Purification of pig brain methionine synthase and its control and regulation by nitric oxide

By

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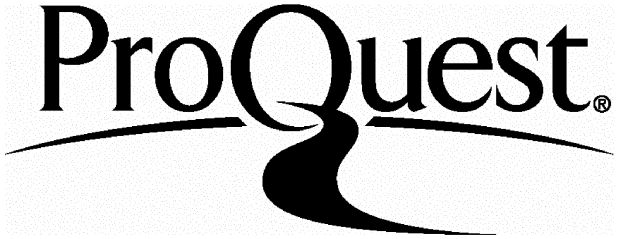

Abstract

The vitamin B_{12} network links the folate and sulphur amino acid pathways with, the synthesis of polyamines and lipid biosynthesis. The role that this vitamin plays within human disease has been linked to a variety of haematological, developmental, neurological and pathological conditions such as cancer and heart disease.

The role of nitric oxide within the body has also been shown to be extensive. The physiological effects range from cardiovascular function, peripheral nervous system, central nervous system, and the immune system. The pathophysiological actions include, septic shock, reperfusion injury, arteriosclerosis, hypertension, and neurotoxicity.

In order to better study and identify neurological disorders linked to B_{12}, the mammalian B_{12} dependent methionine synthase enzyme was purified. The enzyme appears under native conditions to be 155kd in weight composed of several subunits (94 and 36 Kd). The pI for the enzyme was determined to be 4.3 and 5.1.

Concurrent with the purification work, studies were done to determine some of the biological effects of NO. NO inhibits B_{12} MS with an IC50 of 3 μM. A comparison between the effects of NO and N_20 on B_{12} MS inhibition both of which inhibit B_{12} MS in a dose dependent manner and irreversibly. To contrast these studies a comparison of the effects of NO and N_20 on cGMP synthesis. NO stimulates the production of cGMP while NO was found to have no significant effect.

The production of NO was also studied in vivo and in vitro to determine the effect of changes in oxygen partial pressures. NO synthesis in vivo was measured using the production of nitrate and nitrite in plasma and measured by capillary electrophoresis. When human volunteers were exposed to 2.8 bar levels of nitrate increased 4.2 times above background.

Acknowledgements

A wise man once said a journey of a thousands miles begins with but one step, a thesis of 42,686 words begins with a title page, an abstract and many thanks to an awful lot of people.

Firstly I must thank my family for helping through all the pain of writing up a thesis while working full time and still maintaining a family and social life.
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Last but not least I would like to thank my mother and father for all the support they have given me over the years. I hope that one day I will be able to say thank you properly.
This thesis is dedicated to my family: my sons Jack 4 and Harry one and my loving wife whom I could not live without and who has put up with my many tantrums. She is my sunrise and my sunset, the mother of my children and most of all my best friend.

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Glossary

AdoCbl	adenosylcobalamin
AdoHcy	adenosylhomocysteine
AdoMet	adenosylmethionine
AMPA	alpha-amino-3-hydroxy-5-methyl-4 isoxazole proprionic acid
ANF	Atrial naturetic peptide
AqCbl	aquacobalamin
BSA	bovine serum albumin
CNCbl	cyanocobalamin
CSF	cerebro spinal fluid
CVD	cardio vascular disease
Cys	cystathionine
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
DEAE	diethylaminoethyl
DHFR	dihydrofolate reductase
DPM	disintegrations per minute
dcAdoMet	decarboxylated adenosyl methionine
dcSAM	decarboxylated s-adenosyl methionine
dTMP	deoxythymidylate
dUMP	deoxyuridylate
EDRF	endothelial derived relaxation factor
EDTA	ethylenediaminetetraacetic acid
ESR	electron spin resonance
FDA	federal drug administration
FPLC	fast protein liquid chromatograohy
fsw	feet sea water
GABA	gamma amino butyric acid
Glu	glutamic acid
GMP	guanosine monophosphate
GTP	guanosine triphosphate

HCys	homocysteine
HPLC	high pressure liquid chromatography
HTP	hydroxy apatite
IEF	Iso-electric focusing
LTP	long term potentiation
MMA	monometyl arginine
MMCoA	metyl malonyl CoA
MTA	methyl thioadenosine
mTHF	methyl tetrahydrofolate
NADPH	nicotinamide adenine dinucleotide
NMDA	n-methyl-d-aspartate
NO	nitric oxide
NOS	nitric oxide synthase
NSAID's	non-steroidal anti inflammatory drugs
ODC	ornithine decarboxylase
ORF	open reading frame
PMSF	phenylmethylsulphonyl flouride
PNS	peripheral nervous system
RBC	red blood cells
RIA	radio immuno assay
ROS	reactive oxygen species
rRNA	ribosomal ribonucleic acid
SAH	s-adenosyl homocyteine
SDS	sodium dedecyl sulphate
SNP	sodium nitro prusside
TC	transcobalamin
TCA	tricarboxylic acid cycle
TEMED	N,N.N,N-tetramethylene diamine
TLCK	N-P-tosyl-L-lysine chloromethyl ketone
tRNA	transfer ribonucleic acid

Chapter 1 INTRODUCTION

1.1 History of Vitamin B_{12}: General remarks on nutrition and disease.

Normal human development is dependent on a good diet, the essential macrocytic components of which are proteins, carbohydrates and fats. Included in this diet are a variety of macrocytic vitamins and minerals that are important components and take part in many chemical reactions in the body (Stadman, 1971).

Human nutrition and our knowledge of the essential vitamins and minerals came about from the study of pathological conditions associated with poor diet. Sailors would suffer from scurvy, which was finally associated with lack of fresh fruit and vegetables in their diet. The study of nutrition and vitamins has therefore gone hand in hand with the study of the diseases associated with them. The study of vitamin B_{12} began with a study of disease and an attempt to purify the essential factor required preventing the disorders associated with it.

As early as 1926 raw liver was being prescribed in the treatment of pernicious anaemia (Minot and Murphy, 1926), a megaloblastic anaemia associated with neurological deterioration. Later it was proposed that a combination of an extrinsic factor from the liver and an intrinsic factor from the gut were required (Castle 1929). Eventually a red crystalline compound was purified by Merck in 1948 (Rickes 1948), which turned out to be vitamin B_{12}. It was however not until 1955 that Dorothy Hodgkin et al (1956) proposed a structure for vitamin B_{12}. The initial difficulties in purification of vitamin B_{12} lay in the fact that it was active in low concentrations and only the liver contained high levels of storage. It was only by beginning with 1000kg of raw liver that the scientists at Merck were able to purify 15mg of crystalline B_{12}.

The term vitamin B_{12} is used to describe cyano-cobalamin and a group of chemically related compounds, the cobalamins. (fig 1.1) All the cobalamins have the same basic structure of a corrin ring surrounding a cobalt atom. The ring has a nucleotide (5,6-dimethyl-benzimidazole) attached to a sugar (ribose-3-phosphate) set at right angles to the ring. The cobalt atom is coordinated by four nitrogen atoms of the corrin ring. The ring itself is similar in structure to the porphyrins. The cobalt ion can be found in different oxidation states. Those

most commonly found in nature are cyano-cobalamin, methylcobalamin, deoxyadenosyl cobalamin, and hydroxycobalamin. Cyano and hydroxy cobalamins are stable oxidised compounds with the cobalt atom in the Co(III) state (Cob(III)alamins) while the methyl and the deoxyadenosyl are reduced to the Cob(I)alamin. Methylcobalamin can be oxidised by light to the hydroxy form. The two active forms of vitamin B_{12} found in man are methylcobalamin, which is an essential cofactor in the conversion of homocysteine to methionine by the enzyme methionine synthase and deoxyadenosylcobalamin, the essential cofactor for the enzyme methylmalonyl CoA mutase which catalyses the isomerisation of methylmalonyl CoA. Other forms of B_{12} are used in treatment of vitamin B_{12} deficiencies (hydroxycobalamin) and in measuring B_{12} absorption using what is know as the Schilling test (radioactive 57 or 58 Co cyano-cobalamin). The different oxidation states of the cobalamins can be differentiated by their absorption spectra (Banerjee and Matthews 1990).

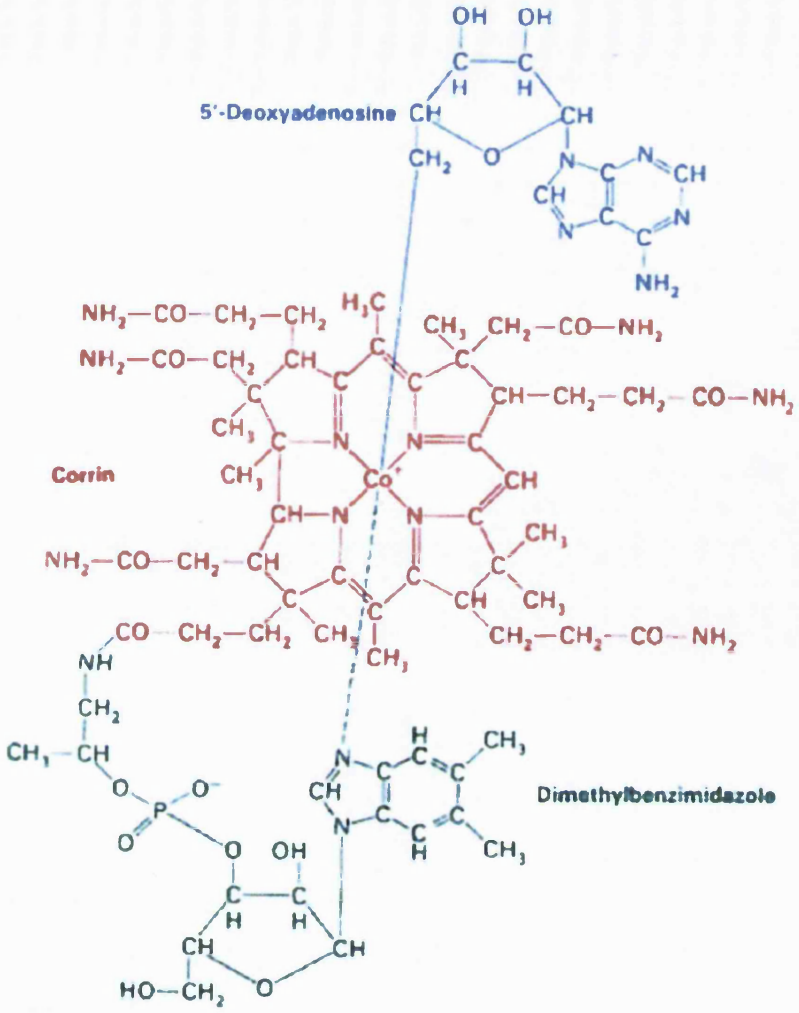


Figure 1.1The structure of coenzyme B12 as the octahedral 5'-deoxyadenosine form.

The central cobalt has six ligands, 4 from the nitrogens of the corrin ring (red),1 axial from the dimethylbenzimidazole group (group), with 5' deoxyadenosine group (blue)as the second axial ligand. The blue ligand can be replaced by others including; CN; OH; CH3; etc, and the green ligand by a group (histidine) from the enzyme. Other B12 forms having only 4 or 5 ligands exist.

Digestion of B_{12} is thought to follow the outline in figure 1.2. In foods vitamin B_{12} usually occurs bound to protein. In order to be absorbed it must first be separated from the protein by acid hydrolysis. Once separated it then binds to gut intrinsic factor (IF) secreted by the parietal cells of the stomach lining, or what are termed 'R' proteins which are collectively known as cobalophilins or haptocorrins (HC). The HC's are found in most body fluids including saliva. The free cobalamin binds to the HC in the stomach and travels into the intestine by

a specific transport system. The HC is eventually hydrolysed by the proteolytic enzymes from the pancreas again freeing B_{12}. IF is able to survive the proteolytic action of the pancreas and binds to the B_{12} in the proximal intestine. Eventually the B_{12}-IF complex is absorbed at the B_{12} receptor sited in the ileum. (Nexo et al, 1994). Once absorbed across the distal ileum the bound cobalamin is transferred to one of three proteins, but mainly transcobalamin II. Transcobalamin II is responsible for transport of vitamin B_{12} around the body. Serum B_{12} levels is considered deficient below 100pg/ml. Approximately 0.1% of the circulating B_{12} is lost per day. This loss is due to faecal excretion and not due to biodegradation of the cofactor. In fact the cobalamins act as a circulating store of B_{12} as well as those stored in the tissues. The recommended daily allowance of B_{12} in the diet is based on 3ug/day in 1980. Care must be taken in dietary supplementation that no interference occurs with other vitamin supplements. It has been shown that 500mg of vitamin C can interfere with the absorption of B_{12}.

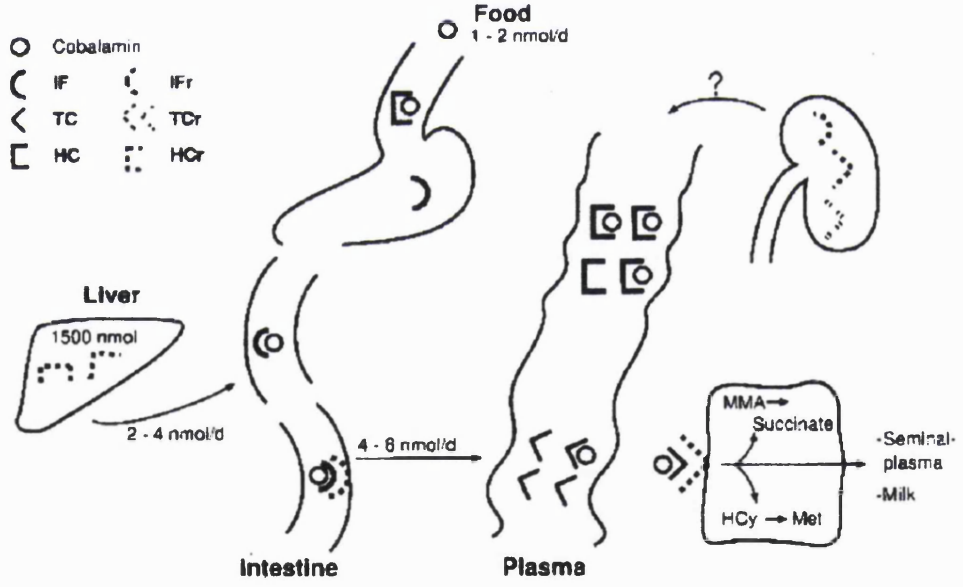


Figure 1.2 Absorption and distribution of cobalamins in mammals.
Cobalamins from the food and bile are transferred from haptocorrin to intrinsic factor which is then absorbed in the intestine where it is complexed with trans cobalamin. Trans cobalamin is metabolised with T1/2= 0.75 days and most cobalamin is transferred to the liver and kidney. Plasma cobalamin mostly exists bound to HC and has T1/2 = to nine days and is delivered mainly to the liver. Cobalamins are transported to other cells as needed and occur in milk seminal fluid and the foetus.

Vitamin B_{12} is almost completely absent from the plant kingdom. Reports of B_{12} in plants have been attributed in most cases to contamination of the samples tested. Studies have shown that a pure vegetarian diet will lead to B_{12} deficiencies (Chanarin 1990). This condition was almost unheard of in the UK until the influx of Hindu immigrants in the early 1960's. As strict vegetarians they have an extremely low source of B_{12} in their diet. The Hindu population is strictly vegetarian and has a low intake of dairy products, which are an alternative source of B_{12}.

As discussed, one cause of this may be due to lack of dietary B_{12}. Other mechanisms may cause lack of available B_{12} including and dysfunction in the mechanism by which B_{12} is absorbed or metabolised in the body and can lead to reduction in available concentrations. Pemicious anaemia is a disease that is responsible for reduced levels of B_{12} in the body caused by lack of intrinsic factor released by the gut. In order to counteract this lack of intrinsic factor regular intravenous injections of B_{12} must be taken by the patient.

Vitamin B_{12} related disorders are many and can be grouped as shown in fig 1.3. (Metz, 1993)

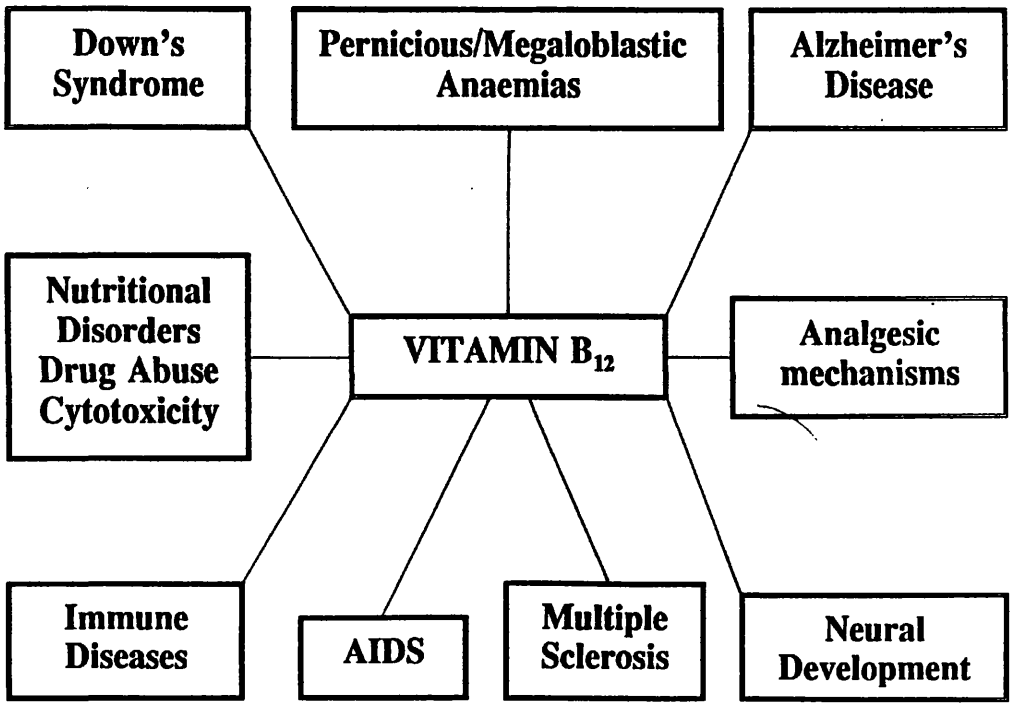


Figure 1.3 Diseases found to be associated with B12 dependent processes and their metabolites.

Megaloblastic anaemia was one of the most easily diagnosed symptoms of B_{12} deficiency. A quick clinical diagnosis can be made by examination of the tongue, which will appear red and inflamed. The other conditions are not as easily recognizable or attribute to B_{12} deficiencies alone. Megaloblastic anaemia is characterised by the release into the blood stream of large immature erythrocytes and is relatively uncommon in the UK outside of strict vegetarians and those suffering from pernicious anaemia. Pernicious anaemia can be brought about by either deficiencies in folate or B_{12} in the diet. Reduction in DNA synthesis leads to a failure of the cells to properly divide that is then coupled with a continued production of RNA, The increased formation of RNA leads to excess production of cellular proteins and the cells often contain large amounts of haemoglobin. The anaemia can be treated with folate supplementation.

As will be discussed later B_{12} dependent methionine synthase is responsible for the conversion of homocysteine to methionine, and is at the centre of what is

called the B_{12} network. Methionine is the main source for the production of S-adenosylmethionine, which is a methyl donor in most systems of the body. Lack of the B_{12} cofactor prevents the conversion and allows the build up of homocysteine to occur, leading toxic levels of homocysteine within the body. The raised levels of homocysteine have been linked to peripheral, cerebral and coronary vascular disease (Ueland 1989).

Homocysteine has also been linked to ankylosing spondulitis, and autoimmune rheumatic disorder. Speculation exists that a free cysteine residue associated with mutations of the HLA B_{27} antigen can react with the homocysteine thus changing the confirmation of the protein and making it antigenic (Gao 1995).

Table 1.1 Representative diseases, symptoms and factors affected by B_{12} related processes

Anaemia
Hypercellular bone marrow
Mal absorption of B_{12}–pernicious anaemia
Autoimmune IF antibodies (Chanarin 1991)
Difficulty walking
Optic atrophy
Lack of concentration
Poor memory
Depression
Paranoid psychosis
Demyelination of peripheral nerves, spinal cord and cerebrum (Lever et al, 1986)
Neural tube defects (Kirke 1993, Scott 1990)
N_2O effects (Deacon 1980)
MS deficiency (Reynolds 1987)
B_{12} and fertility (Kawata 1992)
Homocysteine and MS (Reynolds 1992)
Heart disease (McCully 1996)

There are currently only two established B_{12} dependent enzymes in mammals. These are methylmalonyl CoA-mutase and methionine synthase.
In bacterial micro organisms there are several more enzymes that use B_{12} as a cofactor as shown in Table 1.2.

Table 1.2 B_{12} Dependent enzymes in mammals and micro-organisms

Enzymes	Function
Methionine synthase	Conversion of homocysteine into methionine
Methylmalonyl CoA-mutase	Conversion of methylmalonyl CoA into succinyl-CoA
Glutamate mutase	Conversion of L-glutamate into L-threo-β-methylaspartate
Diol dehydratase	Conversion of diols into aldehydes
Lysine mutase	Conversion of D-lysine into 2,5-diaminohexanoic acid
Ethanolamine deaminase	Cleavage of amino group
Ribonucleotide reductase	Reduction of ribonucleotides to corresponding deoxyribonucleotides

From this it has been proposed that B_{12} and thus cobalt is being deselected by evolution. This is possibly due to the role of transferring methyl groups being taken over by methionyl CoA or iron and manganese containing enzymes. The reason behind the evolutionary change has been attributed to the increase dioxygen in the atmosphere and B_{12} susceptibility to oxidation.

It is now recognised that B_{12}–deficiency is common especially with older persons of whom 10% or more suffer from it. [Although in the past (called pemicious anaemia) these diseases were attributed to cobalamin deficiency in the diet. It is now thought to be either impaired uptake from ingested food or transport deficiency in the body which causes B_{12}-deficiency.] To sustain physical well being an average dose of 6 nanomoles of B_{12} is recommended of which 2 nanomoles/day is usually supplied in food.

TC, and HC in Table 1.3 bind cobalamins strongly and transport them to tissues where B_{12} is needed for activation of the only two intracellular enzymes in mammals that utilise B_{12} as a cofactor. These are methionine synthase found in the cytoplasm and methylmalonyl- CoA mutase located in the mitochondria – called B_{12}MS and MMCoAM respectively in this thesis.

Table 1.3 Proteins involved in B_{12} uptake, transport and storage (Nexo 1994)

B_{12} Transport and Receptor Proteins	Mass (Kd)
Intrinsic factor (IF)	50
Transcobalamin (TC)	45
Haptocorrin (HC)	70
Qp280 receptor for IF	460
Tgp330 (megalin) receptor for TC	600
Asialoglycoprotein receptor for HC	Unknown

Each day 1-2 nanomoles of cobalamin supplied in food is bound first to HC and later transferred to IF after HC digestion by pancreatic and intestinal enzymes, (fig 1.3). Approximately 2-4 nanomoles of cobalamin also re-circulated via the enterohepatic circulation about 6 nanomoles need to be absorbed in the distal intestine each day (Nexo 1994). The most frequent cause of cobalamin deficiency is disturbances in the latter process, which includes age–related problems. If the IF receptor is defective or low in concentration the Immerslund-Grasbeck Syndrome results. The major part of the absorbed 6 nanomoles cobalamin is transported by TC from the intestine to the rest of the body. Cobalamin is redistributed from the liver via the enterohepatic circulation and from the kidney storage either directly to TC or bound to HC from whence it passes the liver prior to re-circulation.

1.2 Structures of cobalamins and B_{12} dependent methionine synthase.

Cobalamins are neither biosynthesised nor occur in the plant kingdom. Although B_{12} is a necessity for good health and for normal growth it is obtained only by diet. Only two forms of cobalamin are used by mammals and these are methylcobalamin (1) and adenosylcobalamin (2). These are the cofactors for methionine synthase and methylmalonyl-CoA mutase respectively. (fig 1.4)

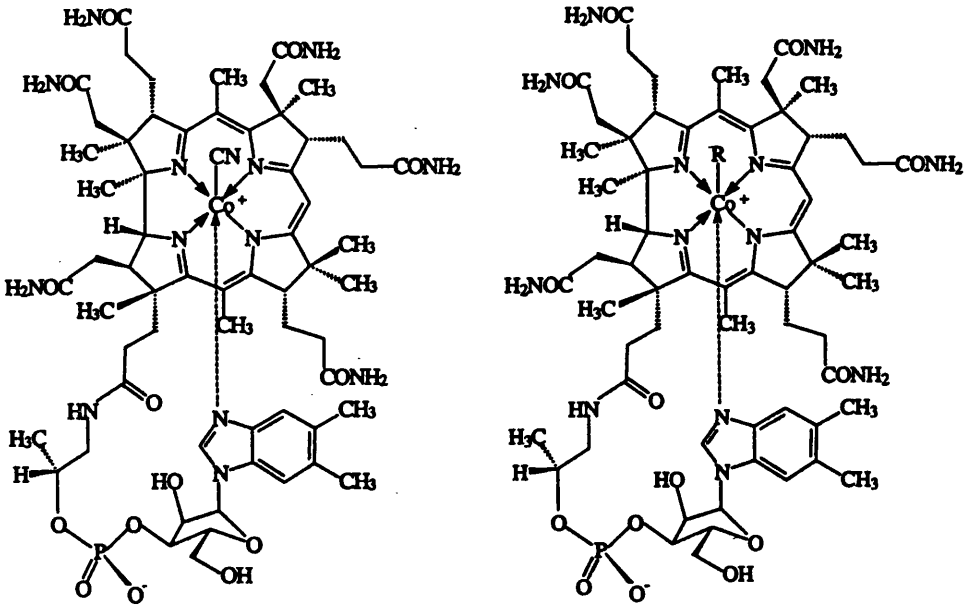


Figure 1.4 Different forms and uses of vitamin B_{12}.
The cyano and hydroxy cobalamins are stable forms of the vitamin that are readily converted to the methyl and adenosyl cofactors in the body. Hydroxy cobalamin is administered for dietary deficiencies and ^{57}Co or ^{58}Co cyano-cobalamin is used for absorption measurements in the Schilling test. Here R=methyl or R=adenosyl in the enzymatic cofactor form of the vitamin (adapted Krautler 1998).

One feature of cobalamin is the oxidation state of their central cobalt atom, which can be Co(I), Co(II) or Co(III). These are readily distinguished by their UV-visible absorption, infrared or ESR spectra. The Co(III) forms have octahedral symmetry a property that is critical when considering the mechanisms of action of enzymes containing cobalamin cofactors. The Co(I) form is diamagnetic and has tetrahedral symmetry. These property of Co(III) and of Co(I) are of fundamental importance since in Co(III) there are two bound axial ligands which are not present in the Co(I) which ligands only with its four N atoms of the corrin ring system. Co(II) corrins are pentacoordinated having four corrin N atoms and one axial liganded. All three forms, Co(I), Co(II) and Co(III) are required to fully explain the mechanism of action of B_{12} dependent methionine synthase. Essentially the Co(III) form in B_{12}–MS is the resting form, which is attacked by the nucleophile. This breaks the two axial bonds to generate Co(I). The nucleophile is the amino homocysteine (in its thiolate form) which is converted to methionine (its methylated form).
Regeneration of the Co(III) form is achieved by remethylation utilizing the cofactor to N5-methyltetrahyrofolate. (fig 1.5).

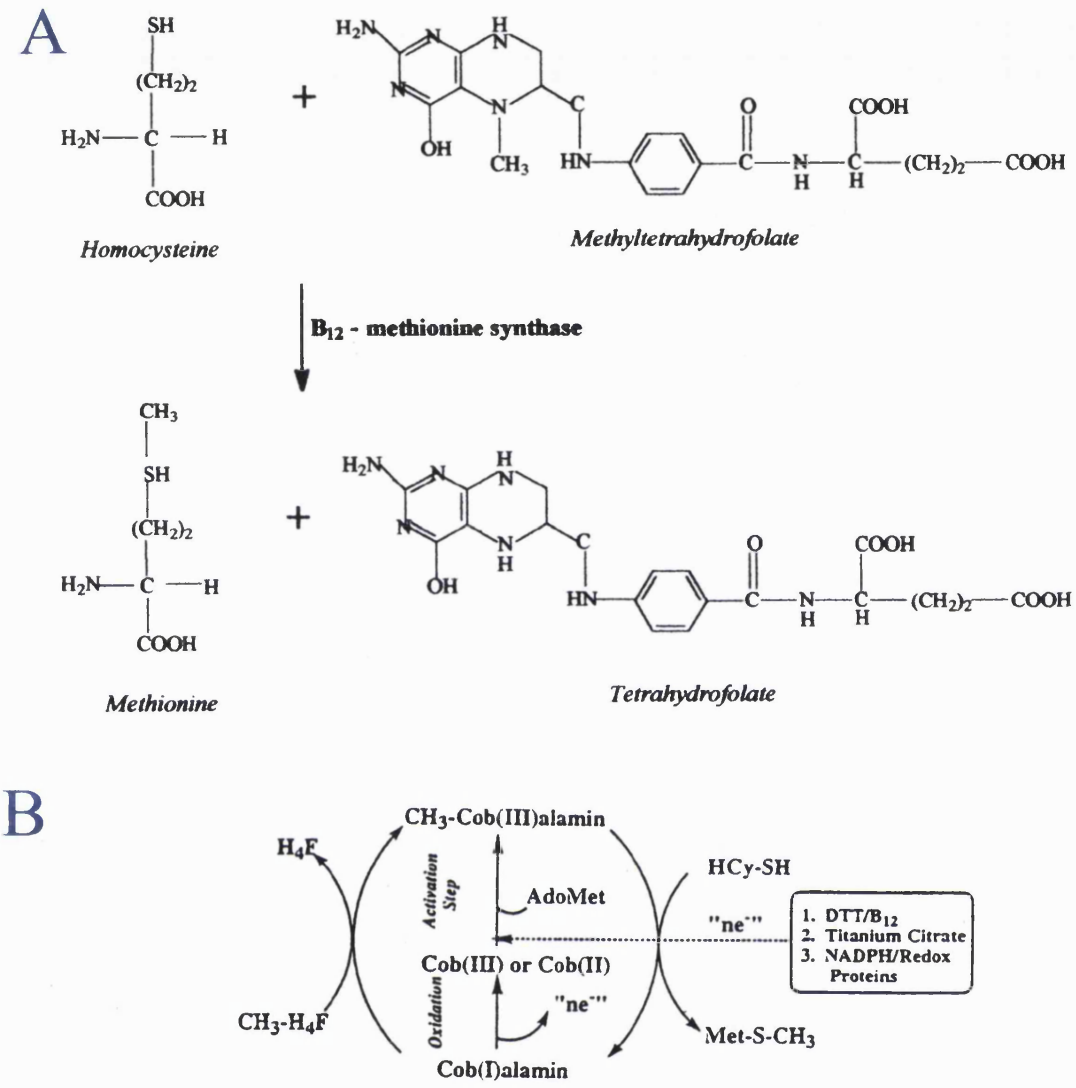


Figure 1.5 Enzymes, substrates, products and factors involved in the conversion of homocysteine to methionine.

