G. Bukovska and J.P. Kraus 1994. Transsulfuration depends on heme in addition to pyridoxal 5'-phosphate. Cystathionine beta-synthase is a heme protein. J Biol Chem. 269:25283-25288.

Kery, V., L. Poneleit and J.P. Kraus. Trypsin cleavage of human cystathionine beta-synthase into an evolutionarily conserved active core: structural and functional consequences. 1998. Arch Biochem Biophys. 355:222-232.

Kluijtmans, L.A.J., G.H.J. Boers, E.M.B. Stevens, W.O. Renier, J.P. Kraus, F.J.M. Trijbels, L.P.W.J. van den Heuvel and H.J. Blom. 1996. Defective cystathionine beta-synthase regulation by S-adenosylmethionine in a partially pyridoxine responsive homocystinuria patient. J Clin Invest. 98: 285-289.

Kluijtmans, L.A., G.H. Boers, F.J. Trijbels, H.M. Lith-Zanders, L.P. van den Heuvel and H.J. Blom. 1997. A common 844INS68 insertion variant in the cystathionine beta-synthase gene. Biochem Mol Med. 62:23-25.

Kozich, V. and J.P. Kraus. 1992. Screening for mutations by expressing patient cDNA segments in E. coli: homocystinuria due to cystathionine beta-synthase deficiency. Hum Mutat. 1:113-123.

Kozich, V., E. Kraus, R. de Franchis, B. Fowler, G.H.J. Boers, I Graham and J.P. Kraus. 1995. Hyperhomocysteinaemia in premature arterial disease: examination of cystathionine β-synthase alleles at the molecular level. Hum Mol Genet. 4:623-629.

Kraft,R., K.S. Krauter and L.A. Leinwald. 1988. Using mini-prep plasmid DNA for sequencing double stranded templates with Sequenase. BioTechniques. 6: 544-547.

Kraus, J.P., S. Packham, B. Fowler and L.E. Rosenberg. 1978. Purification and properties of cystathionine β-synthase from human liver. Evidence for identical subunits. J Biol Chem. 253:6523-6528.

Kraus, J.P. 1987. Cystathionine beta-synthase (human). Methods of Enzymology. 143:388-394.

Kraus J.P., K. Le, M. Swaroop, T. Ohura, T. Tahara, L.E. Rosenberg, M.D. Roper and V. Kozich. 1993. Human cystathionine beta-synthase cDNA: sequence, alternative splicing and expression in cultured cells. Hum Mol Genet. 2:1633-1638.

Kraus, J.P. 1994. Molecular basis of phenotype expression in homocystinuria. J. Inher Metab. Dis. 17:383-390.

Kraus, J.P., J. Oliveriusova, J. Sokolova, E. Kraus, C. Vlcek, R. de Franchis, K.N. Maclean, L. Bao, Bukovsk, D. Patterson, V. Paces, W. Ansorge and V. Kozich. 1998. The human cystathionine beta-synthase (CBS) gene: complete sequence, alternative splicing, and polymorphisms. Genomics. 52:312-324.

Leclerc, D., E Campeau, P. Goyette, C.E. Adjalla, B. Christensen, M. Ross, P. Eydoux, D.S. Rosenblatt, R. Rozen and R.A. Gravel. 1996. Human methionine synthase: cDNA cloning and identification of mutations in patients of the cblG complementation group of folate/cobalamin disorders. Hum Mol Genet. 5:1867-1874.

Lentz, S.R., C.G. Sobey, D.J. Piegors, M.Y. Bhopatkar, F.M. Faraci, M.R. Malinow and D.D. Heistad. 1996. Vascular dysfunction in monkeys with diet-induced hyperhomocyst(e)inemia. J Clin Invest. 98:24-29.

Lentz, S.R. and J.E. Sadler. 1991. Inhibition of thrombomodulin surface expression and protein C activation by the thrombogenic agent homocysteine. J Clin Invest. 88:1906-1914.

Lowenstein, J.M. ed. 1969. Methods in Enzymology. 13: 3-26.

Mackall, J., M. Meredith and M.D. Lane. 1979. A mild procedure for the rapid release of cytoplasmic enzymes from cultured animal cells. Anal. Biochem. 95: 270-274.