A)Detailed structures and structural changes involved in the action of B_{12}MS (adapted from Stryer biochemistry). B)The activation, catalytic and deactivation cycles for the B_{12}MS reaction. CH_3Cob(III)alamin is the enzyme bound form of B_{12} in the resting state. After the catalytic reaction it is converted to the Cob(I)alamin form which requires CH_3H_4folate to effect re-methylation of the enzyme bound cofactor. Occasionally Cob(I)alamin is oxidised to Cob(II)alamin and hence becomes unavailable to recycling by CH_3H_4folate. Redox protein that utilise NADPH are needed in the cell to convert Cob(II) Cob(III). Titanium ion can be used in vitro to replace the redox proteins.

1.3 B_{12}–dependent methionine synthase from micro-organisms

The biosynthesis of vitamin B_{12} in micro organisms has been fully elucidated by several groups including Eschenmoser, Arigoni, Battersby and Scott. The latter, significantly, has demonstrated in vitro biosynthesis using a single pot reaction with 12 bacterial enzymes, which act sequentially to produce cobalamins. Table

1.2 has already listed the remarkably few enzymes from micro organisms to humans, which utilize B_{12} as a cofactor to convert substrate to product. Even more remarkable is that, despite the critical human requirements for dietary B_{12}, only two enzymes in mammals utilize B_{12} as a cofactor. Although the cloning, the expression, genes, protein structure, crystal structure and mechanism of action of B_{12} dependent MMCoA-mutase are well establish, even for this B_{12} enzyme it has not been fully recognised what physiological as distinct from biochemical roles are played in growth, development, health maintenance and disease.

At the beginning of this thesis very little was known about B_{12} dependent methionine synthase from pig brain, the subject of this thesis. However, during the tenure of this research, progress was rapid throughout the world especially on the E.coli B_{12}MS and now on several B_{12} isozymes from various mammalian tissues. Nevertheless a great deal remains elucidated, even today, for the other isoenzymes which are intimately involved in normal cell growth and differentiation and in particular in foetal growth. This is particularly true of the brain enzyme which play such a role in the brain growth and development; other roles in brain pathology are rapidly being discovered or hypothesized based upon the activity of this enzyme and its control of homocysteine metabolism.

The E.coli B_{12}MS protein, when isolated (Paessens, 1980: Rudiger 1970) had a molecular weight similar to that, eventually, derived from the DNA sequence of 1227 residues. Purified B_{12}MS was a single polypeptide chain by SDS gel electrophoresis with a molecular mass with approx. 140 Kd; the latter was supported by gel permeation chromatography. Its pI = 5.1 was determined by gel electrofocussing. The discovery during purification that smaller, catalytically active, forms of E.coli B_{12}MS were present (generated) during the isolation procedures led to systematic proteolytic digestion study. fig 1.6 and fig 1.7

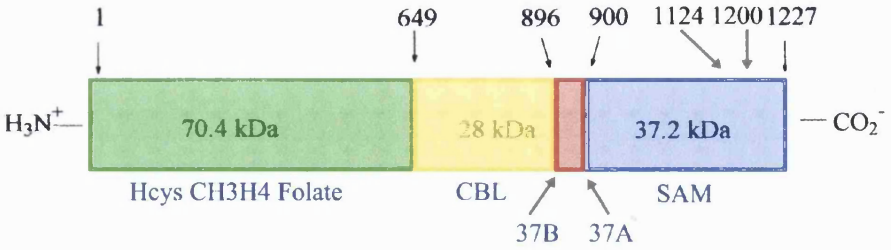


Figure 1.6 Diagram showing sequence of B_{12} MS and fragments discovered by proteolytic digestion, binding and catalysis studies.
(adapted from Drummond 1993 and Banerjee 1989)

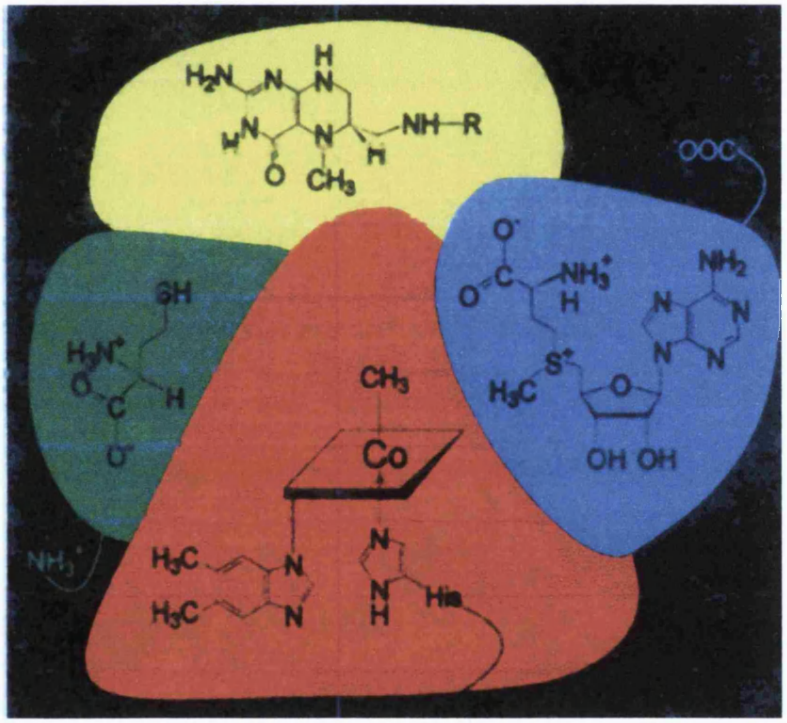


Figure 1.7 Cartoon of functional modules found in methionine synthase.
Proteolysis of the native enzyme by trypsin releases the C-terminal domain (residues 896-1127) that binds SAM and the cobalamin binding fragment (residues 643-896).

Generally the reaction catalysed by B_{12}MS starts with the resting form E – methylcobalamin, which undergoes the nucleophilic displacement at the methyl group by the thiolate form of homocysteine. The product E – cob(I)alamin is

then remethylated by the second substrate, CH_3H_4Folate to regenerate E – methylcobalamin, the starting/resting form of the enzyme. Thus both the E – methylcob(III)alamin and the E – cob (I) alamin forms of the enzyme were kinetically competent and it was concluded that the cleavage of the Co – carbon bond was heterolytic rather than homolytic (the formation of ions not of free radicals)

Proteolytic digestion of B_{12}MS was shown to proceed in several stages: Attack at ARG896 to generate a 38kD C-terminal fragment that binds the SAM/AdoMet. The 98kD fragment, when formed from the 'resting E-methylcobalamin' form of the enzyme, was capable of 'many rounds of turnover' but eventually was slowly inactivated to the E-cob(II)alamin form. The later could not be revived by addition of the SAM methylating agent and a reductant – in vitro.

Further digestion of the 98kD fragment yielded 28kD (residues that lay upstream from the activation domain; it was not able to sustain catalysis. The new N terminal 71kD sequence could be further cleared into a number of pieces which identified, in part, three possible binding sites for cobalamin, CH_3H4Folate and SAM/Ado Met. It has been suggested by the Matthews, Ludwick and Banerjee groups that intact B_{12}MS was evolved by fusion of DNA's that coded for the consensus binding sequences for homocysteine, CH_3H4Folate, cobalamin and SAM/AdoMet.

A truncated one-649 residue fragment of B_{12}MS contained the binding determinants for both CH_3H4Folate and homocysteine. These proteolysis-catalysis-binding studies with the intact B_{12}MS and its fragments combined with a search for a consensus binding sequences (for substrates) in protein databases, lead to the proposed 'domain model' of B_{12} MS fig 1.7.
The crystal structure of a 28kD E.coli B_{12}MS was reported and discussed by Matthews, Ludwick and their co-workers. (fig 1.8 and fig 1.9)

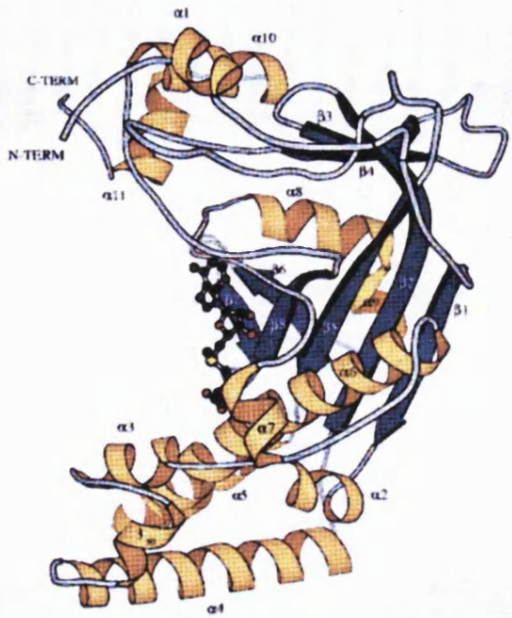


Figure 1.8 Crystal structure of the activation domain of B12 MS from E.Coli;
alpha helices (yellow) and beta sheets (purple) (adapted from Drennan 1994).

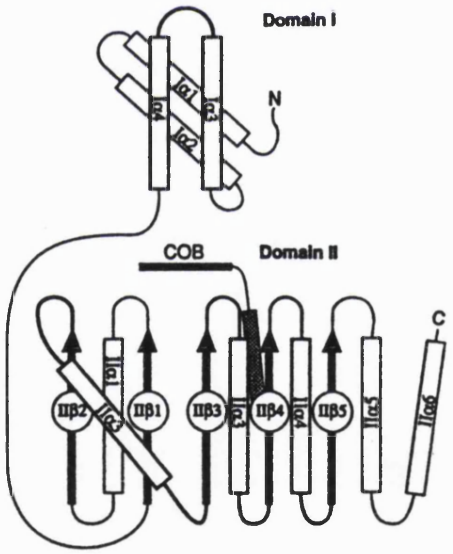


Figure 1.9 Cartoon of two domains of B12 MS showing the 4 helix bundle
(1) and the parallel beta barrel and set of helices that constitute domain (2) (adapted from Drennan 1994)

The yellow N terminus contains four alpha helices, I α1, I α2, I α3 and Iα4 with their appropriate connecting loops. This connects to a α/β nucleotide binding fold of five parallel sheet – strands (designated purple) and a set of six alpha

helices. They proposed a structure – function model in which B_{12}-MS can assume at least three different conformations:

"A resting state in which an α helical cap domain of the cobalamin binding region protects the methyl group of cobalamin and the upper corrin face from interaction with solvent or substrates"

"A conformation for reductive activation, in which the cap is displaced and, with the C-terminal domain, positioned above the cobalamin. In this way the SAM/AdoMet could react with the prosthetic group."

A conformation in which cobalamin can react with homocysteine and/or with $CH_3H4Folate$ and in which the N terminal region of the protein was positioned above the cobalamin.

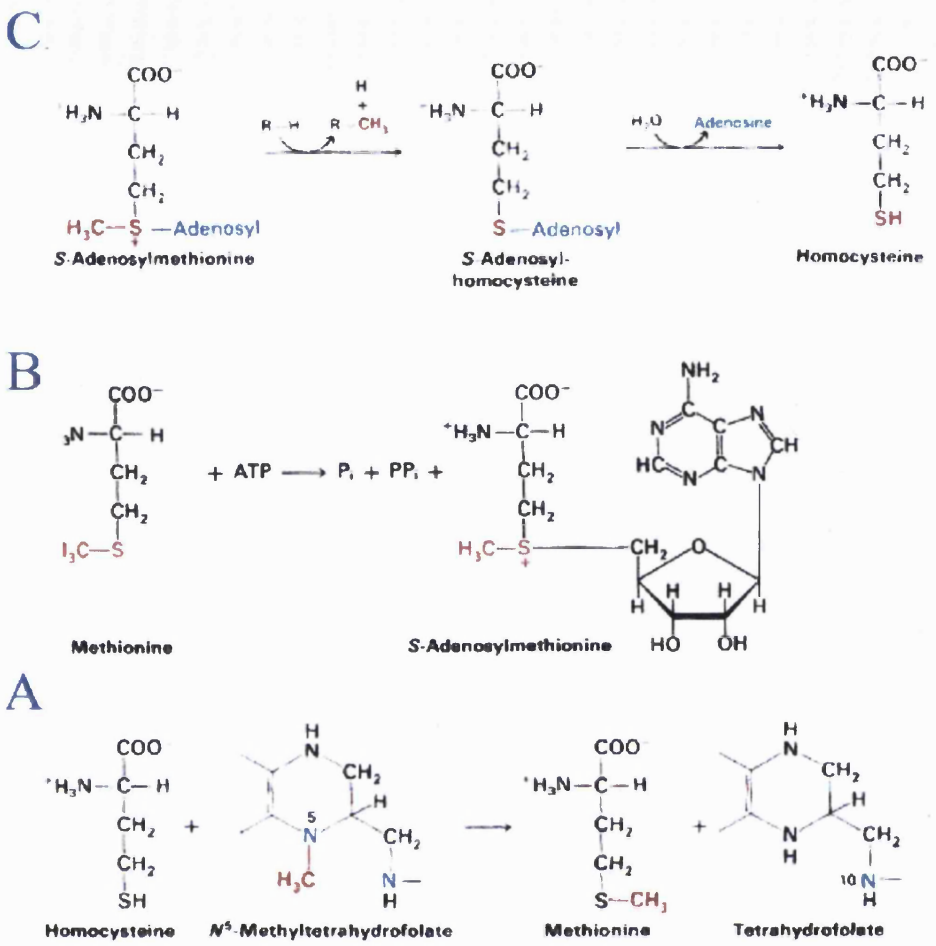


Figure 1.10 Enzymatic interconversion of homocysteine , methionine, SAM and SAH.
(A and B) Two consecutive reactions involving homocysteine → Methionine → SAM. (C) Enzymatic reactions showing conversion of SAM to SAH involved in methylase reactions followed by hydrolysis of SAH to homocysteine and adenosine

Methionine is biosynthesised by two enzymes. The first (**fig** 1.10) is B_{12} dependent methionine synthase and the second is betaine/homocysteine methyl transferase. The latter is a B_{12} independent enzyme.

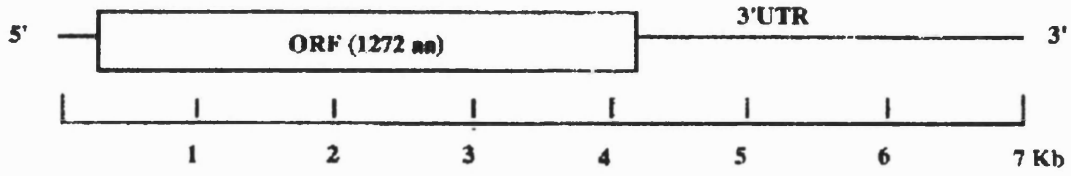


Figure 1.11 The structure of cDNA coding for human B_{12} MS;
ORF, open reading frame; UTR, untranslated region (Li et al 1996)

Banerjee and co-workers (Li et al, 1996) reported the isolation and characterisation of human B_{12}MS cDNA. The latter "contains an open reading frame of 3798 nucleotides encoding a protein of 1265 amino acid residues with a mass of 140kD". (fig 1.11) This amino acid sequence was 55% identical with that of E.coli B_{12}MS and 64% identical with the predicted Caenorhabditis elegans enzyme. In addition, seven peptide sequences derived from purified porcine B_{12}MS bore "substantial similarity" to the human protein.

They carried out Northern blot analysis which indicated firstly that B_{12}MS was present in a wide range of tissues and confirmed the results from B_{12}MS activity studies that the highest B_{12}MS concentrations were principally in heart, skeletal muscle, pancreas and placenta with considerably lower concentrations in brain, liver, lung, kidney, testis etc. In fact, Banjeree et al's blotting experiments revealed two bands of B_{12}MS cRNA at 7.5Kbase and 9.5Kbase respectively. At this moment it is not clear whether the 9.5cRNA corresponds to a pre-processed form of the 7.5Kbase species or an alternatively spliced (or altered) polyadenylated version of the gene. The 7.5Kbase B_{12}Ms was mapped to 1q43 near the telomere of chromosome 1.

A further significant aspect of the cDNA derived protein sequence homology was that 55% identity was not restricted to any single region of the 1265 amino acids but was spread throughout the protein. Furthermore the human protein possessed a 27-residue fragment at the N terminus that was divergent from the bacterial and nematode sequences.

Intracellularly, cobalamin exists in several forms including the two cofactor forms methylcobalamin (CH_3Cbl) and adenosylcobalamin (AdoCbl); the other forms are cyano-cobalamin (CNCbl), aquacobalamin (AqCbl), sulphatocobalamin (HSO_3Cbl) and glutathionylcobalamin. The formation of enzyme bound CH_3Cbl and AdoCbl forms (say) CNCbl occurs in several steps – decyanation, reduction of an internalised cobalamin, synthesis of CH_3Cbl (in cytosol) and of AdoCbl (in mitochondria).

1.4 Human placenta B_{12}-dependent methionine synthase (Utley et al, 1985)

A placental homogenate (1.1Kg) was tested as follows: ammonium sulphate precipitation, dialysis, DEAE cellulose then N5-CH_3H4Folate – cystamine – Agarose affinity, then AdoHcy-Agarose affinity chromatography. This gave B_{12}MS with a 933 fold purification with a 19% recovery and a specific activity of 1381 EU x 10^5 /mg.

A micropak TSK G 3000SW column was used to assess purity and Sephadex 200 chromatography gave Mr = 160,000. Purified B_{12}MS applied to native PAGE resulted in loss of all enzyme activity and the appearance of multiple straining bands of protein. The major band on the unreduced gel had Mr 160,000 with two additional bands at 80,000 and 70,000. Reduction of B_{12}MS in boiling 2-mercaptoethanol resulted in disappearance of the Mr = 160,000 band and the appearance of Mr = 90,000, Mr = 45,000 and Mr = 35,000 protein bands with no sign of the 70kD or 80kD bands on this reduced gel.

The metal contents of the purified B_{12}MS differed from that of B_{12}MS converted fully to the holoenzyme. The latter yielded 1.1 moles Cobalt/mole enzyme whereas the former gave 0.11 moles Cobalt and 2 moles Iron/mole B_{12} MS

1.5 B_{12} methionine synthase from plasmodium falciparum (Krunkrai et al 1989)

A two step purification involving sequential use of high performance size exclusion, then HP anion exchange chromatography gave an enzyme of 105,000 + 8000kD which was purified 300-400 fold. The Km (CH_3 H4THF) = 24.2μm (compared to 60μm and 75μm for E.coli and pig brain determined by Taylor and Weissebach 1967 and Km (Hcy)= 58.3 μm. The predominant form of B_{12}MS in the P falciparum extracts was the isoenzymes (>90%)

1.6 Porcine liver B_{12}-dependent methionine synthase (Chen et al, 1994)

The pig liver enzyme was purified 9000-fold to a specific activity of 1.6 micromoles/min/mg protein at 37^{0}C which was 100 fold higher than that of homogeneous human placental B_{12}MS.

Starting with 3Kg of pig liver, the homogenate was filtered through cheesecloth, followed by sequencial chromatographic separation on DEAE cellulose, Q sepharose, Phenyl sepharose and then passed twice through HTP chromatography which yielded two active bands. The molecular weight was determined under denaturing conditions on a 5% PAGE gel containing SDS to be a 151kD and by Superose 12 FPLC to be 155kD.

The overall yield was 0.9% to 2.5% for the most active fragments. No metals other than cobalt were detected by plasma emission spectroscopy.

Kinetic analysis using Scatchard and Hill plots gave its kinetic parameter of Vmax=101.8-125 nmol/hr, Km ($CH_3H4Folate$) = 142μm. The specific activity was determined as 0.54μmol/min/mg protein and 2.5μg protein was used in each assay. As with the E.coli enzyme the mechanism was established to beordered-sequential with a proposed ternary complex of Enzyme – (Hcy)-($CH_3H4Folate$) (fig 1.12).

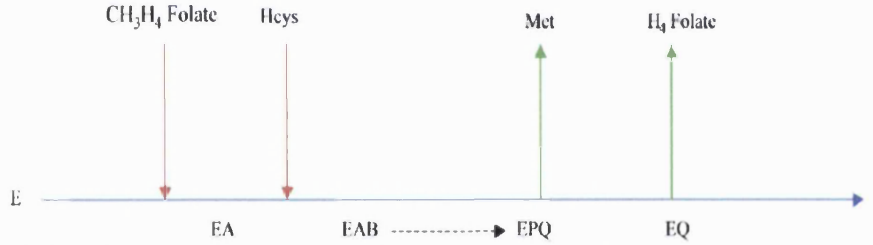


Figure 1.12 Sequence of events involved in the B_{12} MS reaction
The ordered sequential mechanism of action of B12 methionine synthase showing the order of addition of the two substrates (CH_3H_4 Folate and Hcys) to form the ternary enzyme complex (EAB) which is converted to complex EPQ on the enzyme. The subsequent sequential loss of its two products (Met and H4 Folate) to regenerate B12- MS methyl Cob (III)alamin.

1.7 Metabolic pathways, enzymes and metabolites dependent upon B_{12} methionine synthase levels and activity.

Although MMCoA mutase also depends upon B_{12} for its activity only B_{12}MS will be discussed here. The fundamental enzymatic processes are shown in fig's 1.13 and 1.14. Two enzymes effect the conversion of homocysteine to methionine: enzymes 1A uses betaine as its methyl donor and enzyme 1B (B_{12} dependent methionine synthase) uses $CH_3H4Folate$ as its methyl donor. This conversion of Hcys and $CH_3H4Folate$ to methionine and H4Folate is the first step in a variety of metabolic pathways that are essential for cellular function and for cell and tissue growth and differentiation. Enzyme 2 utilises ATP to form

S-adenosylmethionine an essential cofactor in most of these pathways. Enzyme 3 (SAM decarboxylase) converts cofactors SAM to cofactor decarboxyl SAM essential for cellular production of polyamines from ornithine and putrescine (and from cadaverine/lysine). SAM is a ubiquitous cellular methylating agent and is the cofactor in essential biological methylations such as DNA, proteins, phospholipids, catecholamines, indoleamines, ethylene, fatty acids, etc.

Fig 1.13 and 1.14 show diagrammatically and then in detail the metabolic pathways that depend upon the levels of homocysteine and methionine in cells. The latter are controlled (at least in part) by the activity of B_{12} methionine synthase but other controlling factors are dietary sources of these amino acids and betaine dependent MS. Each of these pathways will be described briefly here and in more detail in the sections on B_{12} related pathologies and chemotherapeutic intervention in the actions of these pathways.

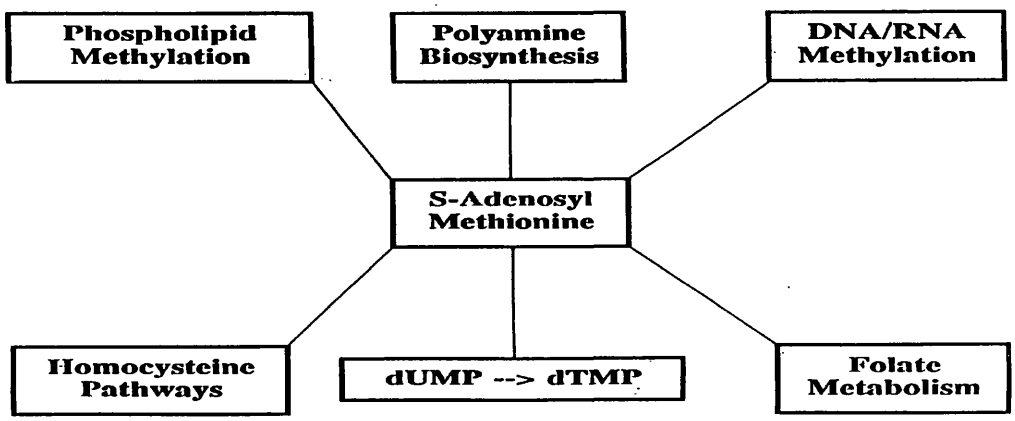


Figure 1.13 Diagram showing the central role played by B12 MS, Homocysteine, and SAM in several metabolic pathways

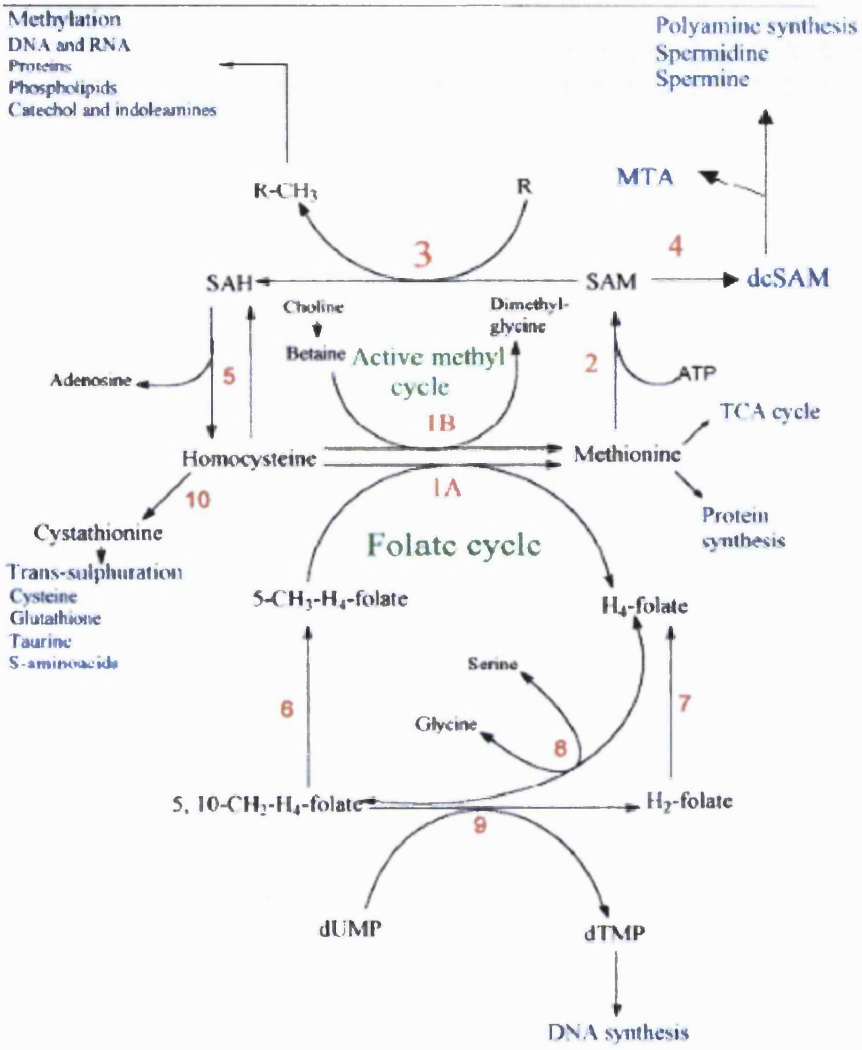


Figure 1.14 Detailed illustration showing the central role of B12 MS, homocysteine and SAM in several metabolic pathways.

1.8 AdoMet, AdoHcy, Folate and Thymidine metabolic pathways and enzymes.

The folates fig 1.15 and 1.16 consists of three parts, a pteridine ring linked to a para-amino benzoic acid, which in turn is conjugated via a peptide bond to glutamic acid polymers. The latter can consist of up to beta-linked residues. Folic acid is reduced in two steps via N5, N10 – methylene-tetrahydrofolate to N5 – methyl-tetrahydrofolate by dihydrofolate reductase (Molloy,1993).

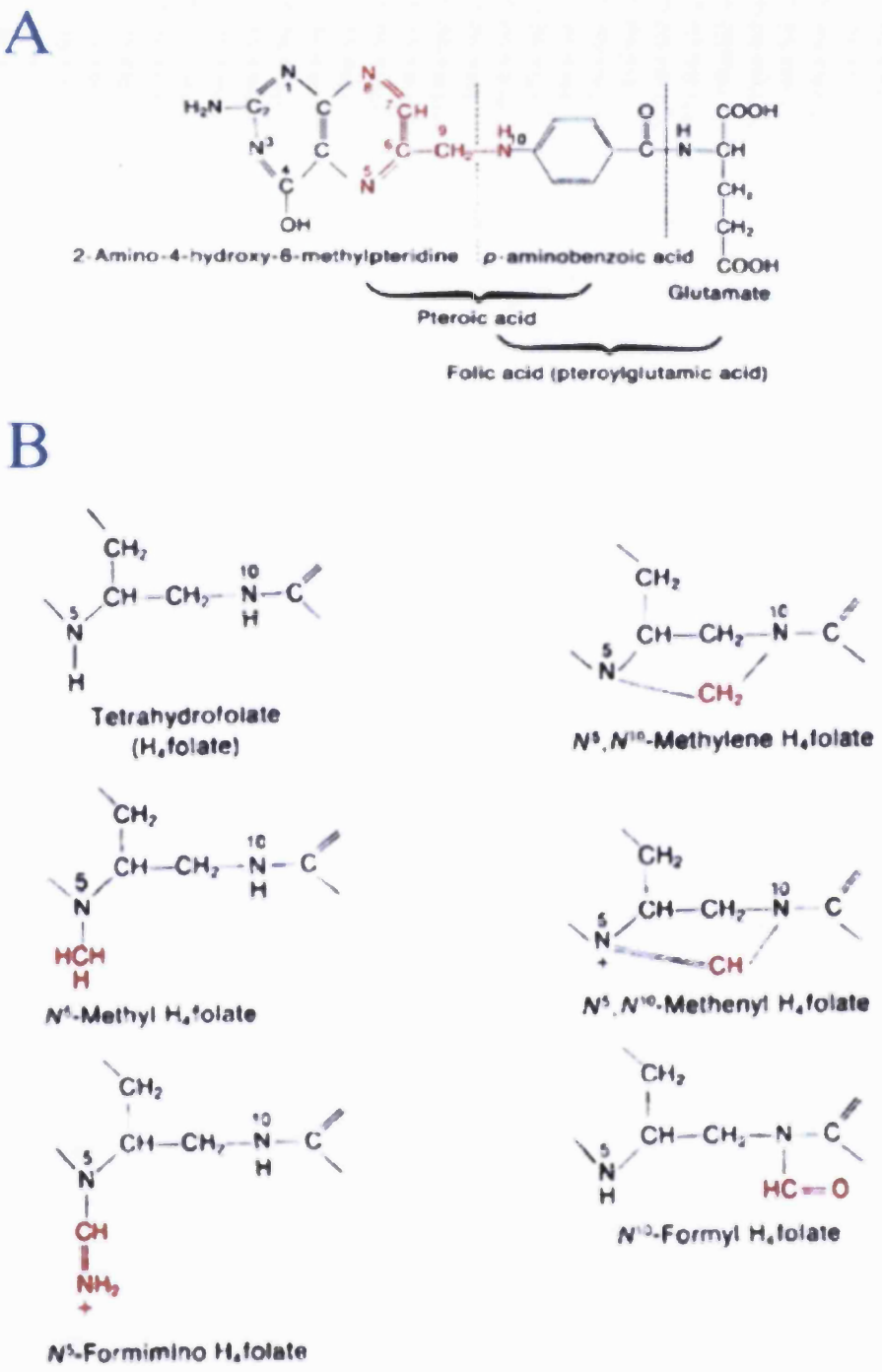


Figure 1.15 Detailed structures of tetrahydrofolate and other carriers of the designated 1 carbon fragments

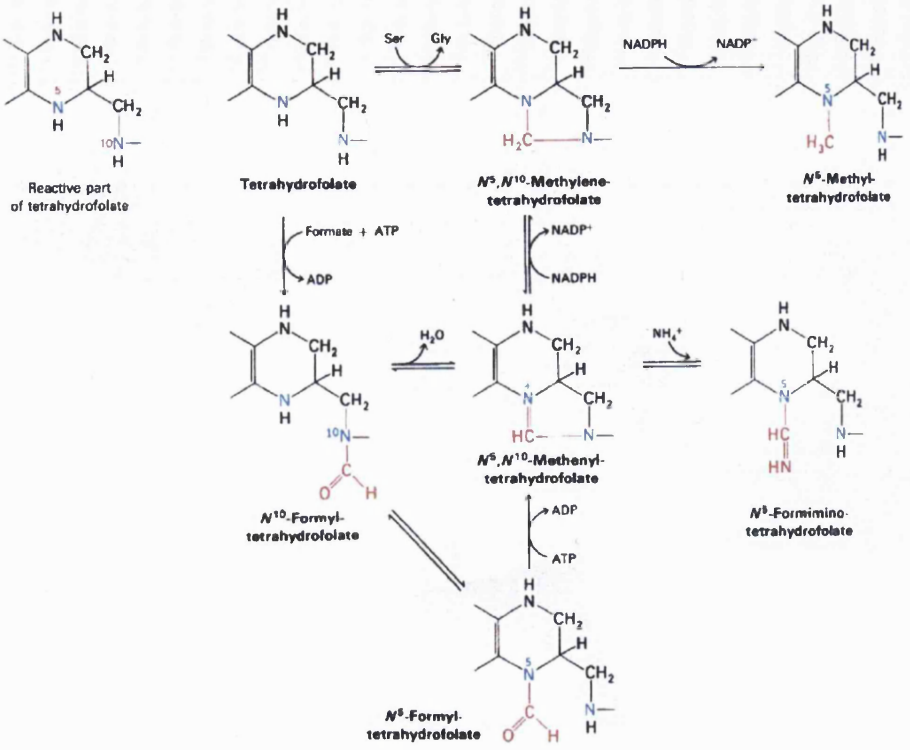


Figure 1.16 The metabolic cycle and pathways involved in the interconversion of the various forms of folate.
(The latter are the carriers/providers of one carbon fragments in cellular reactions.)

These fragments are methylene, methyl, formyl and iminoformyl. Not included here are enzymes that add (and cleave) glutamates to the PABA moiety. Tetrahydrofolate (H4Folate) is provided for this cycle either by diet (as a vitamin) or as a by product of the enzyme B_{12}MS. Dihydrofolate is therefore the only source of N5, N10-methylene H4Folate and N5-methyl H4Folate, the latter being the obligatory second substrate for B_{12}MS. DHFRase has therefore been the principle target enzyme for the design of antigrowth therapeutic substances for cancer and antimicrobial therapy.

Since the liver normally stores enough vitamin B_{12} to last mammals for 'up to six years' both DHFR and B_{12}MS can be modulated by means other than supply of B_{12}MS, Hcys, Met, AdoMet, and AdoHcys to the folate pathways and biological methylation is depicted in the Active Methyl Cycle diagram of fig 1.14

The fourth enzyme in this cycle is AdoHcys hydrolase, which splits AdoHcys into adenosine and homocysteine. The homocysteine is thus recycled but it is not yet established whether or not the enzyme AdoHcys is used to generate adenosine as a messenger (for adenosine receptors) or as a salvage pathway or both?

1.9 AdoMet(SAM), Decarboxy AdoMet(dcSAM), 5 methyl thioadenosine, polyamines; metabolic pathways and enzymes.

Although micro organisms have a variety of structurally different polyamines only four are of major importance in mammals (fig 1.17). The structures of the cofactors involved in polyamine biosynthesis are shown in figure 1.18.

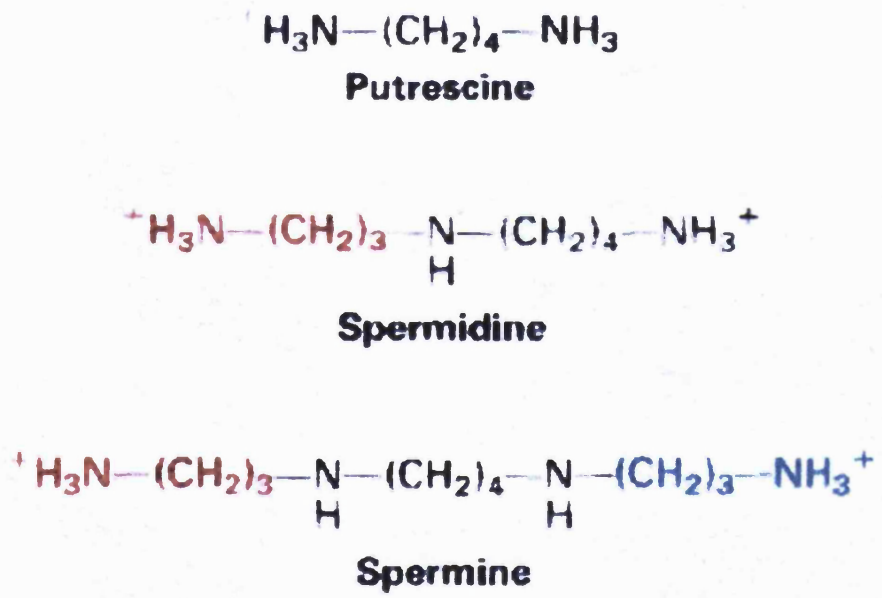


Figure 1.17 The structure of the three main cationic polyamines of mammalian cells

The amino propyl group [$^{+}H_3N(CH_2)_3$–] is transferred from cofactor dcAdoMet to form spermidine then spermine. The different charges (+2, +3, +4) and the biosynthesis by consecutive addition of an aminopropyl group donated by dcSAM is emphasised.

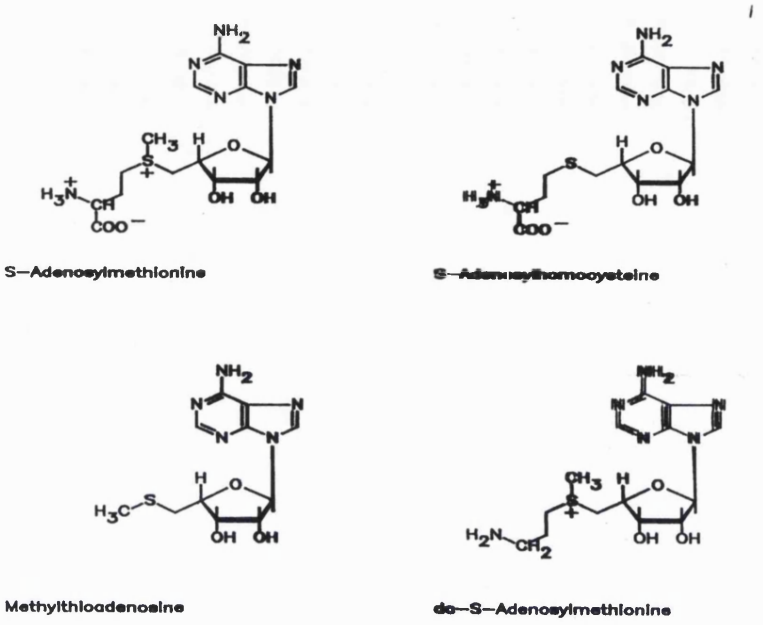


Figure 1.18 Detailed structures of the adenosine cofactors pairs involved in cellular reactions, namely, SAM/SAH and dcSAM/MTA

Cadaverine and putrescine are both derived by the action of appropriate decarboxylases on lysine and ornithine respectively. Putrescine is converted to spermidine and spermine by successive transfers of the aminopropyl group. (NH_2- (CH_2) $_3$-) from decarboxylated AdoMet. The latter is formed by the action of AdoMet decarboxylase. The relationship of the homocysteine to methionine reaction are shown in fig 1.19 (Arillo, 1985).

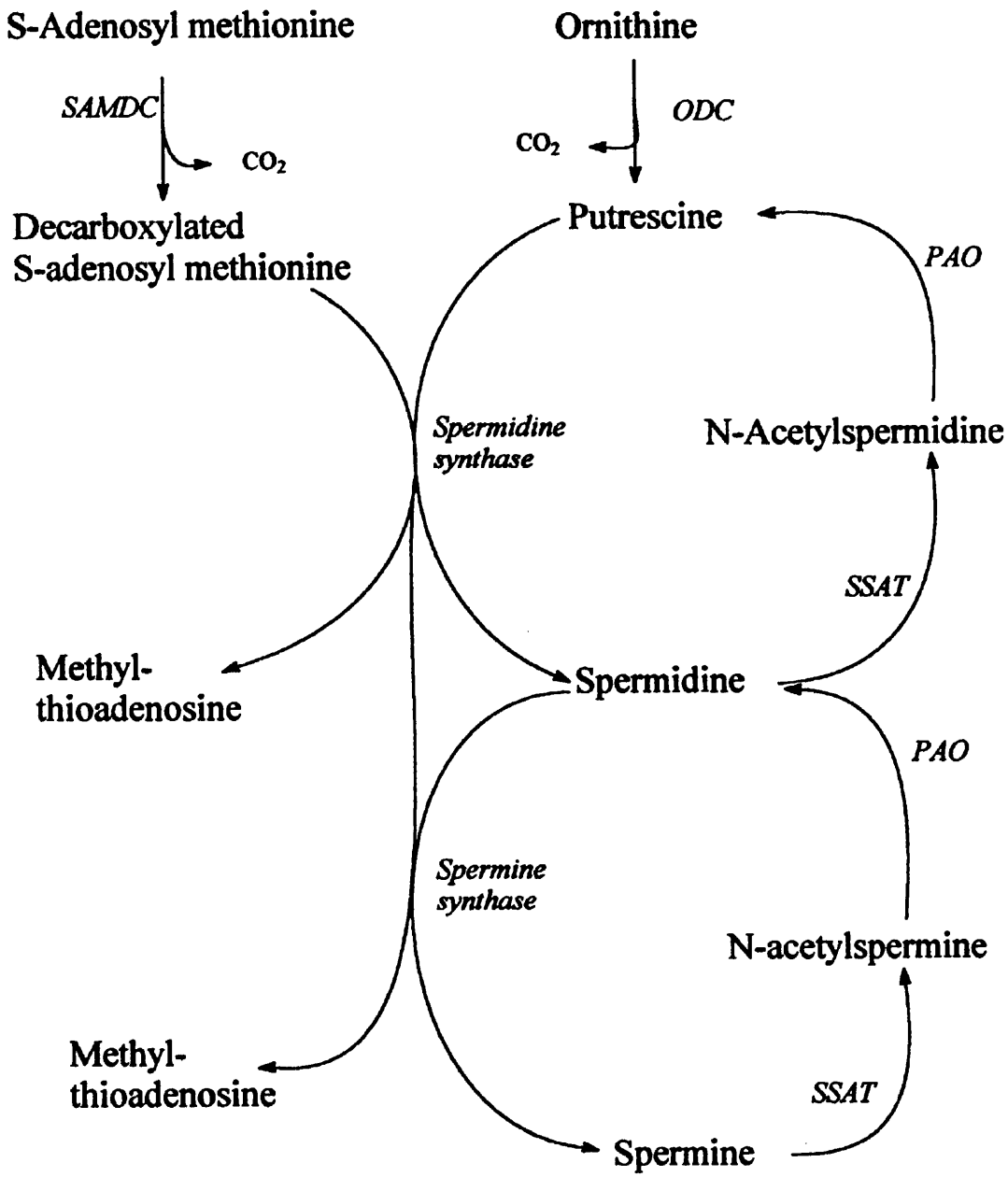


Figure 1.19 The biosynthetic and catabolic pathways involved in polyamine metabolism.
The enzymes SAM decarboxylase (dcSAM), ornithine decarboxylase (ODC), polyamine oxidase (PAO) and spermine/spermidine aminotransferase (SSAT) have been extensively studied as targets for anticancer drug design.

The polyamines, once formed, are deactivated by acetylation of their amino groups and/or by oxidation to aldehydes by amino-oxidases. Because they are polycationic carrying +2, +3, and +4 charges, it was logical at first to hypothesize that putrescine Spermidine and spermine acted at the level of DNA, which is a polymer of phosphates. Binding studies analogous to histones

(which contain extensive percentages of cationic amino acids) and their effect on transcription and translation in specific cells somewhat supported this theory (Pegg, 1982: Rennart, 1978).

Recent research has however indicated that polyamines affect many important biochemical and physiological processes. These include:

- Modulation of mitochondria CA channel proteins, K^+ channels
- Binding to the NMDA and GABA receptor channels and the nicotinic receptor
- Inhibition of signalling kinases, including protein kinases A and C, casein kinase, calcium/calmodulin, dependent kinase
- Phospholipases A2 and C, palmitoyl hydrolase and nitric oxide synthase are all inhibited
- Calcium –ATPase
- Glutamate dehydrogenase
- Insulin production and stimulation of cell growth and differentiation
- Lowering blood pressure
- Relaxation of smooth muscle
- Inhibition of gastric secretion and ulceration
- Elevation in many transformed cell lines and tumours
- Analgesic and anti - inflammatory effects

(Morruzi, 1987;Ficker 1994; Johnson, 1996; Weiger, 1994; Robinson, 1990; Rock, 1992;Scott, 1993; Hughes, 1994; Seller, 1981, 1991; Hu, 1994; Kameji, 1987; Kroes, 1988; Russel 1973)

The levels of the three polyamines in CSF fluid, serum, erythrocytes, fibroblasts and ascites tumour cells are given below: (table 1.4)

TABLE 1.4 POLYAMINE LEVELS IN CELLS AND BODY FLUIDS*

Location	Putrescine moles/ml	Spermidine moles/ml	Spermine moles/ml
Serum	$0.1\text{-}0.5 \times 10^{-9}$	$0.1\text{-}0.3 \times 10^{-9}$	$<0.1 \times 10^{-9}$
CSF	184×10^{-9}	150×10^{-9}	Very low
Erythrocytes	$600\text{-}900 \times 10^{-9}$	159000×10^{-9}	635000×10^{-9}
Fibroblasts	29000×10^{-9}	430000×10^{-9}	602000×10^{-9}
Ascites	3000×10^{-9}	1835000×10^{-9}	694000

*Thanks to Dr. K. Valko, Dr. M Anderson, Dr. S Kenyon and Dr. A Nicolaou for the above information. (Morgan 1990)

Clearly the levels in body fluids, serum, and CSF are in the nanomoles/millilitre range of concentrations but intracellular levels are in the 1-1000 micromoles/ml range. The latter corresponds generally to Ki values found for inhibition of kinases, phospholipases and other enzymes and also satisfactorily accounts for the concentrations at which polyamines modulate ion channels and receptors. The proposal (Gibbons and co-workers 1994) that polyamines may be another class of intracellular signalling molecules is supported by the data above. In the case of cell growth and differentiation, and particularly the cell cycle, systematic changes in polyamine levels have been observed. Thus polyamines stimulate growth and inhibition of their synthesis decreases growth (Danzin 1979). Fredlund et al (1995) examined the levels of all three polyamines throughout the cell cycle of Chinese hamster's ovary cells and found differential changes throughout the S, G1, and G2 phases. Concomitant changes in the levels / activity of ODCase (ornithine decarboxylase) and AdoMet decarboxylase were established, both of which are critical for the synthesis of putrescine and decarboxy AdoMet the substrate for spermidine biosynthesis. The fact that inhibitors of both these enzymes affected the cell cycle and polyamine levels has led to a search for therapeutic suitable inhibitors for microbial and cancer chemotherapy's.

Polyamines have been found to have a dual or biphasic effect on platelet aggregation (Pales 1984, Lee 1991, 1993, Joseph 1985). These effects were observed at polyamine concentrations above blood levels, but again, polyamines are stored and secreted from platelets upon stimulation and thus the effects are possibly real and physiological.

Polyamines were shown to affect neurologically important receptors and ion-channels. The glutamate channels are the most studied and polyamines generally enhance glutamate responses that are modulated by NMDA, Kainate and AMPA; of these, the NMDA sensitive channel was the most studied. Bernardi et al (1996) demonstrated that blockage of polyamine binding to the NMDA channel "prolonged the reaction time to pain and enhanced the effects of morphine". Johnson (1996) and Nankai (1995) have reviewed these effects on the NMDA receptor channel and also the effects of polyamines on the gating and /or conductance of Ca^{2+} channels and inward-rectified K^{+} channels.

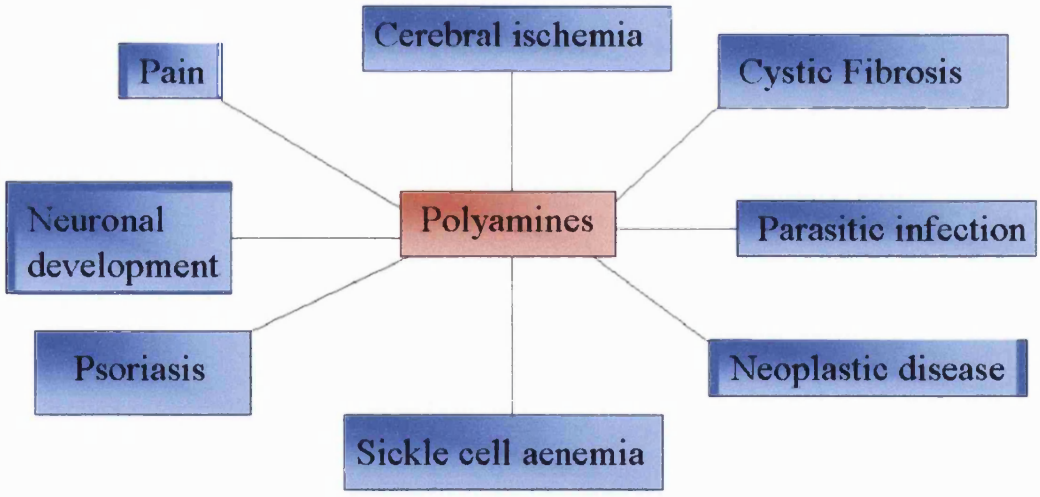


Figure 1.20 Clinical conditions for which there is evidence of a significant change in polyamine concentrations.

The above diagram fig 1.20 delineates those clinical conditions for which there is evidence of a significant change in polyamine concentrations. It also presents examples of therapeutic areas where active research for medicinal substances, both natural and synthetic is currently taking place.

Because of the discoveries of the role of polyamines in neurotransmitter function and signalling and the realisation that ornithine is both the product of NO synthase and a substrate for ODCase (and is involved with enzymes of the urea cycle and N metabolism) more certain intracellular roles for polyamines should become evident. It seems clear that since B_{12}MS is the first step in the pathway leading from homocysteine through decarboxy AdoMet to polyamines, it is important to examine this relationship. It has already been shown that N_2O, nitrous oxide, a specific inhibitor of B_{12}MS affect polyamine levels. The same is true of AdoMet synthase and AdoMet decarboxylase inhibitors.

1.10 AdoMet, AdoHcy and sulphur amino acid pathways, enzymes and metabolites.

The complex network of sulphur amino acid metabolic pathways shown opposite in fig 1.21 is shown principally because the control, regulation and/or inhibition of B_{12}MS (or Betaine MS) affects the cellular levels of Hcys, Met, AdoMet, AdoHcy and other metabolites that depend upon their concentration. Dietary conditions, protein metabolism and activity of the TCA cycle, also affect Hcys and Met levels. Thirdly, inbom errors in metabolism of B_{12}MS and several potent cellular toxins are known to affect these pathways. Finally the sulphur amino acids and their analogs have been proposed to play important roles as neurotransmitters, neuromodulators, anti-oxidants etc., and their cellular and blood levels have been correlated with cerebral, peripheral and coronary vascular disease.

A genetic link has been postulated for B_{12}MS, cystathioninase, and cystathionine β synthase have been found to be defective in some patients and has led to hyperhomocystinuria, cardiovascular and other conditions. At high levels homocysteine itself is toxic.

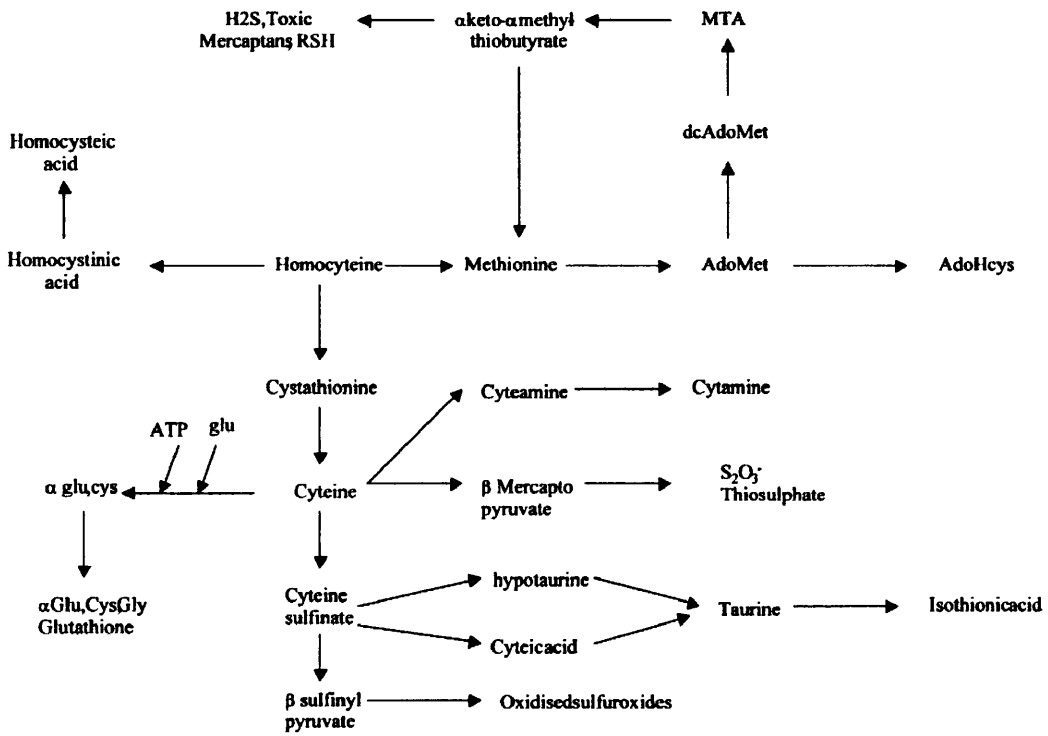


Figure 1.21 The principle pathways and enzymes leading from homocysteine to cystathionine;
cysteine, cysteic acid, taurine, various forms of oxidised sulphur (SO_4^{-2}, $S_2O_3^{-2}$, HSO_3^{-1} etc.) and the important molecules glutathione and reduced glutathione.

Defects in B_{12}MS genes affect Met and AdoMet levels and hence affects the levels of folates in cells. As shown later, low methionine levels can lead to hypomethylation of DNA at "CpG islands" and to transcription of oncogenes and to tumourogenesis.

The oxidised forms of the sulphur amino acids, cysteine and homocysteine, that have – SH converted to SO_2, SO_3 and SO_4. (Such as cysteic, homocysteic, cystinic, homocystinic acids, and hypotaurine and taurine), are all isoteric with common neurotransmitters such as gaba, glycine, glutamate, glutamine and aspartate can in fact act as neurotransmitters/modulators themselves. This has been the basis for the design of powerful agonists and antagonists of the common amino acid neurotransmitters.

The role of taurine in cells is still a matter of debate since it has been proposed as an osmoregulator, neurotransmitter, neuromodulator, and indicator of

cellular toxicity and as a source of the bile acid taurocholine. The trans-sulphuration pathway (Hcys → Cys) and other pathways of fig 1.21 produce a number of acidic sulphur containing amino acids. The latter are structurally related to aspartate and glutamate, and at least structurally, can be considered isoelectronic or isosteric with these. This structural relationship suggested a possible functional resemblance, which has been tested.

The four metabolites, L-Csx, L-Csa, L-HCsa, L-Hcsa, were all shown to mimic the fast-acting neurotransmitters, L-Asp and L-Glu at their excitatory synapses in the CNS and it was proposed that these acidic sulphur amino acids were candidate neurotransmitters or neuromodulators. (Lehman et al 1988; Griffiths 1991; Cuenod et al 1991). Specifically, a large amount of experimental evidence was accumulated to show the following:

L-Saa's elicited excitatory neural responses in electrophysiological experiment at the L-Asp and/or L-Glu synapses.
The above electrophysiological behaviour was attenuated by the same antagonist which were selective for L-Asp and for L-Glu
The L-Saa was transported by the same plasma membrane neuronal and glial transporters for L-Asp and L-Glu.
L-Saa have specific locations in brain tissue.
Chemical binding studies demonstrated competitive binding at L-Asp/L-Glu sites using radioligand binding.
L-Saa's affected neurotransmitter release, both excitatory and inhibitory transmitters

These data encourage the belief that the conversion of Hcys to Met which is coupled to Hcys → sulphur amino acids (and glutathione/taurine) are important for brain function and should be dependent upon activity of B_{12} methionine synthase. The true function of taurine and the Saa's and glutathionine in the brain have yet to be worked out satisfactorily. It may be that they possess both an intercellular signalling / modulatory activity or they may play a role in extracellular (synaptic) phenomena.

1.11 Vitamin B_{12} and B_{12} methionine synthase in health and disease

Fig 1.3 gives a summary of diseases, which are considered to be dependent, in one way or another, on the activity of B_{12}MS and/or the levels of the reactants and products of B_{12}MS namely, homocysteine, methionine, vitamin B_{12} and the folates (H_4 folate and CH_3H_4folate). Experiments were seldom carried out to distinguish between the role of methylmalonyl CoA mutase and B_{12}MS. In the case of MMCoA mutase usually sufficed to measure the levels of methyl malonic acid in body fluids or tissues, otherwise B_{12}MS or B_{12} uptake and transport have to be considered. The measurements of B_{12}, homocysteine, methionine and folate levels were usually the indicators of a correlation between the activity of B_{12}MS and the disease in question. The levels of these molecules could be influenced by diet or by the activity of pathways or enzymes "downstream" from B_{12}MS. Interpretation of clinical data should also bear in mind that betaine dependent methionine synthase also controls homocysteine levels in cells.

Table 1.5 A selection of physiological and clinical effects of B_{12} and vitamin B_6 (pyridoxal) and folic acid for comparison

Vitamin B_6 (Pyridoxal)	Vitamin B_{12} (Cobalamins)	Folic Acid
Boosts immunity	Energizes the body; rejuvenation	Protects against cancer
Protects against cancer	Alleviates neuropsychiatric disorders	Prevents birth defects
Relieves symptoms of pre-menstrual tension	Prevents mental deterioration; improves memory; reasoning concentration	Beneficial in treatment of mental retardation
Cures some forms of infertility	Protects against cancer, especially smoking-reduced cancer	Beneficial in treatment of arteriosclerosis
Has anticonvulsant effects	Protects against toxins and allergies	Toxic in large doses; causes severe neurological problems
Protects against nervous disorders	Needed to supplement vegetarian/macrobiotic diets	Recommended 400 mgs/day
Helps control diabetes	Non toxic	400-800mg supplemented
Protects against imbalances caused by oral contraceptives	Deficiencies produce nervous disorders, brain damage, anaemias	Toxic in high doses, causes nerve damage
Prevents skin diseases	Cofactor for only two mammalian enzymes	Reduces therapeutic effect of Levo-dopa (Parkinson's)
Inhibits cataracts	USDA recommendation	Cofactor for >60 enzymes; required for Normal DNA, RNA, Protein, Pyrimidine, Purine synthesis, and amino acid transformation
Deficiency gives anaemia, nervous disorders, skin problems		FDA 2mgs daily for adults
		Recommended maximum 50mgs/day
		Recommended 5-50mg daily
		400 mg daily cures sulphate toxicity

Significantly, many enzymes are involved in B_6 and folic acid metabolism and one would expect much more specificity to that B_{12} which is only utilized by two enzymes and is metabolised by very few. Nevertheless B_{12} seems to have profound effects on a large number of cellular and body processes and this list is growing all the time (witness cardiovascular and neurological diseases section)

1.12 Nutritional, pernicious and megaloblastic anaemias

All of these conditions are related to the lowering of the number of erythrocytes in the blood. In addition the erythrocytes that are present are macrocytic in

nature. Nutritional anaemias are caused by dietary deficiencies in B_{12}, folates or other related components. They can be alleviated by dietary supplements of the missing components. Pernicious anaemia, which is also macrocytic, results from a genetic deficiency in the protein. The protein, called Intrinsic Factor, is necessary for B_{12} absorption from the gut. The normal method of alleviating symptoms of pernicious anaemia is direct injection of B_{12} into the blood, thus bypassing the IF mechanism and its deficiencies. The biochemical cause of the megaloblastic Anaemias is the depletion of CH_3H4Folate. The latter is converted to N5, N10-methylene tetrahydrofolate, the cofactor involved in deoxyTMP synthesis from deoxyCMP (which uses thymidylate synthase, another important cancer chemotherapy target). Without dTMP, DNA synthesis and hence cell growth and differentiation are prevented.