Mandel, H., B. Brenner, M. Berant, N. Rosenberg, N. Lamir, C. Jacobs, B. Fowler and U. Seligsohn. 1996. Coexistance of hereditary homocystinuria and factor V Leiden – effect on thrombosis. N Engl J Med. 334:763-768.

McCully, K.S. 1969. Vascular pathology of homocysteinaemia: implications for the pathogenesis of arteriosclerosis. Am J Pathol. 56:111-128.

McDonald, L., C. Bray, C. Field, F. Love and B. Davies. 1964. Homocystinuria, thrombosis and the blood-platelets. Lancet. 1:745-746.

McGill, J.J., G. Mettler, D.S. Rosenblatt and C.R. Scriver. 1990. Detection of heterozygotes for recessive alleles. Homocyst(e)inemia: Paradigm of pitfalls in phenotypes. Am. J. Med. Genet. 36: 45-52

Miller, J.W., J.D. Ribaya-Mercado, R.M. Russell, D.C. Shepard, F.C. Morrow, E.F. Cochary, J.A. Sadowski, S.N. Gershoff and J. Selhub. 1992. Effect of vitamin B6 deficiency on fasting plasma homocysteine concentrations. Am J Clin Nutr. 55:1154-1160.

Mudd, S.H., J.D. Finkestein, F. Irreverre and L. Laster. 1965. Transulfuration in mammals. Microassays and tissue ditributions of three enzymes of the pathway. JBC. 240: 4382-4392.

Mudd, S.H., W.A. Edwards, P.M. Loeb, M.S. Brown and L. Laster. 1970. Homocystinuria due to cystathionine synthase deficiency: the effect of pyridoxine. J Clin Invest. 49: 1762-1772.

Mudd, S.H., B.W. Uhlendorf, J.M Freeman, J.D. Finkelstein and V.E. Shih. 1972. Homocystinuria associated with decreased methylenetetrahydrofolate reductase activity. Biochem Biophys Res Commun. 46:413-425.

Mudd S.H, F. Skovby, H.L. Levy, K.D. Pettigrew, B. Wilcken, R.E. Pyeritz, G. Andria, G.H. Boers, I.L. Bromberg, R. Cerone, B. Fowler, H. Gröbe, H. Schmidt and L. Schweitzer. 1985. The natural history of homocystinuria due to cystathionine beta-synthase deficiency. Am J Hum Genet. 1: 1-31

Mudd, S.H., H.L. Levy and F. Skovby. 1995. Disorders of transsulfuration. In Scriver, C.R., A.L. Beaudet, W.S. Sly and D. Valle, eds. The metabolic and molecular basis of inherited disease, 7th ed., McGraw-Hill, New York. 1279-1327.

Münke, M. J.P. Kraus, T. Ohura and U. Francke. 1988. The gene for cystathionine beta-synthase (CBS) maps to the subtelomeric region on human chromosome 21q and to proximal mouse chromosome 17. Am J Hum Genet. 42:550-559.

Nygard, O., H. Refsum, P.M. Ueland and S.E. Vollset. 1998. Major lifestyle determinants of plasma total homocysteine distribution: the Hordaland Homocysteine Study. Am J Clin Nutr. 67:2637-270.

Palareti, G. and S. Coccheri. 1989. Lowered antithrombin III activity and other clotting changes in homocystinuria: effects of a pyridoxine-folate regimen. Haemostasis. 19(S1):24-28.

Pesce, A., R.H. McKay, F. Stolzenbach, R.D. Cahn and N.O. Kaplan. 1964. The comparative enzymology of lactic dehydrogenase. J.B.C. 239: 1753-1761.

Robinson, K., K. Arheart, H. Refsum, L Brattstrom, G. Boers, P. Ueland, P. Rubba, R. Palma-Reis, R. Meleady, L. Daly, J. Witteman and I. Graham. 1998. Low circulating folate and vitamin B6 concentrations: risk factors for stroke, peripheral vascular disease, and coronary artery disease. European COMAC Group. Circulation. 97:437-443.