This mechanism accounts for the effect of anaemias on erythrocyte formation and structure but does not account for the accompanying physical and psychiatric abnormalities, which eventually are manifested during anaemias. Reduced myelin synthesis (myelopathy or neuromyelopathy) a physical effect which has been attributed to changed activities of folate or AdoMet dependent enzymes, to ratios of AdoMet/AdoHcy, to changes in lipid methylmalonyl CoA mutase which yielded larger than normal amounts of propionyl CoA and malonyl CoA and hence to defects in lipid and/or amino acid (e g valine) metabolism. The fact that many neurological symptoms occur in N_2O induced anaemia implies that MMCoA mutase may not play a significant role in these N_2O does not inhibit this enzyme.

1.13 Cardiovascular disease

(McCully et al, 1969,1971, 1983, 1984, 1996; Berwanger 1995)

High homocysteine levels have now been identified as a primary cause in cardiovascular disease and as a potent risk factor of developing arteriosclerosis. Homocysteine as a risk factor is independent of the more studied factors such as cholesterol, blood pressure and diabetes. By taking specific vitamin supplements patients can normalise their Hcys levels. The first indicator of this "homocysteine versus cardiovascular disease correlation" came from an excellent straight line when homocysteine levels and cardiovascular

mortality levels were plotted for each country. Thus Finland, Ireland and Scotland exhibited both high levels whereas Spain, France and Japan had low levels of each parameter. (Dudman et al 1991,1993).

Three forms of homocystinuria occur in children with inherited enzyme deficiencies, the most common of these being a lack of cystathionine - β-synthase. (see fig 1.21) which converts homocysteine to cystathionine using serine and B_6 (PLP) as the cofactor. Such children developed arteriosclerosis at an early age and many died young of coronary heart disease or stroke. The other symptoms of this type of homocystinuria were thromboembolism and mental retardation. Ueland and Refsum (1989) have demonstrated that some patients with a defective gene for B_{12} methionine synthase exhibit similar symptoms.

When plasma levels of B_6, B_{12} and folic acid are low, patients have high Hcys and incidence of CVD (Fortin 1995). In another study (Selhab 1995), high Hcys levels and low B_6 and folate levels indicated a greater chance of carotid artery obstruction and were a warning sign for both stroke and coronary heart disease. A further clue to the role of homocysteine in cardiovascular disease came from experiments, which demonstrated that folate supplementation protected against heart disease and peripheral vascular disease. The relationship between these is seen in **fig 1.19 and 1.21** where, via the one carbon cycle and trans-sulphuration pathway, inhibition of any of the enzymes could increase homocysteine and that added folates would increase Hcys utilisation and decrease its activity.

A second cause of homocysteine elevation has been demonstrated in the genetics of the trans-sulphuration pathway. Genetic defects in cystathionine synthesis (and other enzymes) from serum and Hcys led to increased Hcys levels as well as increased CAD, thromboembolism and neurological symptoms. High levels of homocysteine in diet are also known to be toxic. Just how different homocysteine levels affect heart and blood vessels and toxicity is still a matter of conjecture. (Green 1990; Gillman 1989; Lindenbaum 1988; Nakamura 1983, 1993)

1.14 Neurological disorders

(Alarcon 1985; Algamanolis 1976; Beck 1991)

An array of diseases of the CNS and PNS have been related to the levels of homocysteine, methionine, $B_{12,}$ folates, B_6 by measuring the levels in patients and by nutritional studies. In some cases, these neurological disorders have been discovered by investigating genetic disorders. Alzheimer, multiple sclerosis, Down syndrome, neural tube defects, dementia and aids-related dementia, depression, Huntingdon's, Parkinson's, have all been related by one of the above approaches, to the activity of B_{12}MS especially the levels of the metabolites in its various dependent pathways. Much of this research is still at an early stage but it has led to the suggestion and the use of vitamin $B_{6,}$ B_{12} and folate supplements as a means of prevention and even therapeutically.

1.15 Neural tube disease(s).

(Scott 1981,1990, 1992, 1994; Lever 1986; Sturman 1978)

Because pregnant women usually have low folate levels this led not only to their taking folate supplements, but also to investigation into the role of folates, folate metabolism and associated metabolites in neural diseases. It was clearly established in neural tube defects (such as spinal bifida, anencephalis etc.) (Ehlers 1996) that there were defects in folate metabolism and possibly in DNA synthesis. Later it was also established that homocysteine levels were elevated.

Recently, it has been demonstrated that a similar defect occurs in patients with ankylosing spondulitis. Further indications that homocysteine metabolism was involved came from studies in which N_2O inhibition of methionine synthase increased the frequency of neural tube defects in mice. Embryos when treated with high homocysteine levels showed an increased incidence of neural tube defects whereas, when high homocysteine concentrations were co-administered with higher folate concentrations, the incidence of neural tube defects reverted to normal. Similar defects in the heart and ventral wall were detached in these embryos and were also prevented by folate supplements.

1.16 Multiple sclerosis

(Reynolds et al 1983,1992a, 1992b, 1992c; Ransohoff 1990; Crellin et al 1989, 1993)

MS patients have been shown to have higher Hcys and lower B_{12} levels than normals. In parallel with macrocytic anaemia patients their RBC were macrocytic, and they have lower folate levels. The reasons for these features have not yet been worked out but they do indicate a role for B_{12}MS and possibly for B_{12} levels being related to defects in myelin synthesis. Although B_{12} treatment did improve nerve function, vision and hearing, it did not improve the other MS symptoms (Bo 1994; Kira et al 1994)

1.17 Alzheimer' s disease

(Das 1979; Davies 1976; Furukama 1990; Ikeda 1990)

It is widely recognised that B_{12} deficiencies lead not only to anaemia but are linked to deterioration in mental functioning, to neurological damage and to psychological disturbances. Up to 10% of the elderly have a clear-cut B_{12} deficiencies, which accompany neuropsychiatric disorders. Recent research has shown that B_{12} deficiencies are common in the general population. Further linking of B_{12} and various neuropathies is also evidenced in the occurrence of these in persons who adopt vegetarian/vegan/macrobiotic diets (Ukeda 1990; Kristensen 1993)

After N_2O administration severe neurological problems including N_2O addiction were detected which is an indication not only of a linkage to B_{12} but to B_{12} methionine synthase. There are genetic diseases associated with B_{12}MS, cystathionine and folate metabolism and in each of these there are observed neurological disorders. As with neural tube defects and CAD, folic acid supplements can alleviate some neurological disorders.

The presence of amyloids and amyloid precursors is increased in Alzheimer brains and there is also evidence of DNA and chromosome damage.

Furthermore, studies have demonstrated low cobalamin and folate and high homocysteine levels in Alzheimer's, and that 41% of Alzheimer's dementia

patients had reduced AdoMet levels. The latter is considered causative in DNA hypomethylation and increased DNA transcription.

Although these symptoms and biochemical, cellular and structural observations have implicated B_{12} and its dependent enzymes, pathways and metabolites in Alzheimer's and other diseases, more careful and directed studies are required.

Nevertheless, it is encouraging that in a series of 39 patients, treated for neurological symptoms with B_{12} supplementation (including abnormal gait, memory loss, decreased reflexes, weakness, fatigue, disorientation, psychiatric disorders and impaired touch/perception) 100% showed improvement and in some cases, dramatic improvement.

1.18 Down's syndrome

(Chadefeaux et al 1988)

B_{12}MS has been mapped to chromosome 1 (Li et al 1996) and cystathionine β synthase to chromosome 21 (21q22.3). B_{12}MS activity is decreased and CBS increased in such patients and perturbation of folate metabolism and a methionine deficiency have both been detected (Regland and Gottfries 1992). It has been proposed therefore that the neurological problems in Down's syndrome may have mechanisms in common with Alzheimer's, Multiple sclerosis and Neural tube defects.

1.19 Aids-related dementia.

Patients with HIV exhibit disturbances in:

1) CSF levels of SAM and SAH;

2) Reduced CH_3H4tetrahydrofolate, i.e. reduced methylation;

3) High interferon levels;

4) Myelopathies that resembled the degeneration caused by B_{12} deficiencies.

The use of drugs that affect folate metabolism to alleviate the symptoms of HIV could therefore have a mechanistic foundation in B_{12} dependent pathways and enzymes.

1.20 AdoMet therapy.

Bottiglieri and associates (Bottiglieri 1991, 1992, 1994; Almario 1990; Christa 1988) have based their treatments of some neuropsychiatric abnormalities and folate deficiency with folate supplements and more recently with AdoMet. Levels of the latter, and especially the AdoMet /AdoHcy ratio, have been investigated for example in psychiatric illness and sleep disorders. There have been proposals that AdoMet synthase levels are reduced in schizophrenic patients and that brain methylation levels (due to AdoMet/AdoHcys) change during the sleep-waking cycle. AdoMet levels are also related to the brain levels of catecholamines (NA, dopamine) and serotonin. Decreased methylation in brain was found in aging patients. It has recently been shown that AdoMet metabolites (AdoHcy and 5'-methylthioadenosine) bind to the gaba A receptors in the brain with micromolar affinities and that these molecules are candidate "endogenous benzodiazepines" since the Kd values are lower than that of any other proposed endogenous benzodiazepines. (Tsvetnitsky et al 1994) AdoMet concentrations were found to be reduced in the following disorders: MS; AIDS; Dementia's; Epilepsy; Methotrexate encephalopathy. It seems reasonable that B_{12} related correlations are common to this heterogeneous set of neurological diseases. Bottiglieri et al (Bottiglieri1984) have used AdoMet directly as an anti-depressant and to improve cognitive function in dementia patients.

1.21 Vitamin B_{12}, DNA and RNA methylation and cancer.

(Chiang 1986; Hoffman 1980, 1984)

Examination of the pathways leading from homocysteine enables us to recognise that there are abundant ways in which B_{12} and homocysteine can be related to cancer – in this way cancer has much in common with abnormal brain growth and development and vitamin B_{12} already discussed.

One of the earlier indications of such a relationship came from polyamines which are elevated during cancers (Chadwick 1978; Cullis 1994) and which were known to be affected by ODC, SAM decarboxylase and polyamine synthase of this pathway (Fredlund 1995). A second indication was the widespread use of inhibitors as potential anti-cancer agents (Pascale 1992; Porter 1986; Lipinski 1984).

A second set of pathways that utilize B_{12} and Hcys and have been related to cancer onset and chemotherapy are the pathways involved in metabolism of folates. Thus the enzymes of the One-C-cycle, the enzymes for folate interconversion and biosynthesis and, in particular the enzyme dihydrofolate reductase (which converts deoxy UMP to deoxy TMP via folate dependent methylation) are all critical for DNA synthesis and DNA expression. This enzyme was found to be particularly important in this regard, since it is the only source of H4THF and hence deoxyTMP that is required for DNA synthesis.

However, methylation of nucleosides, nucleotides and nucleic acids is much more widespread than above and methylation plays several critical roles in the structure and biosynthesis of DNA and all forms of RNA. These methylations depend directly on B_{12} (via SAM-methylases) or indirectly (via folate dependent methylases) (Progibny 1995; Wainfau 1992; Weir 1988, 1992; Zapisek 1992).

Discrete "CpG islands" exist throughout the genome and most of these cover one or more exons. Thus CpG islands are landmarks and indicative of gene promoters and enhancers. 5'methyl cytosine is found at many CpG islands and is designated mCpG and about 60%of all CpGs are methylated in animals.

The connection of CpG islands and mCpG with gene expression (Balaski 1993) and with cancer is now well established. Generally tumours exhibit substantial DNA hypomethylation. More specifically, at c-Fos promoter sites, mCpG versus CpG had little transcription activation. Activation of some genes has been attributed to critical mCpG loci; inhibitors of methylases (5'-aza C and 5'-aza-2' deoxy C) activate genes and cellular differentiation, whilst methylated T-factors (DNA binding proteins) specifically recognised mCpG sites and influence transcription.

Many have proposed that mCpG sites should be considered as "mutational hotspots" and discrimination (which converts mC -> T) are found in mutations of human tumour suppressor genes. Immortalised cell lines (from tumours) have high levels of de novo CpG island methylation. The evidence is therefore more than circumstantial that DNA methylation, especially at CpG islands, is related to gene expression, action of transcription factors, and to cell growth and differentiation. Hypomethylation and hypermethylation, often at specific DNA loci, are also related to abnormal cell growth and differentiation and to cell immortalisation and to cancer.

Viral genes are not generally methylated but upon integration into host genomes, they become methylated. Thus viral DNA methylation has been related to viral latency.
RNA methylation is also important for normal and abnormal cell growth and differentiation but is more complex than DNA methylation. AdoMet dependent RNA methylation occurs in rRNA, mRNA, tRNA and small nuclear RNA. Dependent upon the RNA type, it occurs at guanine (m7G), adenine (m6A), ribose ring (2'-O-methyl), and phosphate (α-methyl phosphate); formation of trimethyl-N7-guanosine is also known. The physiological roles of RNA methylation depends on the RNA species and can affect ribosome assembly, function, transport to the cytoplasm, and interaction with cellular factors. In the case of mRNA, the nascent transcript (called hnRNA) is "capped" at its 5'-end with m7G(5')N. These hnRNA transcripts are degraded in the nucleus (80%) but 20% are processed and transported as mature mRNA to the cytoplasm to

be translated into proteins. The methylation of the cap G is carried out by AdoMet dependent methylases. The precise role of base and ribose methylation of RNAs is not fully known but it is important in cell proliferation and differentiation has been confirmed using inhibitors of methylation and genetic manipulations.

It is too complex for this dissertation but other methylated species have been found in various RNAs and have been designated m1A, m2A, m1G, m2G, m_2G, m6A, m_26A, m5C, m3C, m3U, 2-0-CH_3-A, 2-0-CH_3-G, 2-0-CH_3-T, etc.

1.22 Homocysteine and B_{12} related intracellular signalling network.

Examination of the sets of metabolites that depend upon Hcys and B_{12}MS activity fig 1.22, reveals that, at least intracellularly each metabolite set has components that have been proposed as intracellular signals.

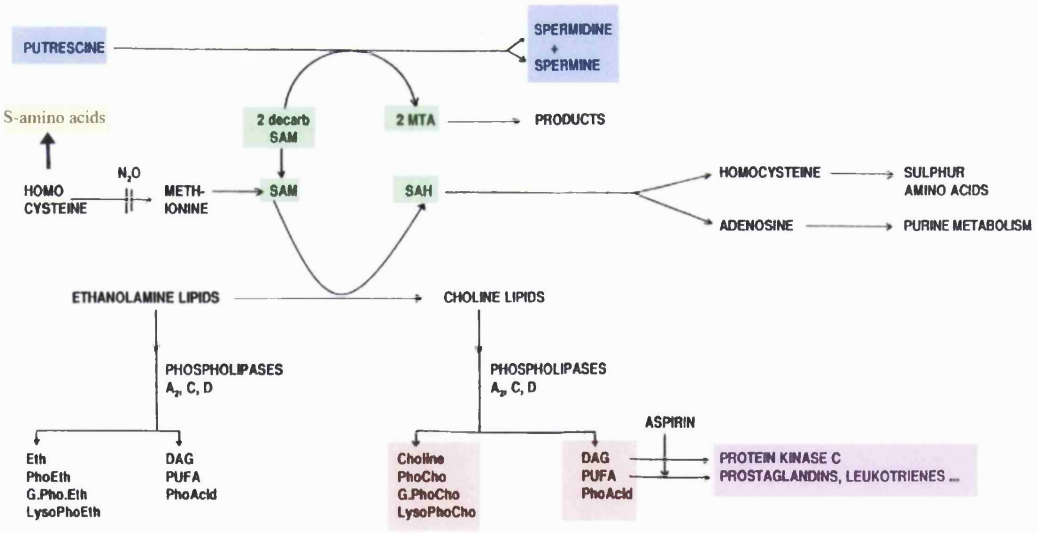


Figure 1.22 Diagram emphasising the several sets of metabolites formed from the b12 ms reaction as the primary step.
Essentially all metabolites in each family (coloured) have proven to be involved as extra cellular and or intracellular signals

Polyamines (section 1.9) are established modulators of some neuroreceptors and ion channels and, furthermore, interact with signalling kinases. That polyamines affect cell proliferation and differentiation and that polyamine levels vary systematically throughout the cell cycle is firmly established. Feedback control of $B_{12}MS$ by polyamines at physiological concentrations was discovered by Kenyon et al. The evidence that polyamines act as intracellular signals is therefore convincing. Inhibitors design to control cell division and cancer by interfering with specific enzymes in the convergent pathways from homocysteine and ornithine to the three polyamines infers the truth of this proposal.

Signalling kinases and phosphates and their effect on the enzymes of the B_{12} network of pathways and enzymes is almost an untouched field of research. Preliminary data from the dissertation of Kenyon (1997) is evidence that, in vitro at least, $B_{12\,MS}$ is modulated by several such kinases.

Lipid and eicosanoid signaliing moiecules are generated from homocysteine and $B_{12}MS$. These are generated, as shown, by a combination of a pathway consisting of $B_{12}MS$, SAM synthase, phospholipid-N-methylase, phospholipases A_1, and A_2(which generate choline lysolipids, polyunsaturated and saturated fatty acids), cyclooxygenases and lipoxygenases (which convert polysaturated fatty acids into eicosanoid signalling molecules). The evidence is therefore convincing that homocysteine and $B_{12}MS$ are the first molecule and enzyme respectively in a pathway that leads, via phospholipid methylation, to a family of firmly accepted lipid cell signalling molecules. The receptors for many of the latter have lately been identified and their genes cloned (**fig** 1.23).

A family of sulphur containing amino acids (Hoshiya 1995; Horowitz 1981; Griffiths 1991; Huxtable 1982; Finkelstein 1990; Pisi 1990; Refsum 1990, 1991) with varying extents of sulphur oxidation is generated from homocysteine via the trans-sulphuration pathway. These sulphur amino acids have been variously referred to as neuromodulators or neurotransmitters. Certainly they affect the action of many of the common neurotransmitter receptors and ion channels. Till recently they also have been referred to as simply "sulphur

containing metabolites" related to the formation and degradation of the amino acids cysteine and taurine and the peptide glutathione. The activity of taurine although present in most cells at concentrations comparable to ATP (millimolar), is controversial for it has been proposed variously as a neurotransmitter, cellular osmoregulator and indicator of cellular toxicity. Glutathionine too has been assigned numerous cellular roles including transport of amino acids, antioxidant and modulator of cellular redox reactions. The family of sulphur amino acids, peptides and the various sulphur moieties generated by these pathways are therefore more than simple metabolites within a pathway, but have important cellular and intracellular roles? One of these roles is probably intracellular signalling for they are found ubiquitously in cells and not simply in neurons and they affect common amino acid neurotransmitter functions.

Controls of gene expression, particularly by cytokines (extemal control) and by transcription factors (internal control) is related to the activity of B_{12}MS and the enzymes and metabolites generated in its network of pathways as shown in **fig**1.19 and 1.22. This type of cell signalling is related in part to homocysteine/B_{12}MS biological methylations of DNA and RNA, particularly at CpG islands and gene control elements (induces and promoters). It may also be related to the set of intracellular signals generated by the B_{12}MS homocysteine network. This latter hypothesis has some evidence, as described above, but much cell cycle, cell signalling and gene expression research focusing on the homocysteine B_{12}MS network is required.

Nitric Oxide (NO) is a gaseous signalling molecule generated by the action of nitric oxide synthase (NOS) on arginine to generate NO and citrulline as products (fig 1.23) (Ignarro 1990, 1994; Garthwaite 1988, 1994, 1995; Knowles 1992; Wood 1994; Zhang 1993, 1995)

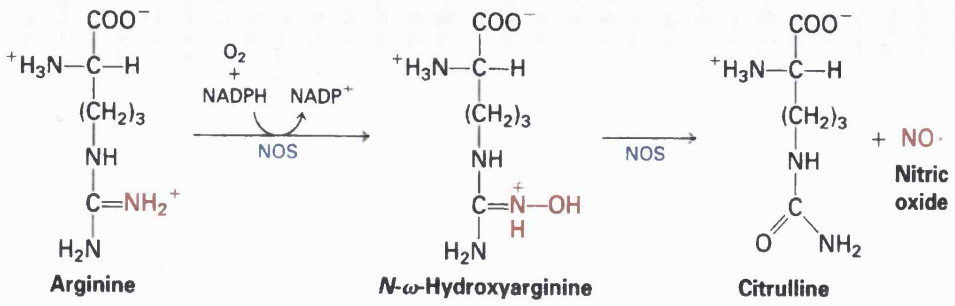


Figure 1.23 The enzymes substrates and products involved in Nitric oxide biosynthesis

NO has many proposed modes of action including (fig 1.24):
activation of guanylate cyclase to increase intracellular cyclic GMP levels;
generation of a family of adducts between different intracellular species of O_2 and NO (for example $NO.O_2^-$) (Beckman 1990; Goldstein 1996; Hansen 1993)
inhibition of metalloproteins and metalloenzymes containing mainly transition metal ions (for example belonging to the mitochondria respiratory and other intracellular redox chains and cytoplasmic enzymes).

Oncogene expression	Calcium flux	NMDA receptor
Kinase C	Kinase A	Ca/CAM Kinase II
Hypertension	Proteinuria	Oedema
Ischemic stroke	Long term potentiation	Learning impairment
Depression	Pain	Anaesthesia
Sleep	Insulin dependent diabetes	Arthritis
High pressure nervous syndrome	Oxygen toxicity	Lung disease

Table 1.6 Selected biological actions of NO

At the cellular and biological levels, NO can affect, directly or indirectly, many processes, such as chemotaxis, secretion, and long term potentiation etc. Not surprisingly the Nobel Prize was awarded for research in this field, although Moncada and co-workers also made pioneering contributions to the discovery, modes of action, and therapeutic importance of the NO signalling pathways (Boulton 1995; Angaard 1994; Brown 1995; Brune 1989; Dawson 1995; Faraci

1994; Hibbs 1987, 1988, 1990; Hobbs 1994; Kendrick 1994; Marletta 1988; Moncada 1991,1993, 1995; Oury 1992; Palmer 1987).
At the writing of this thesis the relationship between the NO signalling and homocysteine/B_{12}MS signalling systems is not yet established, but as seen in the final section of this introduction, that link may well exist, since both NO and N_2O (nitrous oxide) both inhibit B_{12} methionine synthase in a dose-dependent manner.

Common signalling systems that depend on Ca^{2+} concentrations, MAP kinases, oncogenes, antioncogenes and their coded proteins, phosphoinositol and other lipid signalling networks, have not been discussed here for, as yet, no tangible or even tenuous connection to homocysteine, B_{12}MS or the B_{12} dependent network of metabolites has been firmly established or even investigated.

Nitrous Oxide (N_2O) inhibition of B_{12} dependent methionine synthase
Nitrous oxide was known as an anaesthetic but modem research has established that its principal mode of action is that of a gaseous analgesic, the only example of its class. There are many side effects of N_2O such as addiction, tolerance etc. which greatly resemble the side effects of morphine. Equally, antagonists of morphine, such as naloxone, also antagonise the action of N_2O despite the fact that N_2O does not act directly at the morphine beta, kappa and delta receptors. A comparison of the effect of N_2O on morphine-induced cell signalling has not been carried out despite the obvious similarities both in their analgesic properties and physiological side effects.

Scientists in London (Chanarin 1985, 1989, 1991, 1992; Deacon 1980, 1982, 1990) were the first to recognise that the N_2O induced anaemias were similar to other anaemias. They proposed and demonstrated the N_2O acted by inhibiting B_{12} methionine synthase. They took their research further by proposing a "methyl trap hypothesis" to explain that inhibition of B_{12}MS by N_2O affected the levels of biological methylation via folate metabolism, as well as causing anaemias etc. However, no attempts were made to rationalise either: the side effects of N_2O; the resemblance between the various physiological effects of

N_2O and morphine; since aspirin and the NSAID's are also powerful analgesics, no attempt were made to compare and rationalise the efforts of N_2O (and morphine) with those of aspirin and NSAID's.

Morphine acts, through its receptors, to control the levels of intracellular calcium and cyclic AMP. Apart from preliminary experiments demonstrating that cyclic cAMP-dependent kinase affects the activity of B_{12}MS, no relationship between the Ca or cAMP signalling networks and the homocysteine/B_{12} network has been established.

It is however possible, even likely, that the analgesic effects of aspirin and those of N_2O have common elements and that the latter is the existence of their respective target enzymes in a single metabolic pathway leading from homocysteine to the eicosanoids as shown in **fig** 1.25

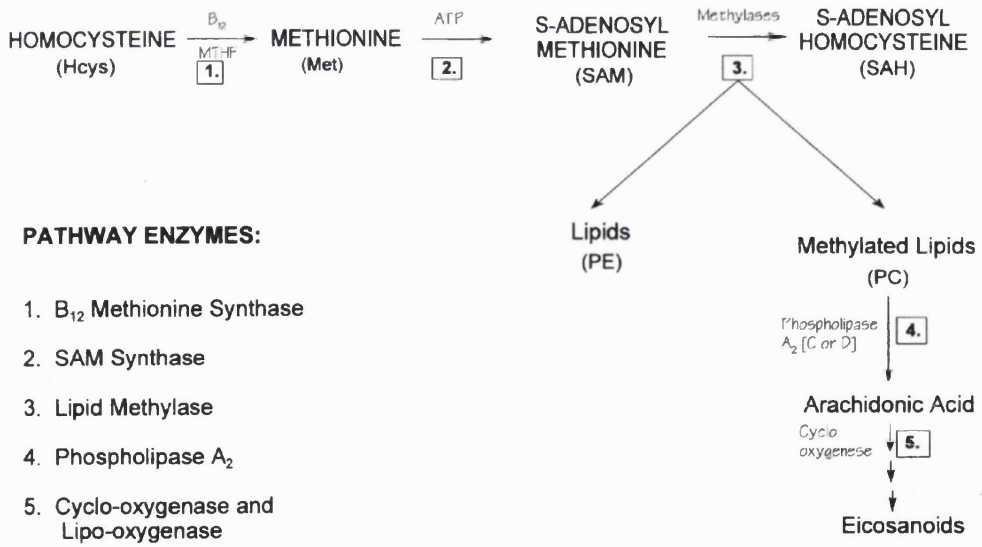


Figure 1.24 Diagram emphasising the biosynthesis of eicosanoids from homocysteine and phospholipids especially unsaturated phospholipids

The mechanisms of action of N_2O on B_{12}MS was confirmed by Matthews, Banerjee and their co-workers. A significant part of this study was to compare

the actions of N_2O and possibly NO on brain B_{12}MS and to compare the effects of NO and N_2O on the levels of cyclic AMP and cyclic GMP, and to investigate the possible role of NO signalling in diving physiology. This necessitated the use of both cellular and purified preparations of brain B_{12}MS, which therefore evolved as a major part of this thesis.

1.23 Objectives

It is intended that this review of the structure and function of vitamin B_{12}-dependent methionine synthase and its dependent or contiguous metabolic pathways, enzymes and metabolites although concise, provide a basis for a better understanding of the role of this enzyme in health and disease. I also attempted in light of this to briefly review the vast field in which vitamin B_{12} and the two mammalian enzymes which utilize it as a cofactor, have become identified as causative or correlative factors in clinical diseases. These fields are expanding rapidly. The primary objectives of this thesis, in view of the above, were therefore:

To isolate and purify B_{12}-dependent methionine synthase from porcine brain and characterize its active forms.

To characterise and compare the mass, pI, Km and Vmax of the brain B_{12}MS with other B_{12}MS enzymes.

Because of its importance in neural cell growth and differentiation, neural development and the many correlation's of its activity with neuropathological conditions, it was considered important to characterise the brain enzyme. Because of its very low concentration in the brain, this was a logistical and slow process. Furthermore, other groups during the conduct of these experiments published the gene and partial protein sequence of the liver enzyme and compared it with the E.coli enzyme. We planned to use antibodies against E.coli and later the liver B_{12}MS to assist in the purification of the brain enzyme but were uniformly assured this has been attempted unsuccessfully in the USA and elsewhere and there was no cross-reactivity. This turned out not to be the case but was too late for this thesis. It took 6 months to obtain synthetic peptides, to obtain antipeptide antibodies and to characterize these antibodies by cross reactivity with mammalian enzymes. Two of the three antipeptide

antibodies were recognised by the liver enzyme, but these came too late to be used in this thesis.

N_2O and NO both have significant neurological symptoms which served as a basis for establishing whether or not some, at least, of these symptoms were explicable by inhibitor of similar enzymes and pathways. This led to the further experiments to compare N_2O and NO as possible inhibitors of cyclic GMP. Finally, since diving physiology and diving are major hobbies, I felt it important to investigate the role of NO in human volunteer divers. The secondary objectives of this thesis were therefore:

To compare the inhibition of brain $B_{12}MS$ by nitrous and nitric oxides and therefore to correlate $B_{12}MS$ with common signalling molecules and systems, especially the NO/cyclic GMP system.

To investigate the possible role of NO in situations related to change in oxygen tension such as SCUBA diving.

Chapter 2 Materials and Methods

2.1 Materials

SIGMA:

DL-homocysteine, S-adenosyl L-methionine (iodide salt), beta-mercaptoethanol, methyltetrahydrofolate (barium salt), DEAE cellulose, sodium nitroprusside, PMSF, TLCK,trypsin inhibitor, aprotinin, Chelating resin (C7901), adenosine, morphine, S-adenosyl homocysteine, methyl-thioadenosine, Fura-2

Amersham:

cGMP radioimmunoassay kit, L-[2,3,4,5-3H]Arginine (53 mCi/mmol), [14C-methyl]tetrahydrofolate (barium salt) (56mCi/mmol)

BioRad:

Hydroxylapatite, TEMED, ammonium persulfate, acrylamide, bis-acrylamide, sodiumdodecylsulfate AG1-X8 resin (200-400 mesh chloride form), Bradford's reagent kit, Bio-lyte ampholyte 3-10 and 4-6

Pharmacia:

Q-sepharose fast flow, phenyl sepharose fast flow, Mono-Q, S300, and Superose 12, Multiphore 7.5% SDS precast gels, molecular weight standards

Amicon:

XM-50 membrane (50kD cut off); YM-100 membrane (100kD cut off); Centricon 30(30 kD); Ultracon 30 kD ultra concentrators

Aldrich:

Coomassie Blue g-250

Pierce:

Silver stain kit

BDH:

All buffers and salt were of Anal-R purity

Millipore:

All water was deionised and had a resistance of 18 Mega Ohm.

Merck:

Nitric oxide gas

BOC:

Nitrous oxide, oxygen, nitrogen, CO_2

2.2 Methods

2.2.1 B_{12} Methionine Synthase (B12-MS) assay

Methionine synthase enzyme activity was assayed by a modification of the method of Weissebach et al 1967. that measured the formation of [^{14}C] methionine after the separation of products and substrate on an anion-exchange column. The difference in charge of the product and reactants was exploited by use of an ion exchange column. Unreacted N5-[methyl-^{14}C] methyl-tetrahydrofolate was retained on the column while the newly formed radiolabelled methionine was eluted. The reaction mixture consisted of: 40 μl DL-homocysteine (400 μM); 20 μl N5-[methyl-^{14}C] methyl-tetra-hydrofolate (100 μM); 10 μl S-adenosyl methionine (300 μM); 10 μl μ-mercaptoethanol (125mM); 100 μl potassium phosphate buffer, pH 7.4 (50 mM); and 100μl of enzyme sample. In order to reduce oxidative inactivation of the enzyme by air, the reaction was performed in sealed serum vials flushed with nitrogen for 30 seconds. As the Vitamin B_{12} cofactor was light sensitive the incubation at 37°C were carried out in the dark for 60 minutes. The reaction was terminated by the addition of 400 μl of ice cold distilled water and then applied to a 0.5 x 5, cm column packed with Bio-Rad AG 1-X8 resin equilibrated with water. The [^{14}C] labelled methionine was eluted 2x 1 ml water. After the addition of 12 ml scintillation fluid the sample was counted using a Beckman scintillation counter.

2.2.2 Preparation of pig brain cytosol

Brains were obtained from twelve male 2-year-old pigs were killed via stunning and ex-sanguination. Immediately after the brains were harvested and placed on ice before transport. 1.87 kg of pig brain was homogenised in ice cold homogenisation buffer (50 mM K_2HPO_4, 0.2 M NaCl, pH 7.4). In order to prevent proteolytic degradation of the enzyme the following protease inhibitors were added to the buffer: 10 μg/ml Aprotinin (serine proteases); 50 μM TLCK (Trypsin like serine proteases); 10 mg Trypsin inhibitor; and 1.0 μM PMSF. The homogenate was then centrifuged at 2000g for one hour, the supernatant was removed and then re-centrifuged at 30,000g for another hour then finally 100,000g for one hour (all centrifugation at 2°C). The cytosol once produced was then immediately transferred for batch chromatography separation.

2.2.3 *S-200 gel filtration*

Fresh pig brain cytosol was prepared as described and immediately run on a Pharmacia S-200 column (100 x 1.6 cm) at 2 ml/min, running buffer 20mM K_2HPO_4, 150mM NaCl, pH 7.2. One ml of sample was loaded and 4ml fractions collected then tested for methionine synthase activity.

2.2.4 *DEAE cellulose chromatography*

500 grams of Sigma DEAE (diethylaminoethyl) cellulose, a weak anion exchanger, was washed and equilibrated with 1 litre of homogenisation buffer (50mM K_2HPO_4, 0.2M NaCl, pH 7.4) and chilled to 4°C. To this slurry was added two 2 litre aliquots of freshly prepared brain cytosol. The mix was stirred with a magnetic stirrer and kept chilled to 4°C throughout the separation. After one hour of equilibration, the unbound protein was removed by vacuum filtration. The cellulose was then washed with a further 2 l of buffer to remove unbound protein. The protein was eluted with 2x 1 litre washes of 0.5 M NaCl (50mM K_2HPO_4, 0.5 M NaCl, pH 7.4) and 2x 1 litre washes of 1.0 M NaCl. (50mM K_2HPO_4, 1.0 M NaCl, pH 7.4). All the fractions were assayed and protein contents determined. The active fractions were then pooled before continuing to the next step in purification.

2.2.5 *Q-sepharose chromatography*

The active fractions from the DEAE batch separation were diluted with 50 mM K_2HPO_4, pH 7.4 to achieve a NaCl concentration of 0.25 M. This solution was then loaded onto a large (100 x 1.6 cm) column packed with Pharmacia Q-sepharose fast flow resin. The sample was chilled on ice and loaded at 10 ml/min over 400 minutes. The column was cooled to 4°C throughout and light excluded to maintain enzyme activity. Once loaded onto the column the enzyme was eluted with a variable linear gradient of increasing concentration of NaCl:

- Buffer **A** was 50 mM K_2HPO_4, 0.1 M NaCl pH 7.2
- Buffer **B** was 50 mM K_2HPO_4, 1.0 M NaCl pH 7.2.

The flow rate was 4 ml/min and 15 ml fractions taken. The gradient profile was controlled by a Pharmacia FPLC and detected at 280 nm using an online

detector. All fractions were tested for methionine synthase activity and their protein content determined.

2.2.6 Ammonium sulfate precipitation

The active fractions from the Q-sepharose column were pooled and an ammonium sulfate precipitation carried out. Previously it had been determined that the enzyme precipitated between 35-45% saturation of ammonium sulfate. The active fraction were made up to 25% (1.0 M) ammonium sulfate by the slow addition of the salt while maintained at 1°C and constantly stirred. After 1 hour equilibration the sample was centrifuged at 50,000g to remove precipitated protein. The still active supernatant was then applied to hydrophobic interaction column.

2.2.7 Phenyl sepharose chromatography

The active supernatant was applied at 6ml/min to a (50 x 1.6 cm) Pharmacia Phenyl sepharose hydrophobic interaction column equilibrated with 0.5 M ammonium sulfate, 50mM K_2HPO_4, pH 7.2 (Buffer **A**) and eluted with a linear gradient of decreasing concentration of ammonium sulfate controlled by a Pharmacia FPLC. The elution buffer was 10 mM K_2HPO_4, pH 7.2 (Buffer **B**). Fractions were taken every 15 ml and were tested for protein content and methionine synthase activity.

2.2.8 Ultra-filtration

The active fraction from the Phenyl sepharose column were pooled and placed in a 250 ml Amicon ultrafiltrator and four times filtered use a 50 kD cut off, filtration membrane (XM50). The sample was filtered at 4°C to remove smaller proteins and finally made up with the equilibration buffer for the hydroxylapatite chromatography. A 100 kD (XM100) Amicon membrane was used previously but methionine synthase activity was found in the eluent passing through the filter at this stage.

2.2.9 *Hydroxylapatite chromatography*

The supernatant retained by the 50 kD ultra-filtration membrane was loaded at 2ml/min onto a (10 x 1.6 cm) BioRad Hydroxylapatite (HTP) column equilibrated with 20 mM K_2HPO_4, pH 7.2 and eluted with a linear gradient of increasing concentration of K_2HPO_4,(500 mM K_2HPO_4, pH 7.2) over 40 minutes. Fractions were collected every 4ml and tested for methionine synthase activity and protein content. The active fractions from the HTP column were filtered using an Amicon 50 kD ultra-filtrator as a method of buffer exchange in preparation for the next step in purification. A sample of each fraction was run on a 7.5% SDS PAGE gel to determine purity and molecular weight of the active fractions.

2.2.10 *Mono-Q chromatography*

The active fractions eluted from the HTP column, having undergone filtration and buffer exchange, were applied at 1 ml/min to a Pharmacia Mono-Q high resolution column (5 x 0.5 cm). The column was equilibrated with 20 mM K_2HPO_4, pH 7.2 and eluted using a linear gradient of increasing concentration of 1.0 M NaCl, 20 mM K_2HPO_4, pH 7.2 over 60 minutes. Fraction were taken every 2 minutes and tested for methionine synthase activity and protein content. A sample of each fraction was run on a 7.5% SDS PAGE gel to determine purity and molecular weight of the active fractions.

2.2.11 *Superose 12 gel filtration*

The active fractions from the Mono-Q column were concentrated to 200□l using a 5ml Amicon ultra-filtrator. 100μl of this sample was then loaded onto a previously calibrated (28 x 1 cm) Superose 12 gel filtration column at 0.25 ml min. The running buffer contained 20 mMK_2HPO_4, 150 mM NaCl, pH 7.2. Fractions were taken every 1ml and tested for methionine synthase activity.

2.2.12 *Protein content*

The dye reagent is a mixture of phosphoric acid and Coomassie stain purchased as a kit from BioRad and based upon the method developed by

Bradford in 1976. The standard reagent was diluted five fold before use and the individual samples prepared as follows: 100 μl of sample and 5ml of diluted reagent was placed in a 5ml cuvette and allowed to incubate for 30 minutes before they were analysed using a Pharmacia UV spectro-photometer at 595nm versus a reagent blank. The unknown concentration of protein in the sample was read from a standard curve calibrated with known concentrations of bovine serum albumin. This assay was used for concentration of protein from 200-1400 μg/ml. When concentrations of protein exceeded this value they were diluted with buffer and re-analysed.

When samples were found to have less than 200 μg/ml an alternative micro-assay was used 0.2 ml undiluted reagent and 0.8 ml of sample were placed in a 1 ml cuvette and incubated for 30 minutes before being read at 595nm. This method permitted measurements between 1-25 □g/ml of protein.

2.2.13 Polyacrylamide gel electrophoresis

Samples throughout the purification were tested for purity and molecular weight using a non-reducing 7.5% sodium-dodecyl-sulfate (SDS) polyacrylamide gel run on BioRad Mini-Protean II slab gels. The gel was cast using a discontinuous method in which a less dense stacking gel (4%, pH 6.8) and a resolving gel (7.5%, pH 8.6) were used to increase the focusing of the protein bands within the gel. The gel was run using known molecular weight standards (Sigma) and each lane was loaded with approximately 5 μg dissolved in 40μl of sample buffer containing SDS. The samples were heated to 95 °C for 10 minutes in an automated heat block to ensure denaturing. The gels were then fixed with a mixture of methanol and acetic acid before staining with Coomassie Blue G. Some gels were then later stained using a Pierce GELCODE silver staining kit to increase sensitivity. Some pre-cast gels were used and run on a Pharmacia Multiphor system. These gels allowed higher levels of protein loading and consistency to be achieved.

2.2.14 Native gels (electro-elution)

A semi-purified sample taken from the Phenyl sepharose step of purification was run on a native discontinuous 7.5% polyacrylamide gel. The gel was then cut into 1 cm vertical sections and the protein eluted using an amicon elctrophoretic protein elutor. The eluted sample was then assayed for methionine synthase activity.

2.2.15 Iso-electric focusing (Rotofor)

The Rotofor produced by BioRad is a preparative iso-electric focusing apparatus capable of handling large volumes of sample and allowing the temperature to be maintained via a cooling rod inserted into the chamber. The separation chamber is rotated to prevent precipitation of the samples prior to their reaching their pI. The sample (10 ml) was first desalted using a Pharmacia G25 column equilibrated with 5 mM K_2HPO_4, pH 7.2. The Rotofor was prepared by prefocusing the Biolyte ampholytes (2% w/v) range between 3-10. Prefocusing was carried out for 1 hour at 550 volts and 25 mA. Once the pH gradient had been formed the sample was injected into the chamber at around pH 7.0. The Rotofor was then run for 6 hours with a final voltage of 1200 volts. The Rotofor, which contains 20 cells, allows the fractions to be harvested quickly by vacuum filtration into individual sample tubes. The samples were tested for pH and then adjusted back to pH 7.2 and the protein resuspended. Methionine synthase activity was then tested for as described earlier. Once the initial focusing using ampholytes with a range of 3-10 and the approximate pI of the protein determined the procedure was repeated using ampholytes with a range of 4-6 to more accurately determine the pI of methionine synthase.

2.2.16 Cyclic GMP radioimmuno-assay

In order to be able to measure any physiological changes involving guanylate cyclase activation a "*Cyclic GMP* [^{3}H] *assay system code TRK 500*" manufactured by Amersham was used.

Four male rats were killed by cervical dislocation and the brains dissected. The brains were placed in ice cold washing buffer (0.32 M sucrose, 10 mM Hepes,

0.1 mM EDTA, pH 7.0) and minced. The tissue was then washed to remove any excess haemoglobin and red blood cells. The tissue was then washed three times in homogenisation buffer (0.32M sucrose, 10 mM Hepes, 1 mM DL-dithiothreitol, pH 7.0). The tissue was made up to 50 ml of homogenisation buffer and homogenised using a Potter Elvehjem homogeniser with a Teflon pestle driven by an electric motor (approx. 20 strokes). The homogenate was then centrifuged at 10,000 g for 15 minutes, the supernatant was kept and then centrifuged at 100,000 g for 30 minutes.

The guanylate cyclase assay was initiated by the addition of 50 μl GTP solution (25 mM Tris, 5 mM GTP, 5 mM $MgCl_2$, 1 mM 3-isobutyl-1-methylxanthine, pH 7.2 at 37 °C) to 150 μl of cytosol solution. The reaction was terminated by the addition of 20 μl of 20% perchloric acid ($HClO_4$) and cooled on ice. The samples were then spun at 10,000 g for 1 minute to remove the precipitated protein.

Samples of cytosol and GTP were incubated in serum vials blown with NO, Nitrous oxide, and nitrogen. Various concentrations of NO solution and SNAP were also used to study their possible dose dependent reaction with guanylate cyclase.

Cyclic GMP levels were measured by radio-immunoassay in aliquots diluted 50:1 in buffer (25 mM Tris, 4 mM EDTA, pH 7.2 at 0 °C). 100 μl of the diluted samples were pipette into the radioimmunoassay solution (50 μl [3H] cGMP (0.008 Ci), 50 μl anti-cGMP antibodies). The assay mixture was then vortexed and incubated at 2°C for 90 minutes. 1 ml of ice cold 60% ammonium sulfate solution ($(NH_4)_2SO_4$) was then added, after 5 minutes of incubation the tubes were centrifuged at 10,000 g for 2 minutes. After all the supernatant had been removed, 1.1 ml of distilled water was added and the precipitate dissolved. Of this final solution, 1 ml of liquid was mixed with scintillant and measured for radioactivity using a scintillation counter. The radioactivity from the unknown samples was then plotted against a standard curve of known concentrations of cGMP to calculate the concentration of cGMP in pico moles. The standard

curve for the cGMP RIA assay allows concentrations of cGMP to be measured between 0.5 - 8 picomoles.
By diluting the sample with an appropriate buffer it was possible to measure the physiological concentrations of cGMP, following a 50-fold dilution of the cytosol giving cellular concentrations of between 25-200 picomoles. When the levels of guanylate cyclase activity was measured in the cell it showed a reproducible level as compared to the assay performed in the absence of GTP. Several experiments were performed using saturated NO solutions (3% = 3μm NO). Serial dilutions of this NO solution were used to study the stimulation of guanylate cyclase by NO. Nitrous oxide gas was used to study any possible inhibitory effects on guanylate cyclase and the sealed serum vials were blown with N_2O gas provided by British Oxygen Corporation

2.2.17 NO and N_2O inhibition of methionine synthase

Inhibition of methionine synthase was studied in a time and dose dependent manner using nitric oxide and nitrous oxide as inhibitors.

A saturated nitric oxide (NO) solution was produced by bubbling NO through a previously de-oxygenated sample of assay buffer (50 mM K_2HPO_4 buffer, pH 7.4) for 1 hour. This gave a saturated NO solution of approximately 3% (v/v) equal to 1.3μm. Different concentrations of NO solutions were achieved by performing serial dilutions of the saturated solution. In order to study the inhibition more accurately later studies involving the rat liver enzyme used degassed homocysteine, and degassed buffer prior to injection of the NO via a gas tight syringe. This ensured that oxidation of NO did not decrease its efficacy as an inhibitor of methionine synthase. The methionine synthase assay was then performed as described previously.

Varying concentrations of nitrous oxide (N_2O) were produced by mixing the gas with pure nitrogen using a commercial anaesthetic trolley. A N_2O: N_2 ratio of 9:1 to 1:9 was achieved by this method. The gas was flushed into the top of a sealed serum vial before incubation. The methionine synthase assay was then performed as described previously.

2.2.18 Capillary zone electrophoresis of proteins

Analysis of purity of the methionine synthase enzyme was determined using a Waters capillary electrophoresis apparatus coupled with a Diode array detector. Detection was at 280 and 365 nm. The samples were injected into a 75 μm internal diameter fused quartz capillary 50 cm in length. The voltage during separation was a linear ramp of 2.5-30 kV. Samples were introduced to the column via electrokinetic injection (2.5 kV for 10 seconds) and run for 40 minutes. The capillary was cooled internally to 4°C and the running buffer was 20 mM Tris , pH 8.6.

2.2.19 Analysis of NO production by mast cells

The effects of pressure and partial pressure of oxygen on the production and release of NO in mast cells was studied by taking cultured mast cells and subjecting them to different concentrations and pressures of oxygen and nitrogen. The cells were grown and harvested over a three day period in individual cell culture dishes. Each dish was then subjected to either to air in a sealed pressure chamber at 1 bar, oxygen at 1bar, oxygen at 10 bar, nitrogen at 1 bar, and nitrogen at 10 bar.

The cell medium was then sampled and analysed for levels of nitrates using Greiss reagent. 250μl sample of media and 250μl Greiss reagent (5% H_3PO_4, 1% sulfanilic acid, 0.1% N-1-napthethylendiamine). The sample was measured in 1ml cuvettes at 554 nm.

2.2.20 NO assay tissue

The assay used to measure nitric oxide synthase was derived from a method developed by Garthwaite et al 1988. By using radio-labelled L-arginine and measuring the conversion to L-citrulline, the level of NO synthesis can be estimated. 2 male Wistar rats (350 grams) were killed by CO_2 asphyxiation and their brains removed. The brains were immediately washed in ice-cold washing buffer (10mM HEPES, 0.32 M Sucrose,0.1 mM EDTA, pH 7.4) to remove excess blood and then homogenised in ice cold homogenisation buffer (10mM HEPES, 0.32 M Sucrose, 1mM DL-dithiothreitol, pH 7.4) using a Potter

Elvehjem homogeniser. The sample was then centrifuged (1400g x 10 min 18,000g x 10 min) to produce a crude synaptosomal pellet. The synaptosomes were then disrupted by the addition of 1mM DL-dithiothreitol and centrifuged at 150,000g for 30 minutes. The supematant was then passed through a chelating resin to remove endogenous divalent cations and arginine.

To an open serum bottle, 50 μl of sample, 20 μl arginine (0.2 μM), 10 μl NADPH (1.0 mM), 10 μl calmodulin (1.2mg 250 μl), 10 μl (1mM $CaCl_2$), and 100 μl Tris buffer (25 mM Tris pH 7.0) were added. The vials were then incubated at 37°C for 10 minutes in a pressure chamber under varying concentrations of oxygen and nitrogen. The reaction was terminated by the addition of 100 μl of stop buffer (pH 5.5 10 mM EDTA, 25 mM Tris). The separation of reactants and products was achieved by applying the mixture to an ion exchange column and scintillant counting of the eluent.

2.2.21 Hyperbaric chamber protocol

Five volunteers were selected according to the following criteria: male; age 20-30; non-smoking; SCUBA divers. All volunteers had recently undergone a diving medical and were physically fit on the day of the trial with no current illness.

All the subjects were briefed as to the safety procedures and had previous experience in the use and operation of hyperbaric chambers. A medical release statement was signed and medical ethics approval gained before experimentation. Before compression all the subjects were fitted with a Venflon venous catheter on their left arm by qualified medical personnel. The catheter was fitted to ease the collection of blood samples while at pressure.

Blood samples were then taken at intervals as described in table 2-1. The total time at a pressure of 2.8 Bar was 115 minutes of which 60 minutes were spent on oxygen at 2.8 Bar. Samples were immediately decompressed and centrifuged at 10,000 g for 10 minutes to remove the cellular fraction.

Run time for dive in minutes	Actual time spent at each stage of the compression schedule	Samples removed
-30	Pre dive breathing air	Blood sample 1
0	Breathing Oxygen at 1 Bar	Blood sample 2
10	10 minutes at 2.8 bar on air	Blood sample 3
30	20 minutes at 2.8 bar on oxygen	Blood sample 4
35	5 minutes air break at 2.8 bar	
55	20 minutes at 2.8 bar on oxygen	
60	5 minutes air break at 2.8 bar	
80	20 minutes at 2.8 bar on oxygen	Blood sample5
85	5 minutes decompression on air	
115	30 minutes post dive	Blood sample 6

Figure 2.1 Compression schedule for exposing volunteers

2.2.22 Analysis of nitrate and nitrite by capillary electrophoresis

The samples taken from the volunteers at pressure were deproteinated using an Amicon 5 Microconcentrator. The molecular weight cut off of 5 kD removed most plasma proteins.

The samples were then diluted 10 fold and analysed using a capillary zone electrophoresis. The optimised method developed allowed a separation time of under 4 minutes at 10 kV. The running buffer was 25 mM sodium tetraborate, 150mM NaCl, and 1 mM TTAB. The samples were introduced using 3 kV for 2 seconds, before being run at 10 kV. Detection was at 214 nm for nitrate and nitrite.

Chapter 3 B_{12} methionine synthase purification and characterisation

3.1 Purification of pig brain methionine synthase

Initially, the starting material for purification was rat brain. However, it was quickly apparent that though we could track the activity of methionine synthase during the purification protocols developed, we would not be able to purify enough material for it show up on an SDS gel even when using silver stain. The choice of pig brain was a matter of convenience and abundance. It did, however, lead to other problems. Handling large volumes of homogenate and centrifuging it was both time-consuming and laborious. The first steps in the purification needed to be done quickly in order to get the volumes involved down to a manageable level quickly.

3.2 Cytosol preparation

When preparing the cytosol, great care was taken to limit the enzymatic digestion of the methionine synthase. All the buffers used were chilled to 4°C and the centrifuge kept at 4°C throughout. The large volumes involved meant that several steps of centrifugation were required. At this point several different peptidase inhibitors were added to limit digestion. The only limitation on this was cost. Again the volumes of liquid and the concentration of protein in the homogenate required large quantities of enzyme inhibitors to be used.

The initial centrifugation removed any undisrupted cells and clarified any particulate matter in the solution. This first step of centrifugation was done at 2000 g for one hour, the supernatant was removed and then re-centrifuged at 30,000g for another hour and finally 100,000g for one hour (all centrifugation at 4°C). The cytosol once produced was then immediately transferred to a batch chromatography separation.

3.3 Chromatography

All the chromatographic steps were initially tested using rat brain as a tissue source. The volumes involved were much smaller as was the total amount of protein. In order to cope with the larger volumes of sample and the increase in total protein, larger columns with greater binding capacities were used. In some

cases these large columns reduced the overall resolution of the eluted peaks but did save time in terms of the number of repeated runs through the columns.

Throughout all the steps of the purification, light was kept to a minimum. This was achieved by wrapping all the column in aluminium foil, sealing all clear plastic tubing in aluminium foil, and reducing the ambient light in the lab (closed curtain, spot lights).

Without a cold room in which to run the FPLC, the columns had to be cooled using water jackets linked to a refrigerated re-circulation pump. Samples were kept on ice during loading and column fractions were collected on ice and transferred to a fridge when collection was completed.

The use of the Pharmacia FPLC during the protein purification simplified method development and allowed for excellent continuity and reproducibility between runs. The FPLC allowed for automated gradient formation and high throughput of sample through the chosen media. The use of an automated fraction collector had drawbacks – on two separate occasions fractions were lost due to malfunctioning of equipment. Detection of methionine synthase activity was always the time consuming step throughout.

3.3.1 DEAE batch chromatography

Sigma DEAE cellulose was chosen as the first step in purification. Although the DEAE did not have a high degree of resolution, as a batch step it did give an 8.9 fold increase in purification and had a yield of 88% (see Table 3-2 for summary). DEAE (diethylaminoethyl) cellulose is a weak anion exchanger. As the published pI of the protein was around pH 5 DEAE was chosen to achieve a good degree of binding without the need to elute with high concentrations of salt. The cytosol produced after centrifugation was loaded onto a 'slurry' of 500 grams DEAE cellulose. This step was most useful in removing the abundant cytochromes and iron-containing proteins that were damaging the Q-sepharose column. Previous purifications had omitted the DEAE step and stained the Q-sepharose making it difficult to regenerate and clean.

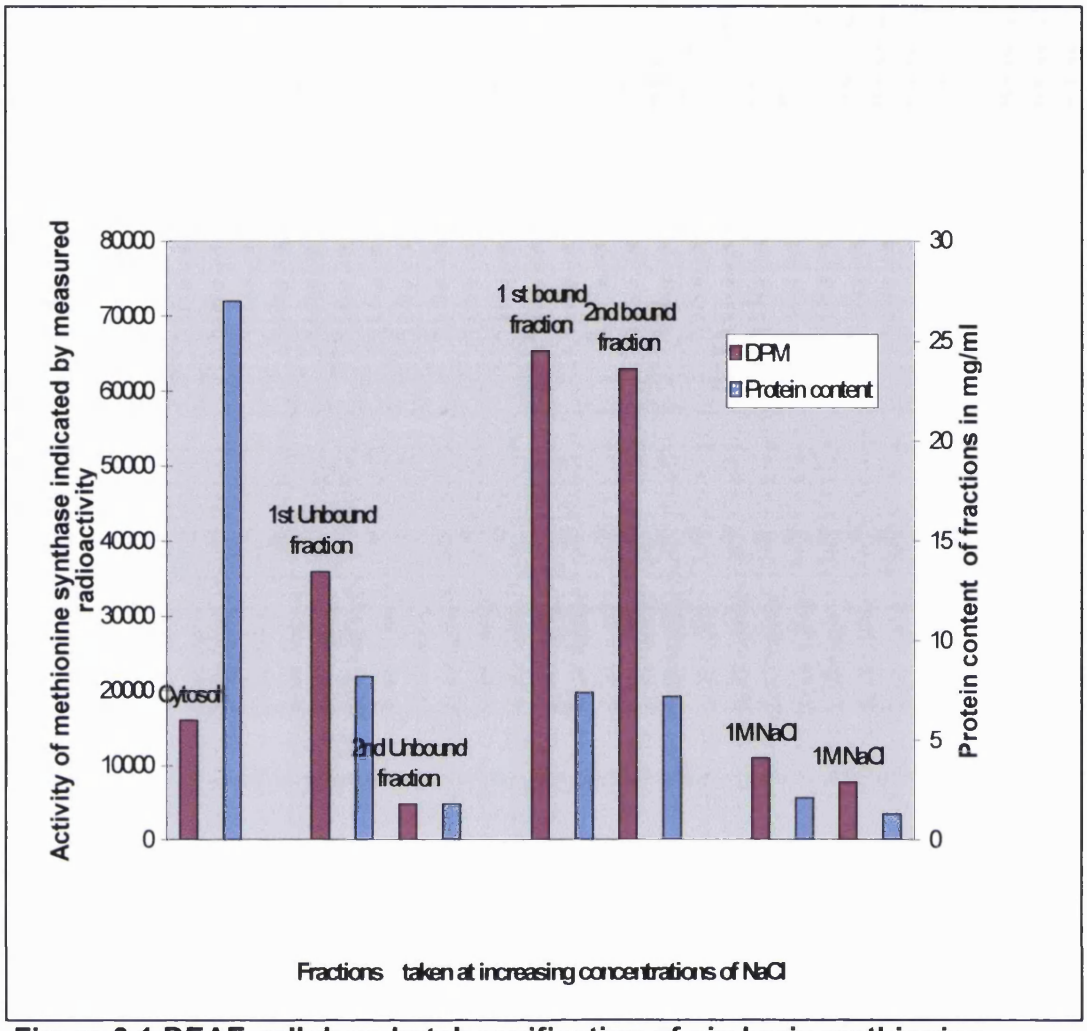


Figure 3.1 DEAE cellulose batch purification of pig brain methionine synthase

The separation was carried out as described in the method. All buffers were kept at 4°C in a chilled cabinet to maintain the integrity of the enzyme and reduce proteolytic degradation. A total of 57 grams of protein in 2 litres were added to the DEAE cellulose. Figure 3.1 is representative of many purifications and demonstrates that B_{12}-MS activity was separated as a first unbound fraction and two bound fractions. The total recovered protein in the active fractions was 7.31 grams with a yield of 88%. The active fraction was eluted with 2 separate washes 0.5 M NaCl.

The slurry of DEAE and cytosol was mixed and incubated for two hours in a cold cabinet kept at 4°C before filtering under vacuum. The slurry was then washed with another litre of homogenisation buffer (50 mM K_2HPO_4, 0.2 M NaCl, pH 7.4) and filtered again to remove all the unbound proteins. The unbound fraction contained a high quantity of red pigmented proteins which had been damaging the Q-sepharose resin in subsequent steps. The next fraction of protein was eluted with 2x 1 litre washes of 0.5 M NaCl (50 mM K_2HPO_4, 0.5 M NaCl, pH 7.4) and 2x 1 litre washes of 1 M NaCl. (50 mM

K_2HPO_4, 1.0 M NaCl, pH 7.4). The results of the batch chromatography can be seen in Fig 3-1. The total protein and pig brain methionine synthase activity are shown in the chart. The purification was effective in terms of yield (88%) and 8-fold increase in purity of the enzyme. The fraction from the DEAE was, however, in a 0.5 M buffered solution of NaCl that would not bind to the Q-sepharose column in the subsequent step. Many different techniques were tried to achieve a reduction in salt concentration. These were: dialysis; ultrafiltration; G25 exchange columns; and gel filtration. The problem at all times was dealing with 2 litres of solution and all of these techniques would have required several smaller repetitions to work effectively. The simple solution, which eventually was used, was dilution of the active fraction with a further 2 litres of 50 mM K_2HPO_4, pH 7.4. This had the advantage over other attempts of being quick, simple, and effective.