Rodgers G.M. and M.T. Conn. 1990. Homocysteine, an atherogenic stimulus, reduces protein C activation by arterial and venous endothelial cells. Blood 75:895-901.

Rodgers G.M. and W.H. Kane. 1986. Activation of endogenous factor V by a homocysteine-induced vascular endothelial cell activator. J Clin Invest. 77:1909-1916.

Roper, M.D. and J.P. Kraus. 1992. Rat cystathionine beta-synthase: expression of four alternatively spliced isoforms in transfected cultured cells. Arch Biochem Biophys. 298:514-521.

Rosenblatt, D.S. 1995. Inherited disorders of folate transport and metabolism. In Scriver, C.R., A.L. Beaudet, W.S. Sly and D. Valle, eds. The metabolic and molecular basis of inherited disease, 7th ed., McGraw-Hill, New York. 3111-3128.

Rubba, P., F. Faccenda, P. Pauciullo, L. Carbone, M. Mancini, P. Striciuglio, R. Carrozzo, R. Sartorio, E. del Giudice and G. Andria. 1990. Early signs of vascular disease in homocystinuria: a noninvasisve study by ultrasound methods in eight families with cystathionine beta-synthase deficiency. Metabolism. 39:1191-1195.

Sanger, F., S. Niklen and A.R. Coulsen. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Nat. Acad. Sci. 74: 5463-5467.

Schimke, R.N., V.A. McKusick, T. Huang and A.D. Pollack. 1965. Homocystinuria. Studies of 20 families with 38 affected members. JAMA. 193:711-719.

Sebastio, G., M.P. Sperandeo, M. Panico, R. de Franchis, J.P. Kraus and G. Andria. 1995. The molecular basis of homocystinuria due to cystathionine beta-synthase deficiency in Italian families, and report of four novel mutations. Am J Hum Genet. 56:1324-1333.

Selhub, J. and J.W. Miller. 1992. The pathogenesis of homocystinuria: interruption of the coordinate regulation by *S*-adenoslymethionine of the remethylation and transsulfuration of homocysteine.

Selhub, J., P.F. Jacques, P.W. Wilson, D. Rush and I.H. Rosenberg. 1993. Vitamin status and intake as primary determinants of homocysteinemia in an elderly population. JAMA. 270:2693-2698.

Shih, V.E., J.M. Fringer, R. Mandell, J.P. Kraus, G.T. Berry, R.A. Heidenreich, M.S. Korson, H.L. Levy and V. Ramesh. 1995. A missense mutation (I278T) in the cystathtionine beta-synthase gene prevalent in pyridoxine-responsive homocystinuria and associated with mild clinical phenotype. Am J Hum Genet. 57:34-39.

Skovby, F., J.P. Kraus and L.E. Rosenberg 1984 Biosynthesis of cystathionine beta-synthase in cultured human fibroblasts. J Biol Chem. 259:583-587.

Skovby, F., J.P. Kraus and L.E. Rosenberg 1984 Biosynthesis and proteolytic activation of cystathionine beta-synthase in rat liver. J Biol Chem. 259:588-593.

Stabler, S.P., P.D. Marcell, E.R. Podell, R.H. Allen, D.G. Savage and J. Lindebaum. 1988. Elevation of total homocysteine in the serum of patients with cobalamin or folate deficiency detected by capillary gas chromatography-mass spectrometry. J Clin Invest. 81:466-474.

Starkebaum, G. and J.M. Harlan. 1986. Endothelial cell injury due to copper-catalyzed hydrogen peroxide generation by homocysteine. J Clin Invest. 77:1370-1376.

Stampfer, M.J., M.R. Malinow, W.C. Willet, L.M. Newcomer, B. Upson, D. Ullmann, P.V. Tishler and C.H. Hennekens. 1992. A prospective study of plasma homocyst(e)ine and risk of myocardial infarction in US physicians. JAMA. 268:877-881.

Svardal, A.M., R. Djurhuus, H. Refsum, P.M. Ueland. 1986. Disposition of homocysteine in rat hepatocytes and in nontransformed and malignant mouse embryo fibroblasts following exposure to inhibitors of *S*-adenosylhomocysteine catabolism. Cancer Res 10:5095-5100

Swaroop M., K. Bradley, T. Ohura, T. Tahara, M.D. Roper, L.E. Rosenberg and J.P. Kraus. 1992. Rat cystathionine beta-synthase. Gene organization and alternative splicing. J Biol Chem. 267:11455-11461.