Column material	Column Dimensions (cm)	Column volume (ml)	Pore size (μm)	Binding capacity (ml of gel)	Total binding capacity of column
DEAE cellulose		500 grams		1 meq/gram	
Q-sepharose fast flow	1.6 x 34	54 ml	45-165	37.5 mg	2025 mg
Phenyl sepharose fast flow	1.6 x 22	35 ml	45-165	30 mg	1056 mg
Hydroxylapatite	1.6 x 10.5	17 ml	20-80	25 mg	336 mg
Mono-Q	0.5 x 5.7	2.85 ml	10	20-50 mg	20-50 mg
Superose gel filtration	28 x 1	28 ml	10 +/- 2	10 mg	10 mg
S-300 gel filtration	100 x 2.6		20		

Table 3.1 Column dimension and binding capacity

The table shows the chromatographic material used in the purification. The column dimension and volumes were used to calculate the maximum binding capacity of the column when planning the separation. All the columns were equipped with water jackets to allow maintenance of a constant temperature throughout the separation. The columns were connected to a refrigerated circulation pump, which maintained a temperature of 4°C. The columns and tubing were wrapped in aluminium foil to prevent the ingress of light. The separation was monitored at 280 nm absorbance by an inline absorbance spectrometer. Salt gradients were produced using a Pharmacia FPLC 5000 controller. This allowed high flow rates with a precise control of mixing and gradient composition

3.3.2 Q-sepharose chromatography

The selection of Q-sepharose as resin for the separation was based on its properties. The Quaternary anion is a strong anion exchanger, which has been coupled to sepharose as a matrix. The material allowed for high flow rates, good binding capacity and reasonable resolution. It had a bead size that ranged from 46-165 microns that, although it did not give extremely high resolution of peaks, did have the benefit of speed and bulk flow. During the early stages of the separation the optimal gradient for separation was developed. This combined the need for resolution of peaks coupled with speed and dilution of fractions. The protein was found to bind to the column at less than 0.25 M NaCl. Once the protein was loaded, the gradient was increased to 0.25 M NaCl and held for 40 minutes. After this time the gradient was increased from 0.25 M NaCl to 0.5 M NaCl

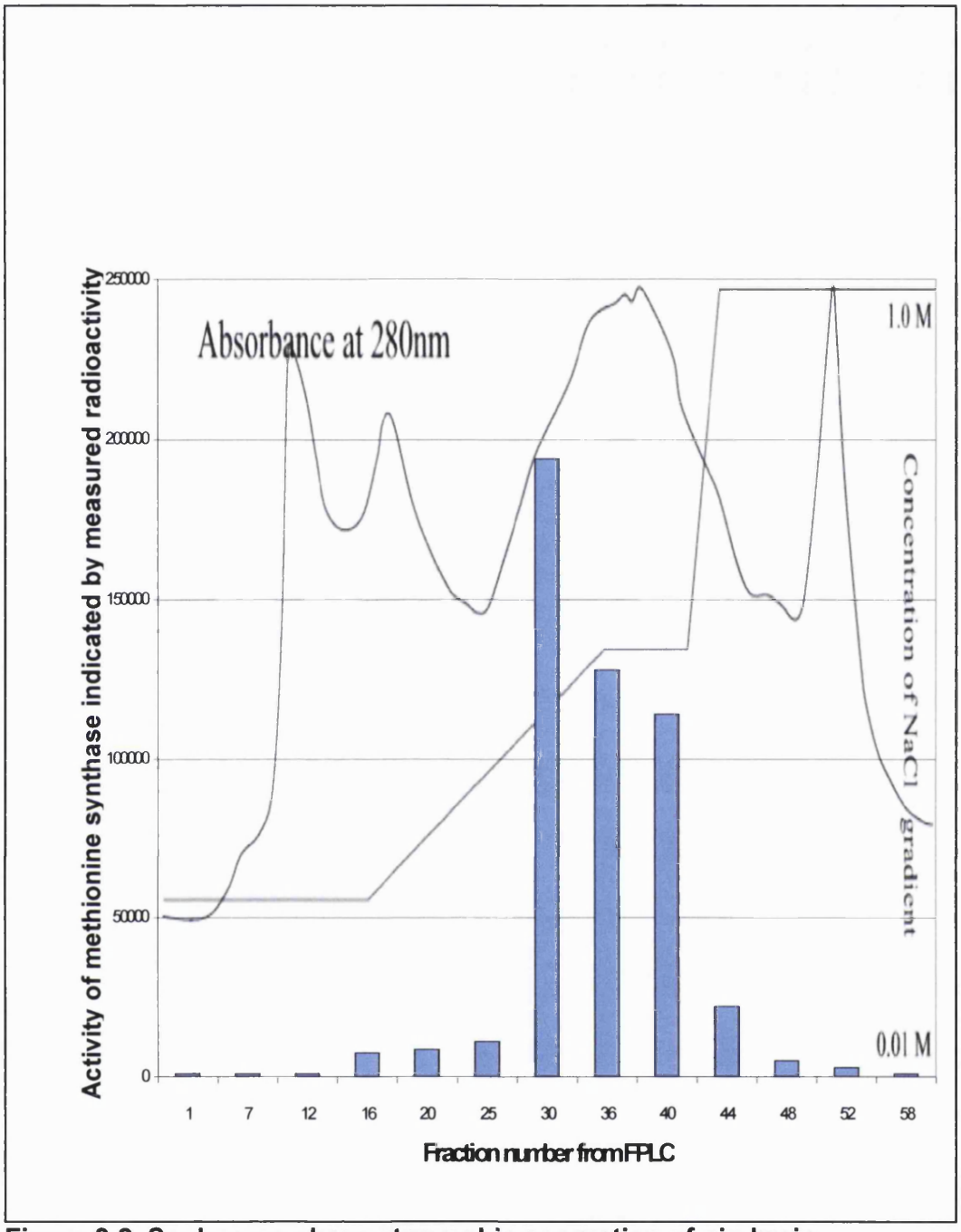


Figure 3.2 Sepharose chromatographic separation of pig brain methionine synthase

The active fractions (2 litres) from the DEAE cellulose separation were pooled and diluted in 2 litres of 50 mM K_2HPO_4, pH 7.2 (total volume 4 litres), and loaded onto a 54 ml Q-sepharose column previously equilibrated with 50 mM K_2HPO_4, pH 7.2 at 10 ml/min at 4°C. The protein was eluted with a linear gradient of 0.2 M-0.5 M NaCl (buffered with 50 mM K_2HPO_4, pH 7.2) at 4 ml/min over 60 minutes. Fractions were taken every 15 ml and tested for methionine synthase activity and protein content. The active fractions were found to elute above 0.4 M NaCl. The total protein loaded onto the column was 7.31 mg and the active pooled fractions contained 0.7 mg of protein giving a tenfold increase in purity of the enzyme and a marked reduction in the total volume of sample from 4 litres to 210 ml. The yield from this step in purification was 75%.Fig 3.2 is representative of several purifications attemps.

over 60 minutes. Once the concentration of NaCl reached 0.5 M the gradient was again held for 30 minutes. At this point the column was then regenerated with 1.0 M NaCl. The Q-sepharose material offered excellent binding with partition coefficient for methionine synthase equal to one (a=1) where it bound strongly to the material and no breakthrough was detected at the high flow rates used for the loading. The flow rate during elution was a compromise between the theoretical ideal value and a practical value considering time for the elution to occur.

The protein eluted from the column in a reasonably concentrated peak but was poorly resolved from other proteins in this region. The use of two anion exchange resins might seem superfluous. However, the first step of batch DEAE allowed for excellent cleaning of unwanted material, where the Q-sepharose allowed for a reduction in sample volume from 4 litres to 210 ml. The yield from this step in purification was 75% with a 10.3-fold increase in purity (see Table 3-2 for summary).

3.3.3 Phenyl sepharose chromatography

The use of hydrophobic interaction chromatography (HIC) resin was invaluable in the purification. The active fractions taken from the Q-sepharose were combined and then made up to 25% using a saturated solution of ammonium sulphate. From earlier work it was known that the pig brain methionine synthase did not precipitate until a 37% saturated solution was reached. The sample with ammonium sulphate (AS) kept at 2°C and incubated for one while agitated with a magnetic stirrer. The sample was then centrifuged for 1 hour at 50,000 g to remove any precipitated protein. The clarified solution was then loaded onto a Pharmacia phenyl sepharose column.

As can be seen from the chromatogram, Figure 3-3, the active fraction from the phenyl sepharose did not elute until the end of the gradient. The degree of purification achieved was 6.5 with a yield of 54 % and a final volume of active fractions of 150 ml. Initial problems with this step of purification were in getting the protein off the column. The benefit of the use of HIC is that samples do not

need to be desalted before addition to the column and the use of AS gave a degree of purification by precipitation.

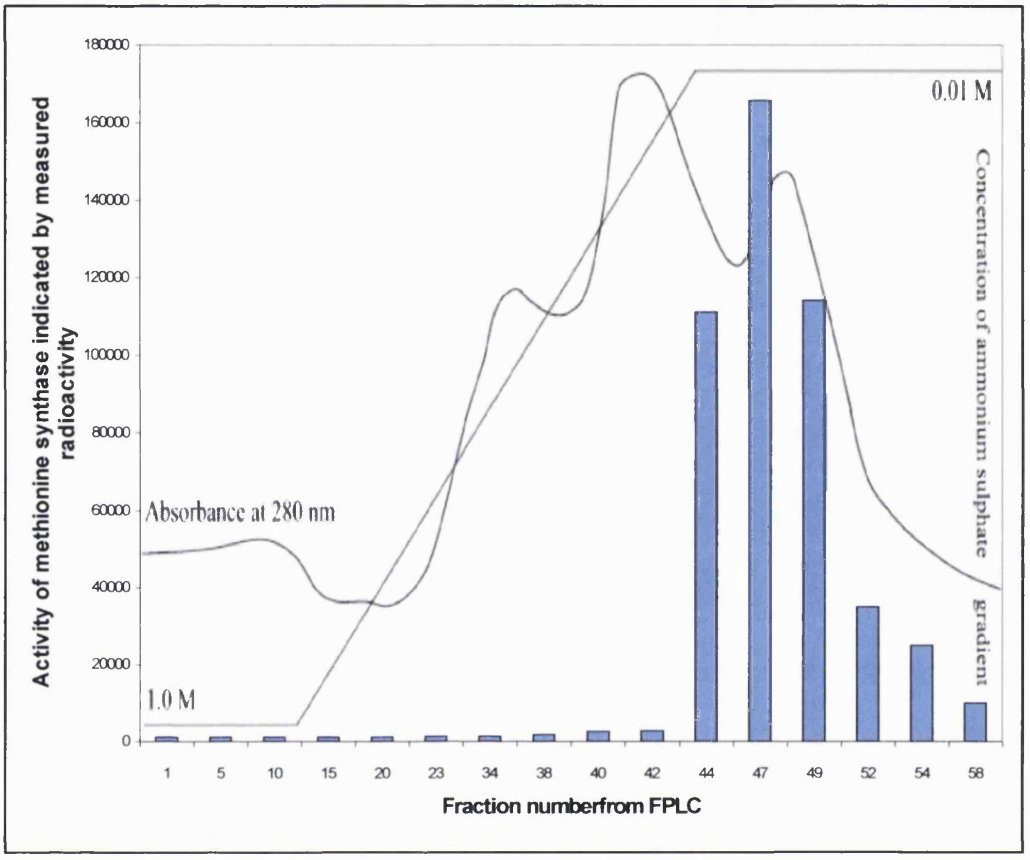


Figure 3.3 Phenyl sepharose separation of pig brain methionine synthase

Before loading onto the phenyl sepharose (high sub units) hydrophobic interaction column the active pooled sample from the Q-sepharose was made up to 25% (1.0 M) ammonium sulfate and centrifuged to remove any particulate matter and precipitated protein. The column was loaded with 235 ml of sample at 4 ml/min. The methionine synthase was eluted at 4 ml/min with a decreasing gradient of 1.0 M (10 mM K_2HPO_4, pH 7.2) Ammonium sulfate to 10 mM K_2HPO_4, pH 7.2 over 60 minutes. Fraction were taken every 15 ml and tested for methionine synthase activity. The yield from the purification step was found to be 54% with an 8-fold increase in purity. The hydrophobic nature of the protein meant that the enzyme was only eluted at the lowest ionic concentration. Fig 3.3 is representative of several purifications attemps.

3.3.4 Hydroxylapatite chromatography

Hydroxylapatite uses a combination of properties to achieve binding and separation. It is also known as calcium hydroxy phosphate and is part of a family of inorganic absorbents. The exact mechanism of action is still uncertain but may be a combination of electrostatic charge, or dipole-dipole bonding.

Prior to loading onto the hydroxylapatite (HTP) column, the sample was filtered through an 'Amicon ultrafiltration' membrane with a cut off of 50 kD (see method) once to remove smaller proteins and perform buffer exchange to 20 mM K_2HPO_4, pH 7.2.

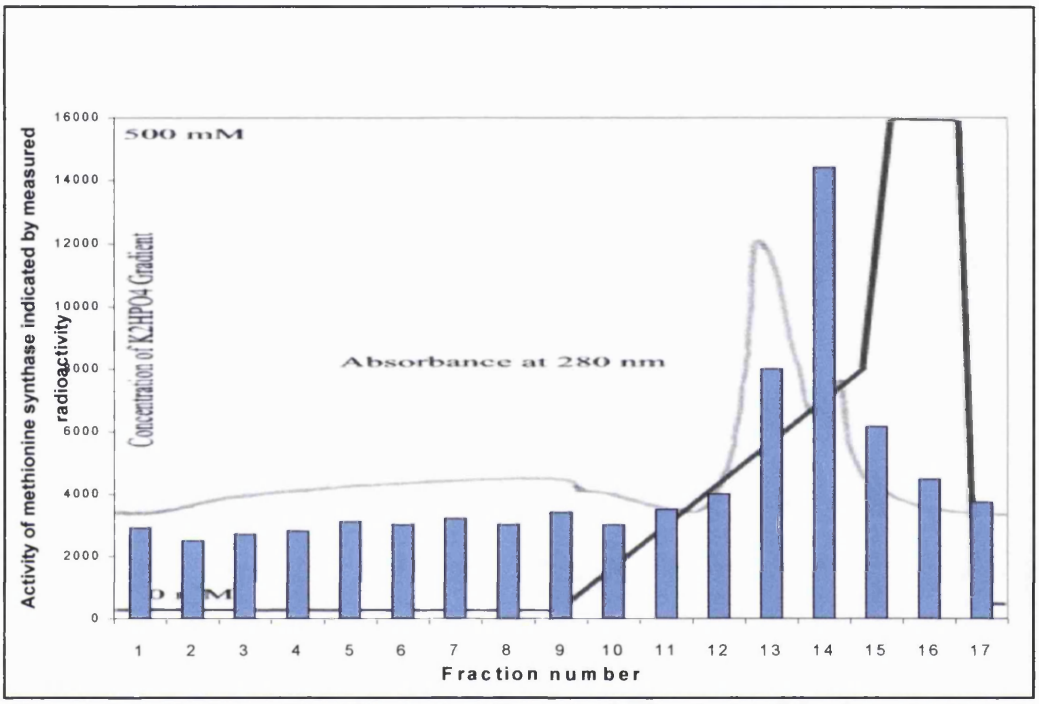


Figure 3.4 Hydroxylapatite separation of pig brain methionine synthase
The pooled fractions from the phenyl sepharose column were filtered using an Amicon Ultrafiltrator with a 50 kD cut off membrane before loading on a 17 ml HTP column equilibrated with 50 mM K_2HPO_4, pH 7.2 at 2 ml/min. The protein was eluted using an increasing linear gradient of 500mM K_2HPO_4, pH 7.2 over 40 minutes with fractions taken every 4 ml. Fig 3.4 is representative of several purifications attemps.

This step in purification gave the worst yield (14%) but was essential in the purification process. The yield at smaller volumes of sample were excellent; the problems occurred when the purification was scaled up. Possible reasons for this might be overloading of the column, and too high a flow rate during loading. This was discounted as the unbound fraction had extremely low methionine synthase activity. The protein could have been sticking to the column and not eluting but examination of the chromatogram revealed that the methionine synthase activity was coming off at 0.25 M K_2HPO_4 and the gradient went up to 0.5 M. The last possibility is that the protein was breaking down on the column

or during filtration and the column was separating the two products of the breakdown. This may have been what was occurring, as, at this stage of the purification loss of a 140 kD band as seen on SDS gels and also the loss of the higher molecular weight species of the methionine synthase protein as detected during gel filtration.

3.3.5 Mono-Q chromatography

The use of Pharmacia mono-Q column as a final step in purification gave extremely high resolution of peaks and hence excellent separation due to the bead size of 10□m and homogeneity. The resin material uses the same anion exchanger as the Q-sepharose but has a much higher resolving power due to the homogeneity of the beads and their small diameter. The sample was pre-filtered using Amicon ultrafiltration membranes with a molecular weight cut-off of 50 kD. The chromatograms showed the main activity of methionine synthase coming off the column at 0.3 M NaCl. Several distinct peaks of proteins were observed.

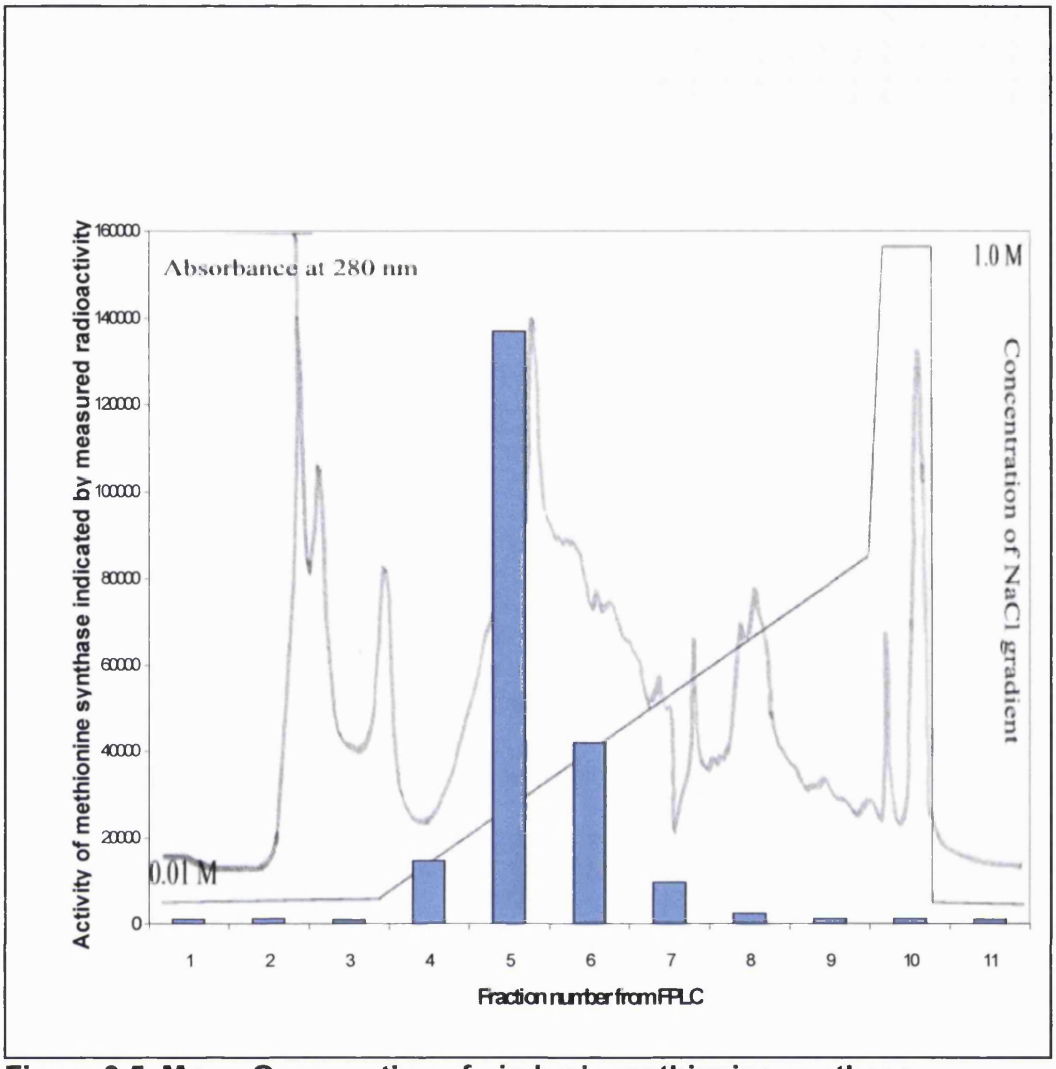


Figure 3.5 Mono-Q separation of pig brain methionine synthase
The active fractions from the HTP separation buffer exchanged in an Amicon ultrafiltrator and loaded onto a 0.5 x 5 cm Mono Q column equilibrated with 50 mM K_2HPO_4, pH 7.2 at 1 ml/min. The protein was eluted from the column using an increasing linear gradient of 1.0 M NaCl (buffered with 50 mM K_2HPO_4, , pH 7.2) over 40 minutes. Fractions were collected every 4 ml and tested for methionine synthase activity and protein content. Fig 3.5 is representative of several purifications attemps.

3.3.6 Gel filtration chromatography using Superose 12 and S-200 gels

In order to estimate the approximate molecular weight of pig brain methionine synthase, gel filtration chromatography was performed at several different stages of purification. Two different resins were used: Pharmacia S-200 and Superose 12.

The Superose 12 resin had an effective filtration range of 1,000-300,000 Daltons for globular proteins. It was pre-packed into 1 x 28 cm column. The

bead size was 8-12 μm allowing a high degree of resolution. However, due to the tight packing, the increased back pressure meant a low flow rate through the bed had to be maintained.

The S-200 resin had an effective filtration range for globular proteins of 5,000-250,000 Daltons and a bead size of 25-75 μm. The resin was packed in a 100 cm column with a diameter of 2.6 cm. The flow rate was set at 1.0 ml/min and protein was detected at 280 nm. To limit any interaction with the column other than size exclusion the buffer also contained 100mM NaCl buffered with 50 mM K_2HPO_4.

Gel filtration was found not to be an effective purification step for methionine synthase but was useful as an analytical tool to monitor changes in the enzyme throughout the purification. Initially, pig brain methionine synthase was found to elute in two distinct peaks from both the S-200 and the Superose 12 column. These two peaks of activity remained until just before the HTP chromatographic step. At this step in chromatography, the yield dropped significantly and the higher molecular weight species of the enzyme disappeared. Table 3-2 summarises the different molecular weights determined by gel filtration.

Gel filtration step	1st peak of activity	2nd peak of activity
Superose 12 of cytosol	155400	101,000
Superose 12 of Q-Sepharose	155400	101,000
Superose 12 of Phenyl Sepharose	155400	101,000
Superose 12 of HTP	N.A.	101,000
Superose 12 of Mono-Q	N.A.	101,000
S-200 of cytosol	162,000	97,000

Table 3.2 Summary of molecular weights as determined by gel filtration chromatography

The table shows that during the early stages of purification methionine synthase activity can be found at two different molecular weights. However by the last steps in purification, the higher molecular (approx. 155 kD) weight protein disappeared leaving the lower molecular weight protein (101 kD). The methionine synthase activity of the higher molecular weight species was found to disappear after HTP chromatography.

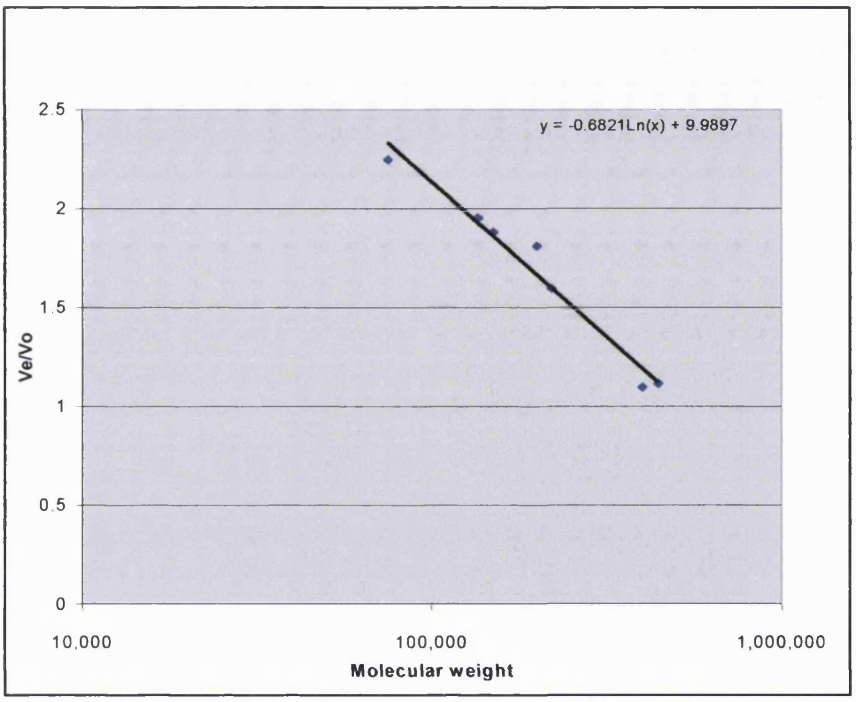


Figure 3.6 Superose 12 calibration with molecular weight standards
The Pharmacia Superose 12 gel filtration column (1 x 28 cm) was calibrated with standard proteins of known molecular weight. The column was run at 0.25 ml/min with a mobile phase of 50 mM K_2HPO_4, 100 mM NaCl, pH 7.2 and monitored at 280 nm. The Superose 12 gel filtration media has a molecular weight fractionation range of 1,000 - 300,000 Daltons and a bead size of 8 - 12 μm allowing for a high degree of peak resolution.

Table 3.3 Calibratio of Superose 12 gel filtration column

Protein standard	Molecular weight	Ve/Vo
Blue dextran	2,000,000	1
Apoferritin dimer	886,000	1.18
Apoferritin	443,000	1.48
Beta amylase	200,000	1.6
BSA dimer	134,000	1.62
BSA	67,000	1.84
Carbonic anhydrase	29,000	2.14
Cytochrome C	12,400	2.3

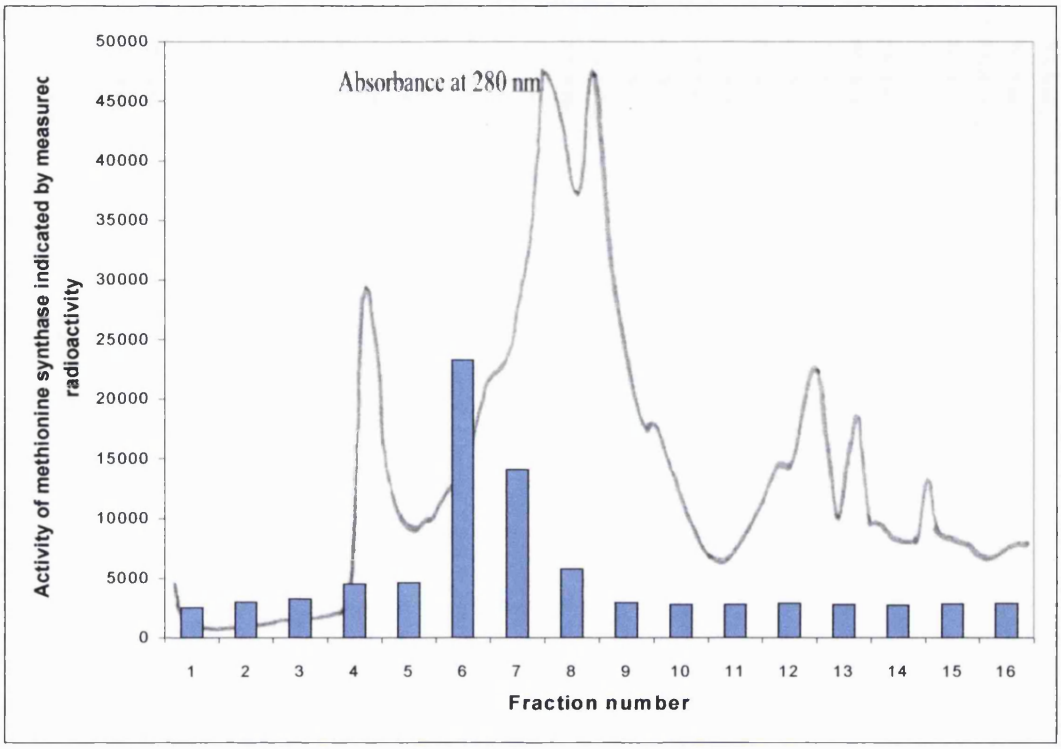


Figure 3.7 Superose 12 fractionation of pig brain cytosol
The sample was concentrated down to 1 ml and 100ml of sample applied to the Superose 12 column at 0.25 ml/min. The major peaks of pig brain methionine synthase corresponded to a molecular weight of 155 kD and 101 kD.

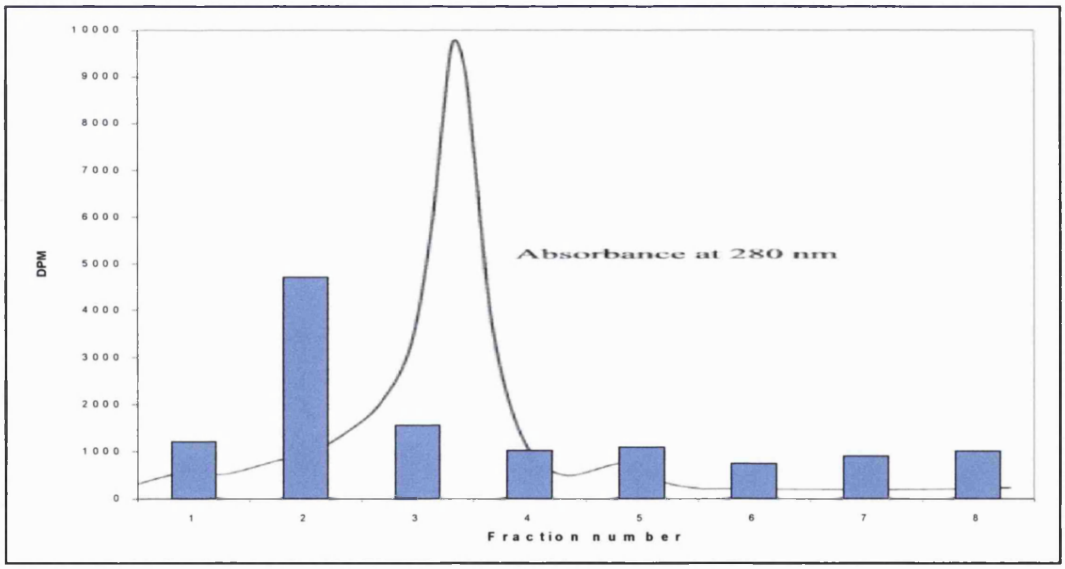


Figure 3.8 Superose 12 separation of active fractions from Mono Q
The active fractions from a Mono-Q separation were pooled and filtered using an Amicon ultrafiltrator with a molecular weight cut off of 50 kD. The sample was concentrated down to 1 ml and 100ml of sample was applied to the Superose 12 at 0.25 ml/min. The major peak of pig brain methionine synthase corresponded to a molecular weight of 101 kD.

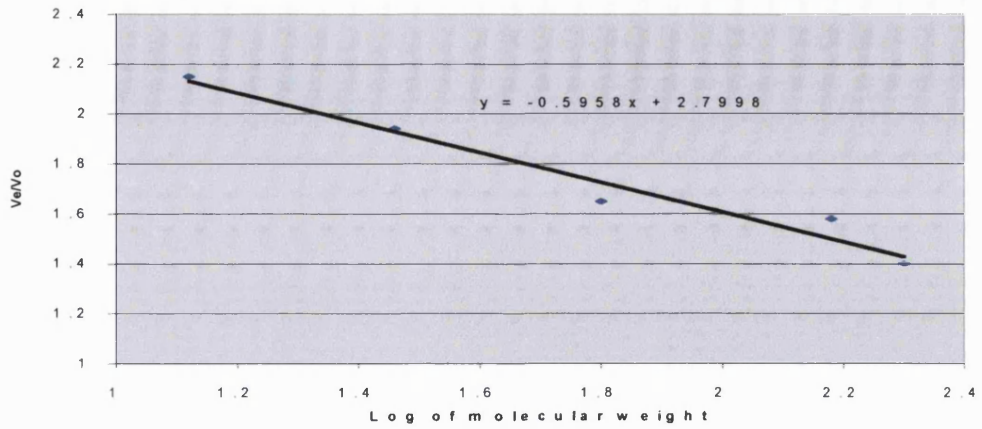


Figure 3.9 S-200 gel filtration column calibration using molecular weight standards

Molecular weight standards were used to calibrate a 100 x 1.6 cm column filled with Pharmacia S-200 HR media. The column had a molecular weight fractionation range of 5,000 - 250,000 Daltons and a bead size of 25 - 75 □m. The column was run at 1.0 ml/min with a mobile phase of 50 mM K_2HPO_4, 100 mM NaCl, pH 7.2 and monitored at 280 nm.

Table 3.4 Calibration of S-200 gel filtration column

Protein standard	Molecular weight	Ve/Vo
Blue dextran	2,000,000	1
Apoferritin dimer	886,000	1.18
Apoferritin	443,000	1.48
Beta amylase	200,000	1.6
BSA dimer	134,000	1.62
BSA	67,000	1.84
Carbonic anhydrase	29,000	2.14
Cytochrome C	12,400	2.3

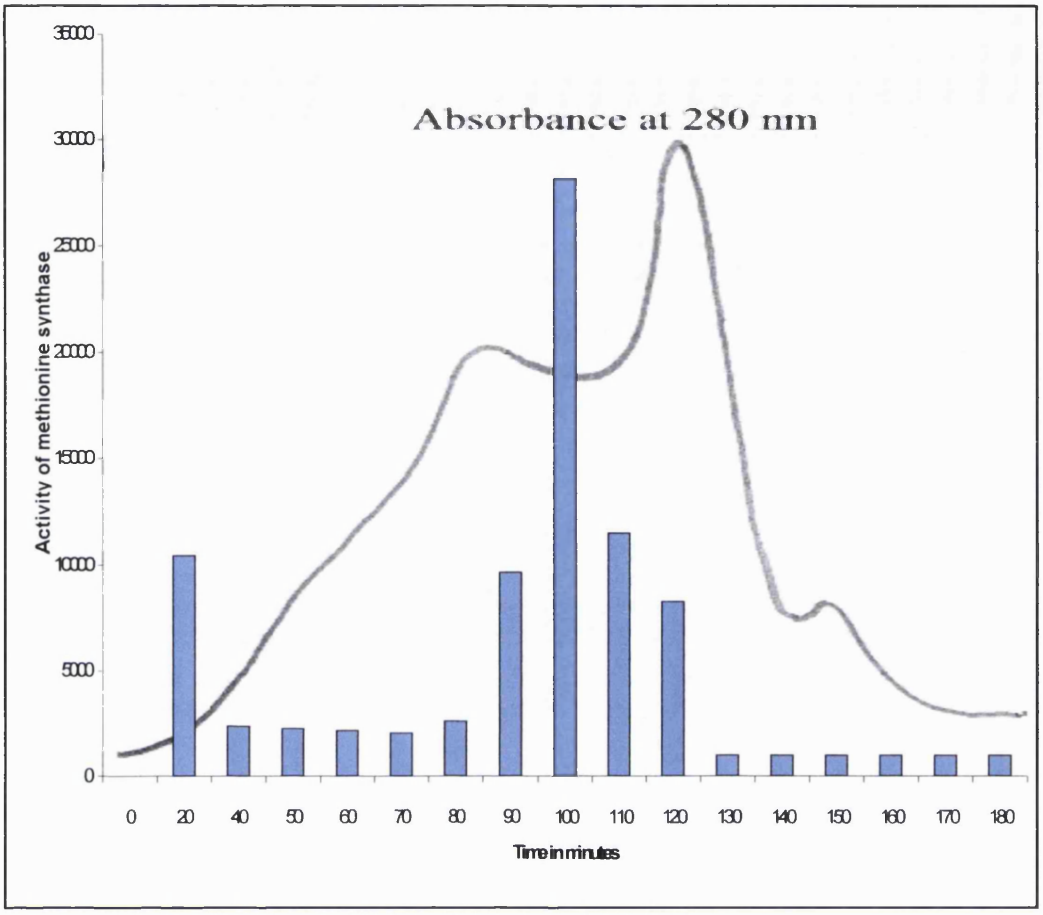


Figure 3.10 S-200 gel filtration of methionine synthase from pig brain cytosol

This sample added was freshly prepared the morning of the separation and then immediately added to the column. The two peaks of activity shown clearly correspond to 162 kD and 97kD. The column was run at 1.0 ml/min with a mobile phase of 50 mM K2HPO4, 100 mM NaCl, pH 7.2 and monitored at 280 nm.

The gel filtration consistently showed two molecular weight species of the enzyme. Whether these were naturally occurring or due to the conditions, and effects of the columns on the enzyme is not able to be determined at this time.

3.4 Electrophoresis and electrofocussing purification and characterisation of B_{12} methionine synthase

In order to determine the purity and molecular weight of the purified pig brain methionine synthase several different types of electrophoresis were used. The method used for each one is described in more detail in the material and methods section.

At each stage of the purification a sample of the pooled fractions from the previous step were loaded onto a 7.5% discontinuous SDS (sodium dodecylsulfate) polyacrylamide gel. The degree of cross-linking for the gel was determined through experimentation with known molecular weight standards and using information published conceming the approximate molecular weight of the previously purified E.coli. A 7.5% gel was used because it had the best separation in the molecular weight range characteristic of the E.coli enzyme.

The pI of the enzyme was determined using a preparative IEF apparatus manufactured by BioRad. It had been hoped that this method could be used as a step in the purification of methionine synthase. However, due to the poor yield of enzyme, the IEF method was abandoned. It did, however, give a good estimation of the pI of the pig brain methionine synthase. A possible reason for the poor yield is that the B_{12} cofactor is only weakly bonded to the pig brain methionine synthase. During electrophoresis the cofactor can be removed from the enzyme resulting in its inactivation. Attempts to reconstitute the enzyme by further addition of B_{12} met with failure.

In the first step of IEF the chamber was pre-focused using ampholytes with a pH range of 3-10. Initial studies showed that ampholyte had no effect on the activity of the enzyme. The maximal enzyme activity was found around pH 3.5-6.5 (Figure 3-12).

To increase the resolution of the pI, a narrower range of ampholytes was used between pH 4-6. Once focused and readjusted to pH 7.2, the samples were tested for methionine synthase activity. The activity was found in a range of fractions with two maxima of activity. The range was 4.3-5.1.

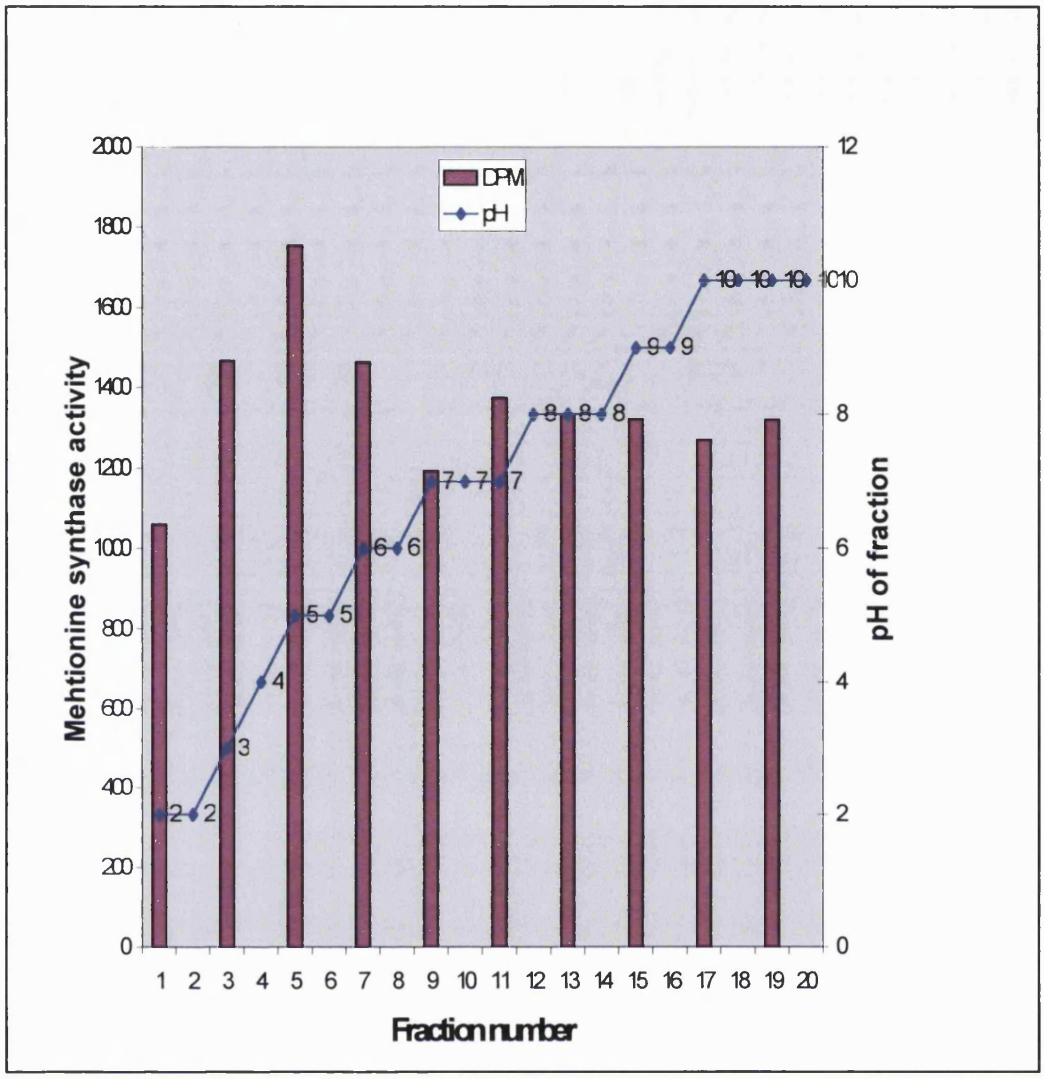


Figure 3.11 Iso-electric focusing using a BioRad Rotofor

A semi-purified sample of pig brain methionine was focused in a BioRad preparative iso-electric focusing chamber. The range of ampholyte used was from 3-10. The final voltage during focusing was 12,000 volts and the total run time was 6 hours. The chamber was pre-focused and the sample was added to the chamber in a region that approximately corresponded to the published isoelectric point. Due to the broad range of ampholyte used the pI could only be estimated between 3.5 and 6.5.

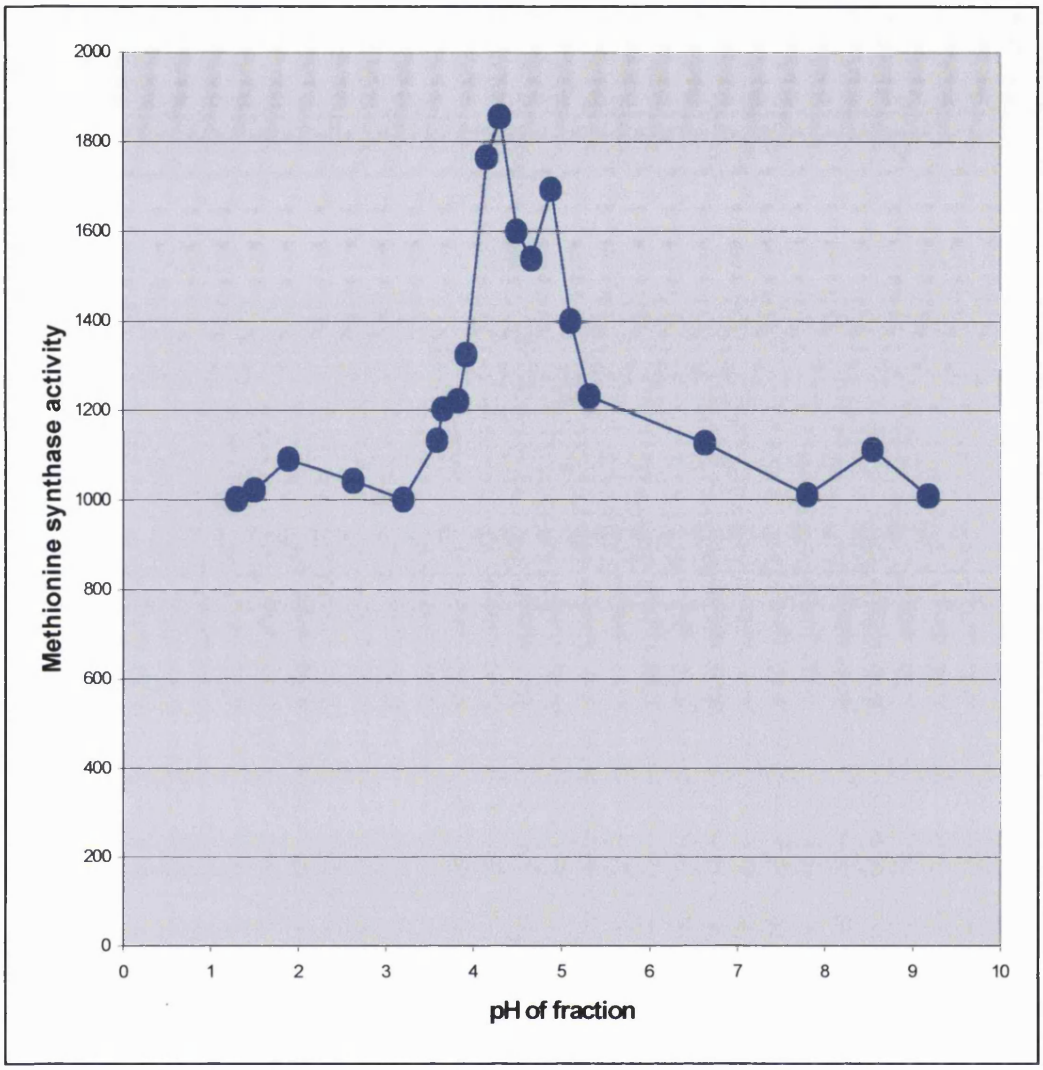


Figure 3.12 Iso-electric focusing of pig brain methionine synthase using ampholyte with a pH range of 4-6

A semi-purified sample of pig brain methionine was focused in a BioRad preparative iso-electric focusing chamber. The range of ampholyte used was from 4-6. The final voltage during focusing was 12,000 volts and the total run time was 6 hours. The chamber was pre-focused and the sample was added to the chamber in a region that approximately corresponded to the published isoelectric point. Once focused the fractions were tested for there pH and readjusted to pH 7.2. From the chart it can be seen that the pig brain methionine synthase activity shows two maxima, one at a pI of 4.3 and the other around 5.1.

Throughout the purification many attempts were made to elute the pig brain methionine synthase in an active form from a native gel. Again this proved (similarly to the IEF) to be extremely difficult. A combination of factors may have contributed to the difficulty. As previously said the B_{12} cofactor is only weakly bound to the pig brain methionine synthase protein and this made it

extremely unstable. The enzyme itself had been found to be unstable breaking down into smaller subunits during certain purification steps. Elution of the enzyme from the electrofocussing gels again resulted in loss of activity. Three methods were therefore tried to detect activity on the gel, but only partial success was achieved.

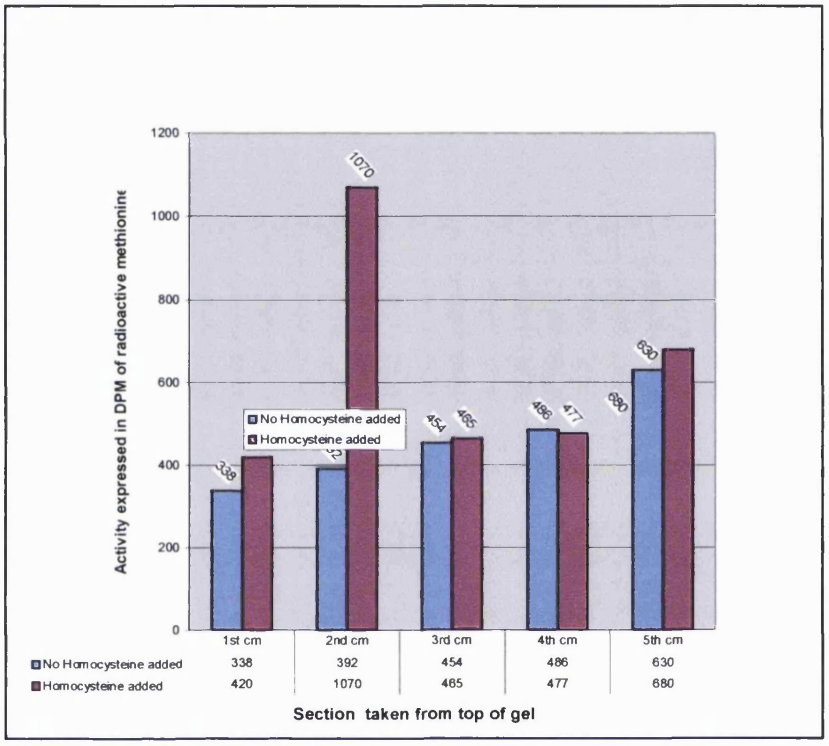


Figure 3.13 Methionine synthase activity eluted from a 7.5% native polyacrylamide gel

The gel containing a semi-purified sample of pig brain B12-MS was run in native conditions. The gel was then sectioned and placed in an Amicon electro-elutor. The protein eluted from the gel was then assayed for methionine synthase activity. The activity eluted from the native gel corresponded to molecular weight range of 80 - 110 kD.

The first method used was to load a sample of the enzyme that had been part way through the assay for the detection of methionine synthase activity. As the reaction to produce methionine is a two-step process involving the donation of a radioactive methyl group to homocysteine from the B_{12} cofactor and the subsequent regeneration of that cofactor by mTHF, it was hoped that by labelling the pig brain methionine synthase that it could be detected using

autoradiography on the gel itself. This was found to be ineffective. All the radioactivity was eluted along the gel front with none remaining on the enzyme.

The second method used was to load the sample on to a native gel and then try running the methionine synthase assay on the gel itself. Again this proved unsuccessful.

The last method used involved the elution of protein from the gel using an Amicon Protein Electroelutor. This method proved to be partially effective and activity from the gel was found to be approximately 3 times the background level for the rest of the gel. The section of the gel from which the protein was eluted was between 80 and 110 kD (Figure 3-13)

SDS Polyacrylamide electrophoretic determination of molecular weight
The level of purity of the active protein was monitored via a non-reducing SDS discontinuous polyacrylamide gel. The protein was denatured in sample buffer containing the detergent SDS and heated to ensure complete integration of SDS with the protein. SDS gels work by ensuring that the protein remains solubilised in the gel without blocking the pores and by ensuring that the entire polypeptide chain is negatively charged. This removes the problem associated with native gels in which migration through the gel is dependent not only on size but also the charge that the protein carries. SDS associates in approximately one SDS molecule for every two peptide residues and will far outweigh any net charge that the protein may be carrying.

The analysis of each gel is described in the caption below it in the following sections. Each gel was fixed and stained in Coomassie R-250 or fixed and stained using silver stain. The gels were then dried using cellulose backing before analysis. For analysis the gels were scanned and saved as tagged image format files before being imported into Molecular Dynamics 'Image Quant' software programme. Using this programme an extremely accurate measurement of the Rf could be determined.

The initial choice of gel and testing of the separation protocol was carried out using known molecular weight standards. Several different standards were tried, the best results being obtained with a mixture of Sigma low molecular weight and high molecular weights, optimised for SDS gels (Figure 3-14).

Figure 3-15 shows the analysis of pooled fractions taken from each step in the purification. Enrichment of a band at 94 kD is clearly visible up to the phenyl sepharose step of purification. This band is still visible in the HTP and Mono-Q lanes of the gel under a light box. In the lane containing the HTP sample a band corresponding to a 36 kD band also becomes enriched. Although this band does not appear to contain methionine synthase activity it is probably an inactive subunit of the enzyme lost in the denaturing conditions of the gel.

Sequential fractions taken throughout the gradient profile of the HTP chromatography step were filtered and concentrated using Amicon Microcon Ultrafiltrators and run on a precast Pharmacia 7.5% SDS-PAGE. The gel shows that the lanes staining for a protein band at 94 kD have the highest enzyme activity, while the lanes that have only the 36 kD band show only a low activity of enzyme. The protein is possibly aggregated or bound to another protein that is co-purifying with the protein, or it is a subunit which is breaking down and denaturing.

In the next step of purification the procedure outlined above was also followed. Sequential fractions from the Mono-Q column were run on precast Pharmacia 7.5% SDS-PAGE. The gel (Figure 3-17) shows the active fraction from the Mono-Q with bands at 94 and 36 kD.

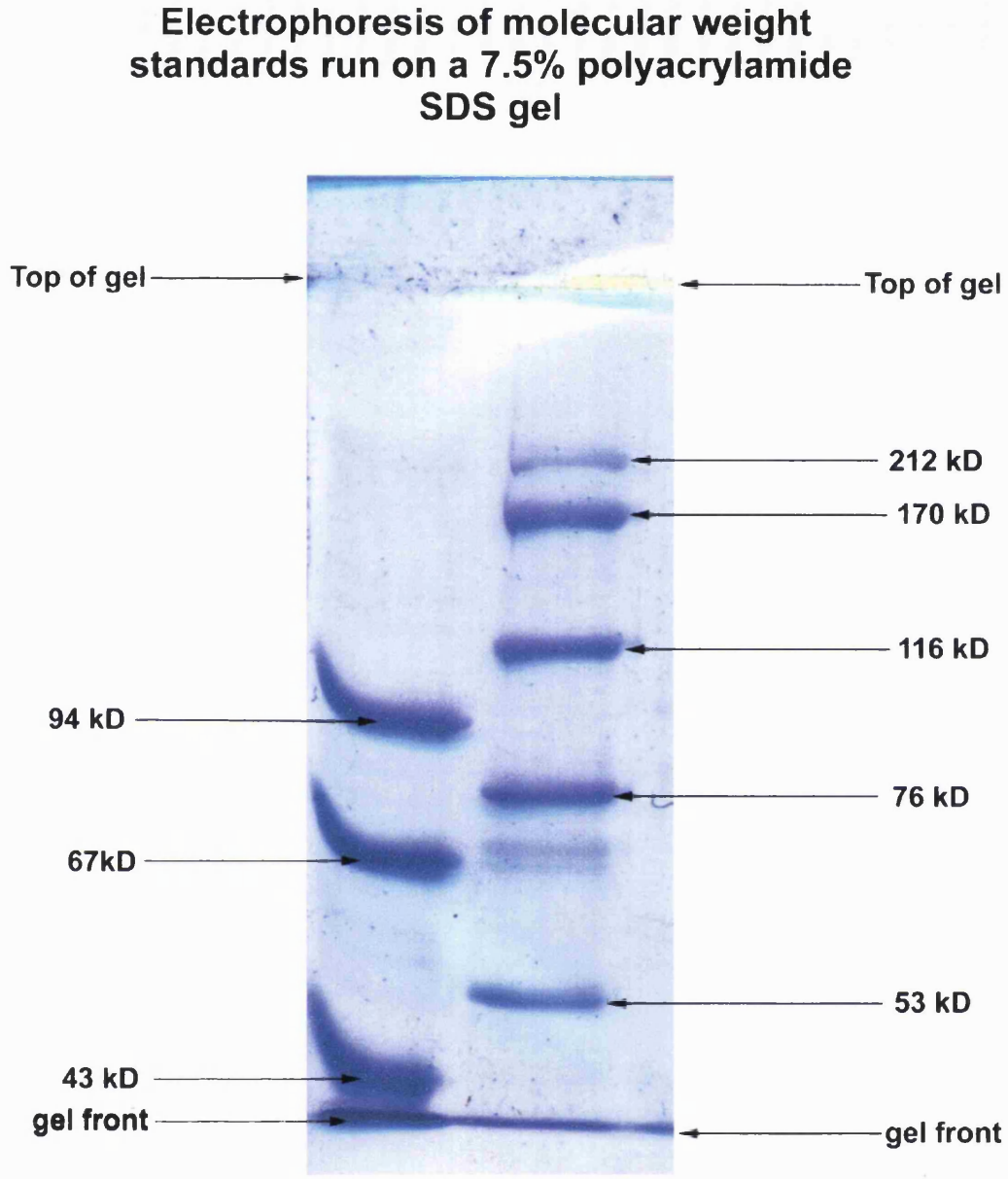


Figure 3.14 7.5% discontinuous SDS polyacrylamide gel of known molecular weight standards

(Gel 3.1) The gel was run at 200 volts for 30 minutes using Sigma molecular weight markers. The stacking gel was 4% cross linked with a pH of 6.8 and the separating gel was 7.5% and a pH 8.6. The gel was fixed with 10:30:50 acetic acid: methanol: water and then stained with Coomassie blue R250. In lane 'A' low molecular weight standards were run, while in lane 'B' high molecular weight standards were used.

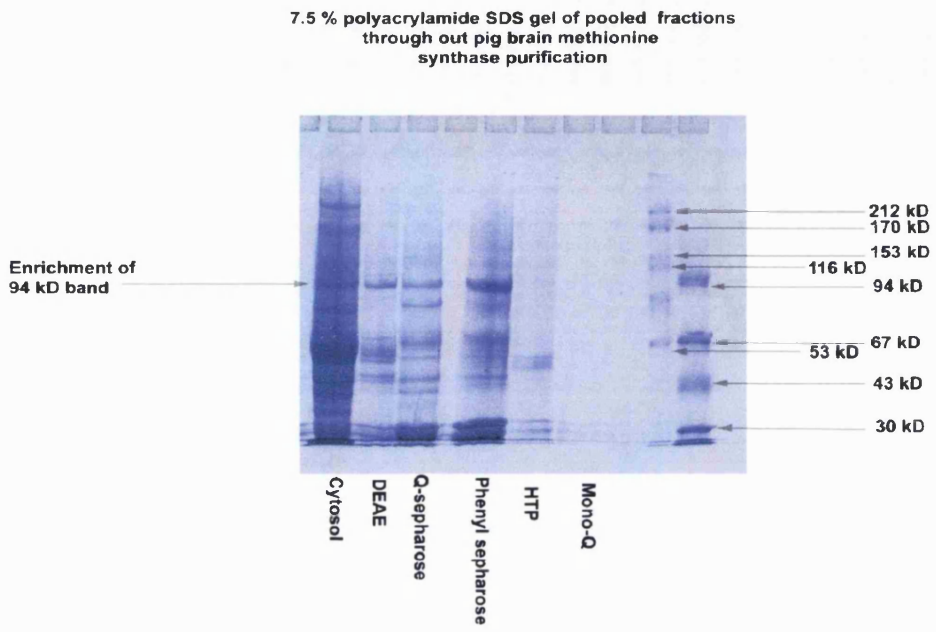


Figure 3.15 7.5% discontinuous SDS polyacrylamide gel of various pooled active fractions throughout pig brain methionine synthase purification

(Gel 3.2) The stacking gel was 4% with a pH of 6.8 and the separating gel was 7.5% and a pH 8.6. Running time for the gel was 30 minutes at 200 volts. The gel was fixed with 10:30:50 acetic acid: methanol: water and then stained with Coomassie blue R250. From the gel we can observe enrichment of a band corresponding to 94 kD up to the HTP chromatographic step. After the HTP step an increase in enrichment of some lower molecular weight proteins was observed. These bands are not visible with Coomassie stain until the samples were re-run after concentration using an Amicon 30 microcentrator. Representative gel of several purification attempts.

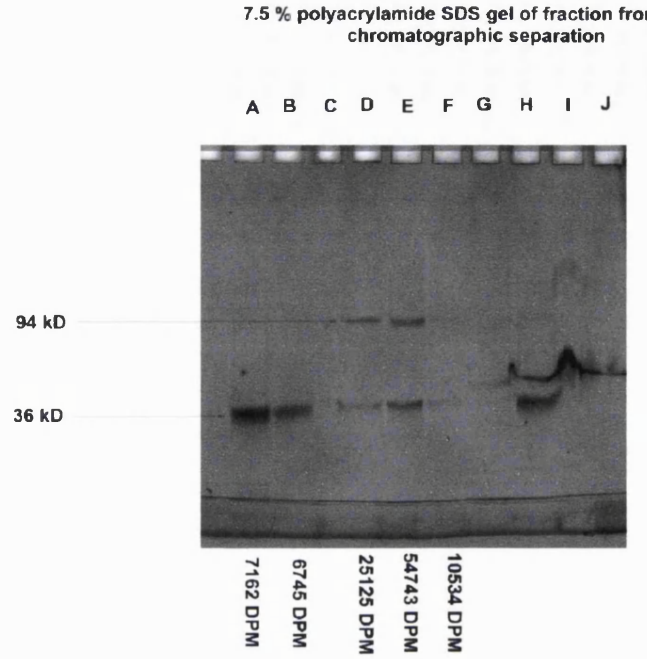


Figure 3.16 7.5% discontinuous polyacrylamide SDS gel of individual fractions taken from HTP chromatographic separation of pig brain methionine synthase

(Gel 3.3) The samples from the HTP column were taken and tested for methionine synthase activity. Once tested, the samples were placed in a sample buffer containing SDS and heated for ten minutes at 99 ^{o}C. Once denatured, 50 μl of sample/sample buffer mixture was loaded onto a pre-cast Pharmacia 7.5% gel and run at 200 volts for 40 minutes. Once finished the gel was fixed and silver stained using Pierce Code silver staining kit. Bands of protein can be identified at 94 and 36 kD. The maximum (54,743) of methionine synthase activity corresponds to lane 'D' which has the highest concentration of staining of the 94 kD band. Representative gel of several purification attempts.