Tsai, J.C., M.A. Perrella, M. Yoshizumi, C.M Hsieh, E. Haber, R. Schlegel and M.E. Lee. 1994. Promotion of vascular smooth muscle cell growth by homocysteine: a link to atherosclerosis. Proc Natl Acad Sci USA. 91:6369-6373.

Tsai, J.C. M. Bignell, K. Schwichtenberg and N.Q. Hanson. 1996. High prevalence of a mutation in the cystathionine beta-synthase gene. Am J Hum Genet. 59:1262-1267.

Ubbink, J.B., W.J. Vermaak, A. van der Merwe and P.J. Becker. 1993. Vitamin B-12, vitamin B-6, and folate nutritional status in men with hyperhomocysteinemia. Am J Clin Nutr. 57:47-53.

Ubbink, J.B., A. van der Merwe, A. Delport, R.H. Allen, S.P. Stabler and W.J. Vermaak. 1996. The effect of subnormal vitamin B-6 status on homocysteine metabolism. J Clin Invest. 98:177-184.

Ubbink, J.B. 1997. The role of vitatmins in the pathogenesis and treatment of hyperhomocyst(e)inaemia. J Inherit Metab Dis. 20:316-325.

Ueland, P.M., H. Refsum, S.P. Stabler, M.R. Malinow, A. Andersson and R.H. Allen. 1993. Total homocysteine in plasma or serum: methods and clinical applications. Clin Chem. 39:1764-1779.

Uhlendorf, B.W. and S.H. Mudd. 1968. Cystathionine synthase in tissue culture derived from human skin: enzyme defect in homocystinuria. Science. 160:1007-1009.

Uhlemann, E.R., J.H. TenPas, A.W. Lucky, J.D. Schulmann, S.H. Mudd and N.R. Schulman. 1976. Platelet survival and morphology in homocystinuria due to cystathionine beta-synthase deficiency. N Engl J Med. 295;1283-1286.

Upchurch, G.R. Jr, G.N. Welch, A.J. Fabian, J.E. Freedman J.L. Johnson, J.F. Keaney Jr and J. Loscazo. 1997. Homocyst(e)ine decrease bioavailable nitric oxide by a mechanism involving glutathione peroxidase. J Biol Chem. 272:17012-17017.

Verhoef, P., M.J. Stampfer, J.E. Buring, J.M. Gaziano, R.H. Allen, S.P. Stabler, R.D. Reynolds, F.J. Kok, C.H. Hennekens and W.C. Willett. 1996. Homocysteine metabolism and risk of myocardial infarction: relation with vitamins B6, B12, and folate. Am J Epidemiol. 143:845-859.

Wall, R.T., J.M. Harlan, L.A. Harker and G.E. Striker. 1980 Homocysteine-induced endothelial cell injury in vitro: a model for the srudy of vascular injury. Thromb. Res. 18: 113-121.

Wilcken, D.E.L. and B. Wilcken. 1976. The pathogenesis of coronary artery disease. A possibel role for methionine metabolism. J Clin Invest. 57:1079-1082.

Wilson, A. D. Leclerc, F Saberi, E Campeau, H.Y. Hwang, B. Shane, J.A. Phillips, D.S. Rosenblatt and R.A. Gravel. 1998. Functionally null mutations in patients with the cblG-variant form of methionine synthase deficiency. Am J Hum Genet. 63:409-414.

Woo, K.S., P. Chook, Y.I. Lolin, A.S. Cheung, L.T. Chan, Y.Y. Sun, J.E. Sanderson, C. Metreweli and D.S. Celermajer. 1997. Hyperhomocyst(e)inaemia is a risk factor for arterial endothelial dysfunction in humans. Circulation. 96:2542-2544.

Zhou, J., S.S. Kang, P.W. Wong, B. Fournier and R. Rozen. 1990. Purification and characterization of methylenetetrahydrofolate reductase from human cadaver liver. Biochem Med Metab Biol. 43:234-242.