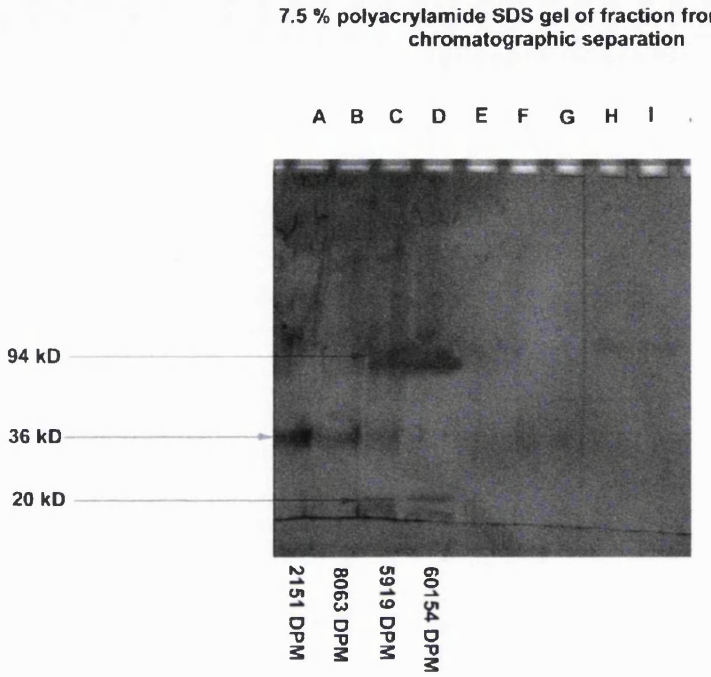


Figure 3.17 7.5% discontinuous polyacrylamide SDS gel of fractions taken from the Mono-Q chromatographic separation

(Gel 3.3) Following separation using the Pharmacia Mono-Q column the samples were desalted using an Amicon 30 microconcentrator before being denatured in sample buffer containing SDS. The samples were heated at 99°C for 10 minutes before 50 μl was added to the Pharmacia precast gel. The gel was then run at 200 volts for 40 minutes on a Pharmacia Multiphore flat-bed which was cooled via a recirculating water refrigeration system. Lanes 'C' and 'D' show a protein at 94 kD and 20 kD. Lanes 'A' and 'B' show a protein at 36 kD. The fractions loaded onto lanes 'C' and 'D' have the highest activity of methionine synthase and this corresponds to the 94 kD protein band. Representative gel of several purification attempts.

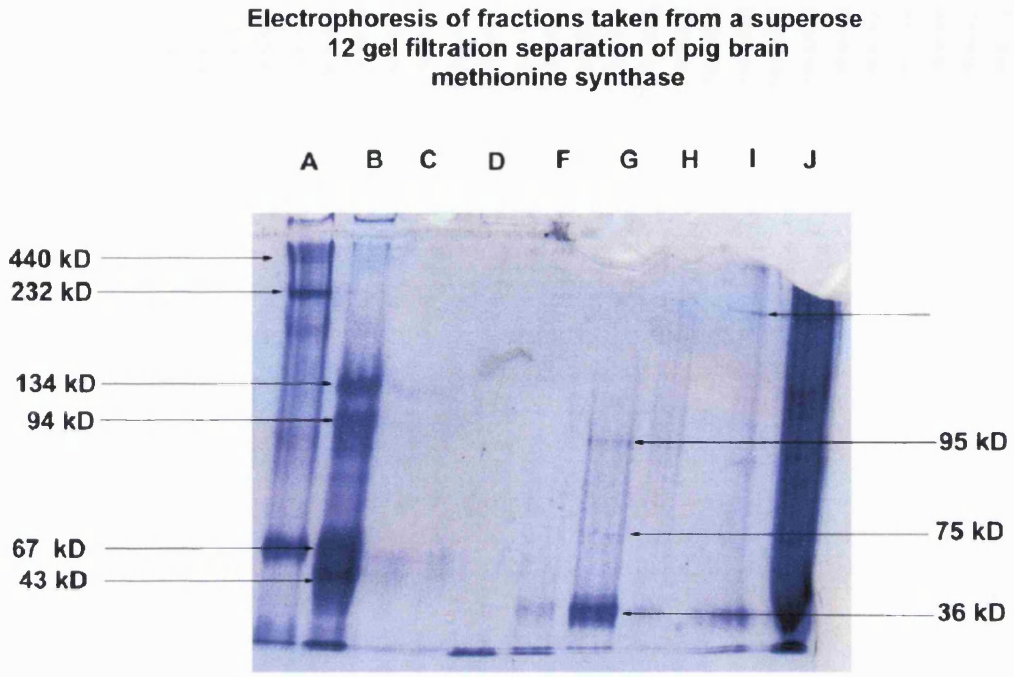


Figure 3.18 7.5% discontinuous SDS polyacrylamide gel of various fraction taken from a Superose 12 gel filtration column
(Gel 3.4). The sample loaded onto the Superose 12 column taken from the phenyl sepharose step of purification were loaded. The fractions were added to sample buffer containing SDS and heated from 10 minutes at 99 oC to denature. The gel was fixed and stained with Coomassie R250 stain. The active fraction contained protein bands corresponding to 95 kD, 75 kD and 36 kD. Representative gel of several purification attempts.

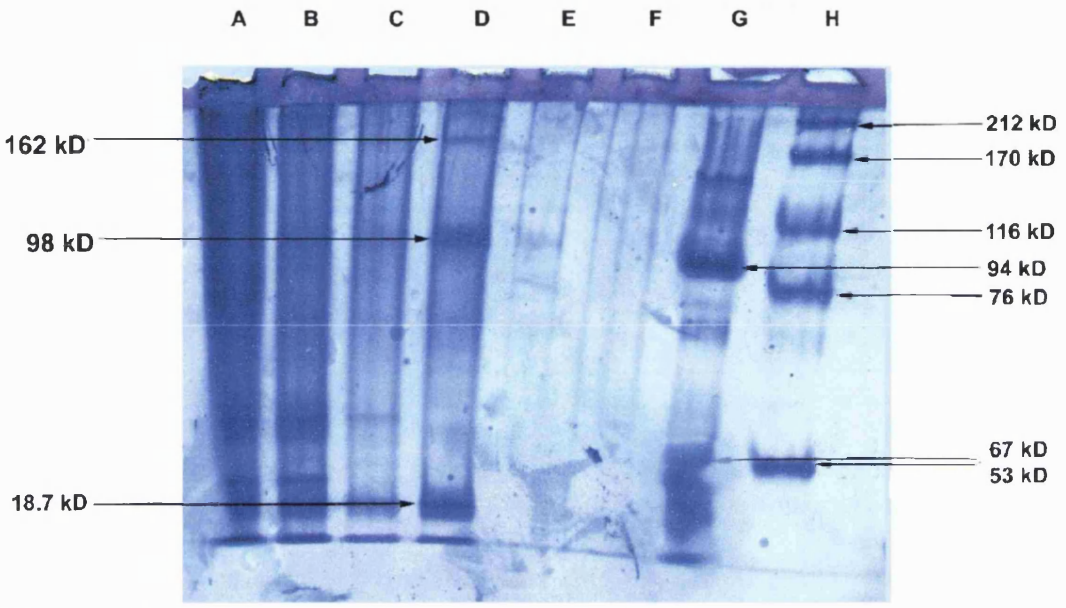


Figure 3.19 7.5% native discontinuous polyacrylamide gel of various pooled fractions from pig brain methionine synthase purification

(Gel 3.5) The gel was run at 200 volts for 30 minutes in non-reducing conditions. Enrichment can be seen of three bands at 162 kD, 98 kD and 18.7 kD at the HTP step in purification but are lost subsequently until the samples were re-run after concentration using an Amicon 30 microcentrator. Representative gel of several purification attempts.

The purification of pig brain methionine synthase was difficult due to the instability of the enzyme as well as the range of molecular weights already reported in the literature for mammalian enzymes. At the beginning of the purification it was found that there were two molecular weights at which pig brain methionine synthase was active. These were determined from gel chromatography using two different resin. The Superose 12 gave molecular weights of 160 and 101, while the S-200 gave molecular weights of 162 and 97 kD.

Purification step	µg/ml of protein	Volume of fraction (mls)	Total protein µg	Percentage yield	Activity in DPM	Activity in DPM/mg/h
Pig brain cytosol	28,800	2,000	57,600,000	100	17,209	6.0
DEAE	3,655	2,000	7,310,000	88	15,227	41.7
Q-sepharose	3,375	210	708,750	75	107,230	317.7
Ammonium sulfate	3,225	235	757,875	95	113,995	353.5
Phenyl sepharose	774	150	116,100	54	42,127	544.3
HTP	77.4	70	5,418	14	12,000	1,550.4
Mono-Q	43	4.5	193.5	10	47,000	10,930.2
Superose	10	1	10	*	12,475	58,625
	A 28,000x purification was achieved					

Table 3.5 Summary of purification steps and yields.
The table shows the degree of purification of methionine synthase from pig brain cytosol. The initial volume of sample was 2 litres which was quickly reduced to a more manageable volume. The yield from each step in the purification was reasonable except in the HTP and Mono-Q. These steps also were the steps where loss of the high (155 kD) molecular weight form of the enzyme occurred. The 28,000 times purification is approximate as not all of the samples were taken to the final step of purification. The specific activity of the enzyme was calculated as 58,625 DPM/mg/h.

This corresponds with the previous reported molecular weight of the mammalian enzyme. Utley (1985) purified methionine synthase using human placenta as a source for the enzyme and got a 160 kD enzyme, which upon analysis had subunits composed of 90, 45 and 35 kD. Chen (1994) purified a single protein of 155 kD from pig liver. Work carried out in our lab by Dr T. Ast and Dr S Kenyon on the rat liver enzyme showed an active enzyme with a molecular weight of 160 kD and subunits of 95 and 60 kD. These results were determined by a combination of calibrated gel filtration chromatography and SDS-PAGE analysis.

The results determined by SDS-PAGE for the pig brain methionine synthase enzyme back up the results from the gel filtration and show a progressive breakdown of the enzyme into two sub-units, one at 36 kD and the other approximately 94 kD. Loss of enzyme at the HTP chromatographic step may be partly due to breakdown of the enzyme and separation of its subunits. Loss

of a 45 kD subunit would explain differences in the reported molecular weights and those determined for the pig brain enzyme.

The pI determination was not carried out for any of the mammalian enzymes and only values for the bacterial enzymes have been reported. Dr Ast did report a pI of 5.1 for the rat liver enzyme (unpublished data T. Ast 1994) in agreement with these data. The bacterial enzymes had several reported pIs between 5.2 and 4.8 (Frasca et al 1988)

The degree of purification and yields are summarised in Table 3-3. The large quantity of starting material allowed for analysis of the enzyme and determination of molecular weight. Pilot studies made it clear that the ability to test for the enzyme activity was in no means linked to its detectability on SDS-PAGE even with silver staining.

Now that the brain B_{12}-MS or at least an enzyme fragment has been purified and the molecular weight and pI determined, the next logical step would be for tryptic digestion and sequencing. Following this a partial sequence can be determined and compared with existing published sequences to confirm that some degree of homology exists between species. Since completion of the purification of the pig brain methionine synthase enzyme a full sequence has been determined for the pig liver enzyme (Chen 1997) and a DNA sequence determined. Using this sequence and the DNA sequence located on chromosome 1 the purification sequence and steps should be much simpler. Production of a monoclonal affinity column and the use of elisa techniques for identification will speed purification and remove the need for such large volumes of starting material.

Chapter 4 Nitric oxide inhibition of methionine synthase

4.1 Vitamin B_{12} and nitric oxide

Vitamin B_{12} is of vital importance for the health and proper function of the nervous system. B_{12} deficiency causes neurological abnormalities such as poor memory, spinal cord damage and demyelination (Surtees 1993, Healton 1991, Duffield 1990). In mammals, B_{12} is an essential cofactor for only two enzymes: a) cytosolic B_{12}- dependent methionine synthase (EC 2.1.1.13) that requires methylcobalamin for the methylation of homocysteine to methionine; and b) methylmalonyl CoA mutase (EC 5.4.99.2) that catalyses the conversion of methylmalonyl-CoA to succinyl-CoA and uses adenosylcobalamin as cofactor.

Of these two enzymes, the first plays a critical role in the nervous system directly through its control of the levels of homocysteine and methionine. In contrast to tissues such as liver, that are potentially capable of synthesising methionine from homocysteine and betaine, the availability of methionine in the central nervous system is totally dependent on the B_{12} dependent methionine synthase. A known property of this enzyme is its inactivation by the anaesthetic/analgaesic gas N_2O, The inhibition is believed to occur through the oxidation of its methylcobalamin cofactor (Nunn 1985, Banerjee 1990). Studies on animals exposed to various concentrations of N_2O proved that this inactivation results in decreased levels of methionine and 5-adenosylmethionine and in alteration of the levels of folates (Vina 1986, Home 1989, Wilson 1986, Koblin 1989). Prolonged exposure to N_2O gives rise to symptoms and neurological changes similar to those observed during vitamin B_{12} deficiency (Flippo 1993, Nunn 1987), and to the side effects of morphine.

This investigation aims to study the *in vitro* inactivation of brain B_{12}-dependent methionine synthase in the presence of another nitrogen gas, nitric oxide, (NO) (Nicolaou 1994), and to compare this inactivation with that by N_2O. Nitric oxide, "the 1992 molecule of the year" affects blood pressure, neurotransmission, immune mechanisms, respiration and lung disease. In fact, it was because of NO's involvement in so many systems that it was awarded its title. NO is a diffusible messenger that mediates cell-cell interactions,

cytotoxicity, and neuronal signaling and is ubiquitous throughout the brain (Southam 1993, Moncada 1991, Schmidt 1993). Its biosynthetic pathway from arginine and its degradation to higher oxidized nitrogen oxides, such as nitrate ion, are known. When overproduced in pathological conditions, or by over administration, NO can produce toxic affects and terminal illness. To explain the natural, beneficial effects of NO as well as its role in disease and toxicity, a knowledge of the cellular target macromolecules and the effect of NO macromolecular complexation on metabolic pathways will have to be elucidated.

Since its identification as EDRF, the endothelium dependent relaxation factor, and the recognition of its role in cellular signalling, nitric oxide has been extensively studied. The L-arginine-nitric oxide pathway has served as an explanation for NO-mediated synaptic transmission, neurodegeneration, long-term potentiation (Moncada 1991, Schmidt 1993, Galley 1990). Its production by NO-synthase, a Ca^{2+} dependent enzyme, is believed to be regulated through a rise in Ca^{2+} influx caused by NO activation of NMDA receptors. The constitutive isoform of NOS releases NO in the initial period and acts as a mechanism for cell communication. The inducible isoform produces NO for longer periods and strengthens the cell resistance to invading microorganisms and tumor cells. The observed cytotoxicity of NO has been variously attributed to its inactivation of glycolytic, TCA cycle and respiratory enzymes that contain transition metals, usually iron, or to its stimulation of cGMP production and hence signal transduction mechanisms. Various redox forms of NO or formation of RONO and dioxygen adducts have also been proposed as important aspects of cellular NO mechanisms (Stamler 1992).

NO has been implicated in neurotransimission and neuromodulation (Bredt 1994). In the cardiovascular system it induces relaxation of the vascular smooth muscle and inhibits platelet aggregation and adhesion. In the immune system it participates in the non-specific immune responses to various pathogens, exhibits cytotoxic activity and has been shown to arrest tumour cell growth (Nathan 1992). In pathological conditions or in the immune responses to various pathogens, NO production exceeds the normal physiological levels

necessary for signal transduction responses. Being cytotoxic and cytostatic in its own right. NO may affect and damage the cells that produce it (Moncada, 1991). As a consequence, NO has been implicated in a variety of pathological situations, including septic shock, inflammation, hypertension, stroke and neurodegenerative diseases (Moncada 1993, Henry 1993).

4.2 Targets for NO inhibition/modulation

Biomolecules possessing transition-state metals are targets for NO. The formation of iron-nitrosyl complexes in the target proteins alters their functional response (Stamler 1992). Characteristic examples are: the activation of the soluble guanylate cyclase (Schmidt 1993), an effect that is considered to be the major biochemical pathway for the NO-mediated cell signalling; the inactivation of cytochrome c oxidase in macrophages (Cleeter 1994); and the arrest of DNA synthesis in the invading pathogens by inactivation of the ribonucleotide reductase (Knowles 1992).

Candidate target macromolecules have been proposed; (HS) groups on proteins and glutathione have been proposed as NO carriers, from-NO complexes with haemoglobin, and porphyrin proteins are known to form, but no fully rational or acceptable correlation between protein-Fe-NO complex formation and NO effects in cells and tissues have been proposed. NO definitively produces increases in cGMP and hence affects cGMP-mediated processes. This, too, has been attributed to NO complexation with the transition metal, Mn, of guanidylate cyclase.

4.3 Methionine synthase inhibition by NO

Here an additional mechanism of NO- mediated cellular reactions is proposed which utilises an altemative transition metal protein containing cobalamin as the target molecule, provides a rational cellular basis for NO signaling and NO toxicity, and resembles the intra-cellular mechanism of N_20 action.

Methionine synthase (N5 -methyltetrahydrofolate-homocysteine 5 - methyltransferase, EC 2.2.2.13) is one of only two mammalian cobalamin-dependent enzymes. It catalyses the production of methionine from

homocysteine using N^5-methyl tetrahydrofolate (MTKF) as cosubstrate and methyl donor. Through its reaction products this enzyme connects two major biochemical pathways: S-adenosyl methionine (SAM)-dependent methylation reactions and the tetrahydrofolate-dependent one-carbon unit transfers. Pathological conditions such as megaloblastic anaemias and homocystinuria have been related to the malfunction or inhibition of methionine synthase (Banerjee 1990, Luschinsky-Drennan 1994a, Luschinsky-Drennan 1994b).

Methionine synthase is known to be inactivated *in vivo* and *in vitro* by nitrous oxide (N_20) (Frasca 1986, Drummond 1994) and preliminary reports of the liver enzyme inhibition by NO (Ast 1994, Nicolaou 1994a, 1994b) and stimulation by polyamines (Kenyon 1995) have appeared. The inhibition of methionine synthase by NO may contribute to the further understanding of biological mechanisms related to methionine synthase activity and could add another dimension to the understanding of NO-induced cytotoxicity. Homocysteine is a substrate for methionine synthase and its inactivation by NO is expected to result in elevated homocysteine levels. Since plasma homocysteine has atherogenic properties and is considered to be a risk factor for vascular disease (Ueland 1989), this research adds a possible important connection between the latter and the NO-mediated effects.

4.4 Network of B_{12} dependent pathways and enzymes

Methionine synthase converts homocysteine (HCys) to methionine (Met) which in turn is converted to 5-adenosylmethionine (SAM) by SAM synthase. SAM is the cell's ubiquitous methylating agent for DNA, RNA, proteins, lipids, and numerous metabolites, including catecholamines and steroids. It also is the carrier for the aminopropyl groups needed for conversion of putrescine to spermine and spermidine.

The inhibition of methionine synthase by N_20, the gaseous analgesic/anaesthetic, and commencing with homocysteine, is a well-known and understood cellular pathway. Inhibition of methionine synthase has been shown to raise levels of homocysteine and hence increase the cellular concentrations of oxidised cysteines and homocysteines including taurine.

What effect this will have on oxidized S-amino acids in neurotransmission and cellular toxicity needs to be explored further. N_20 toxicity and its effects on cell growth and differentiation are mediated by the lowering of methionine and SAM levels and hence of folate metabolites. These factors modulate the biosynthesis of DNA, RNA, and proteins via methylation and via availability of dTMP formed from dUMP by thymidylate synthase. There are therefore not only structural parallels between NO and N_2O but parallels may be drawn with their cellular, physiological and toxicity effects. These conjectures prompted us to purify brain mammalian synthase to compare its inhibition by NO and N_20 at the level of the purified enzyme.

The inactivation of B_{12}-dependent brain methionine synthase may occur under either physiological or pathological concentrations of NO and is expected to result in an enzyme inactivation similar to that induced by N_2O. It therefore offers another possible mechanism for NO mediation of brain functions and abnormalities, including growth and differentiation, memory and learning, neural signaling, analgesia, neurotropic and neurodegenerative diseases. Garthwaite et al (Southam, 1993) have pointed out that more than one NO-dependent mechanism will be needed to rationalise many of these functions and abnormalities. The correlation between vitamin B_{12} and NO involvement in brain functions and diseases also supports a role for NO mediation of B_{12} enzymes and pathways as well as the more traditional NO mediation of guanylate cyclase in these processes.

4.5 B_{12}-dependent methionine synthase inhibition studies

B_{12}-dependent methionine synthase activity was measured at different times and under different gases, by equilibrating the deoxygenated assay solutions in a) N_2; b) N_2O; and c) NO authentic gas. The gases were inserted into the sealed assay vials using a gas tight syringe.

For the NO dose-dependence studies aliquots of a 3% (v/v) NO solutions (-1.3 mM) prepared in deoxygenated 50 mM potassium phosphate buffer, pH 7.4 by the method of Ignarro et al (Ignarro, 1987) were used and diluted as required. The NO solutions were kept on ice and used within one hour of their

preparation. The aliquots were added into the deoxygenated assay mixtures with a gas tight syringe.

For the sodium nitroprusside (SNP) dose-dependent study, aliquots of a solution in 50 mM phosphate buffer, pH 7.4, of the appropriate SNP concentration were added into the deoxygenated assay mixtures prior to incubation.

The *in vivo* inactivation of methionine synthase by N_2O has been detected in mammalian tissue during the animal's exposure to various concentrations of the gas (Nunn, 1985, Nunn, 1987). The *in vitro* inactivation of the bacterial isoform of the B_{12} dependent methionine synthase from *E.Coli* as well as the pig liver enzyme have also been demonstrated (Banerjee, 1990)(Frasca, 1986)(Ast, 1994).

Our studies were initiated to compare the *in vitro* inactivation of brain methionine synthase by N_2O and NO, both in a time and concentration dependent manner. As demonstrated in the time-dependent study (Figure 5-1) almost complete inhibition of the enzyme with N_2O occurred within 5 min.

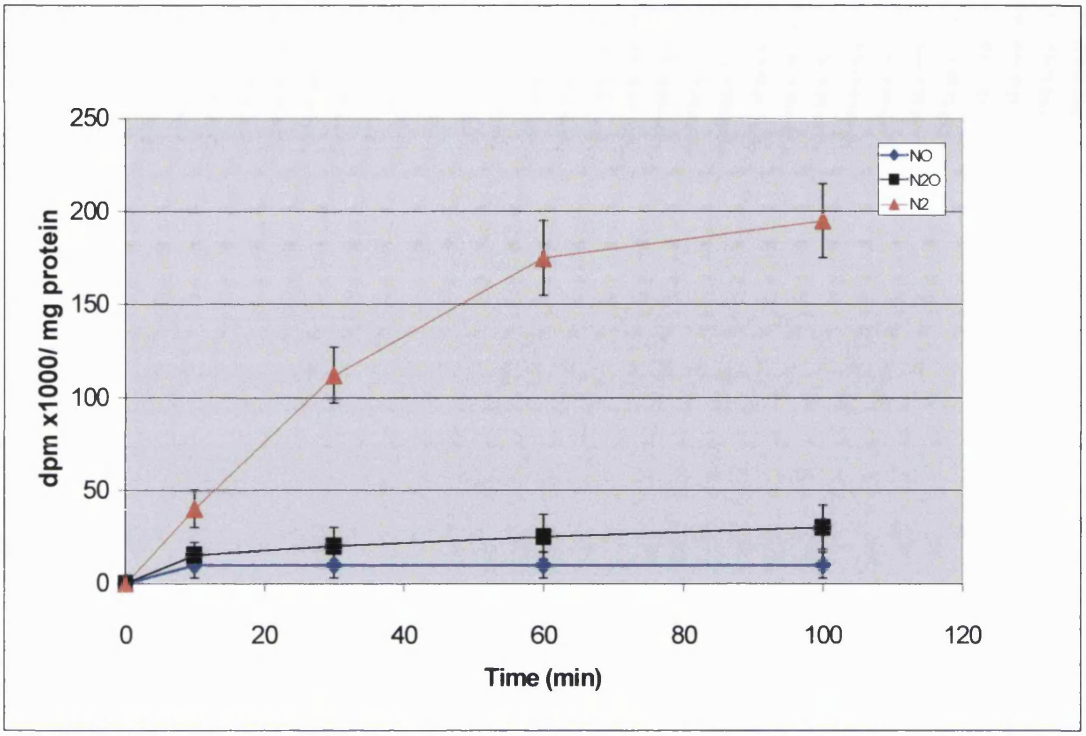


Figure 5.1 Time-dependent study of the inactivation of brain methioninine synthase by N_2O and NO gas.
The results are expressed as dpm/mg of protein. The control levels of the enzyme activity were measured under N_2. The methionine synthase activity was assayed using a modified method of Weissbach et al. Assays were carried out in sealed serum vials and incubated at 37°C. Complete inhibition of methionine synthase is seen immediately and does not recover with extended incubation. The maximum rate of enzyme activity occurs between 5-30 minutes. Each data point represents 5 separate assays averaged and SD calculated.

The N_2O inactivation of the enzyme was also dose-dependent as shown in Figure 5.2. For this experiment the enzyme was exposed to an increased concentration of gaseous N_2O by varying the ratio of N_2/N_2O in the incubation mixture. To date, for the mammalian and *E.Coli* enzymes, similar dose-dependent studies have not been reported.

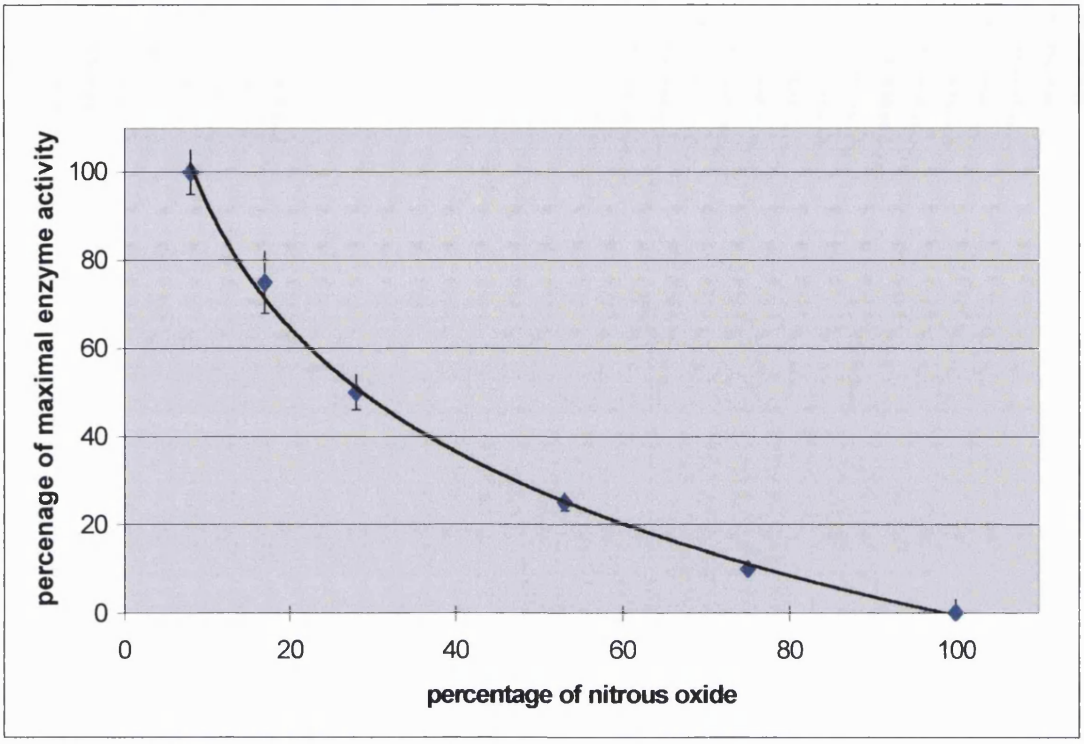


Figure 5.2 Dose-response curve for the in vitro inactivation of the brain methionine synthase by N2O

The results are expressed as percentage of remaining enzyme activity. Mixtures of N_2/N_2O of different ratio were mixed using a surgical anaesthetic trolley. A decrease in the enzyme activity was observed as the percentage of N_2O increased in a dose dependent manner. Due to the difficulties in creating a saturated N_2O solution a molar value cannot be calculated for the IC50. Each data point represents 5 separate assays averaged and SD calculated.

The time-dependency of the NO-induced inhibition was examined by two different experiments. The first approach used aliquots of NO saturated solutions prepared in fully deoxygenated buffer saturated with authentic NO gas (Ignarro, 1987). This study gave an apparent IC_{50} of 20μM (Fig 5.3). Care was taken to carry out these experiments in the absence of haemoglobin and other proteins that can utilise NO as a ligand. Consequently, these values should be taken as an upper concentration limit for NO inhibition.

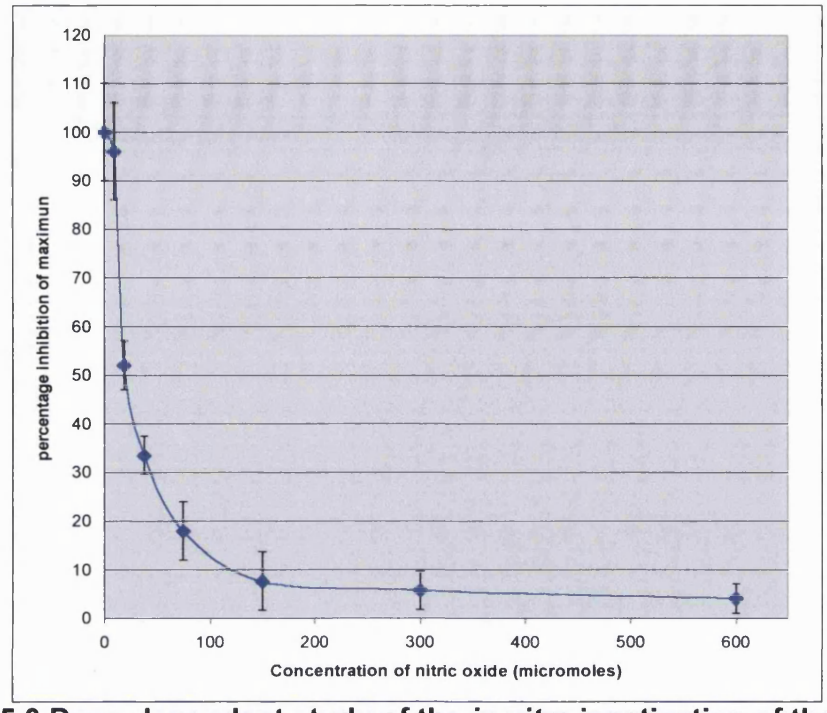


Figure 5.3 Dose-dependent study of the *in vitro* inactivation of the brain methionine synthase by NO

Serial dilutions of 3% saturated NO solution in buffer were used. Methionine synthase activity was assayed using a modified method of Weissbach et al. The results are expressed as percentage of remaining enzyme activity. The IC50 for the brain enzyme was found to be 17.8 μm. Each data point represents 5 separate assays averaged and SD calculated.

Since NO is highly reactive and can be readily oxidised even in the presence of traces of oxygen, the next approach was to confirm its inhibitory activity by using a NO-releasing substance, e.g. sodium nitroprusside (SNP). Aliquots of a 1.6 mM solution of SNP in 50 mM phosphate buffer, pH 7.2 were utilised in the dose-dependent study. As shown (Figure 5-4), SNP inactivated the brain methionine synthase with an IC_{50} of 50 μM.

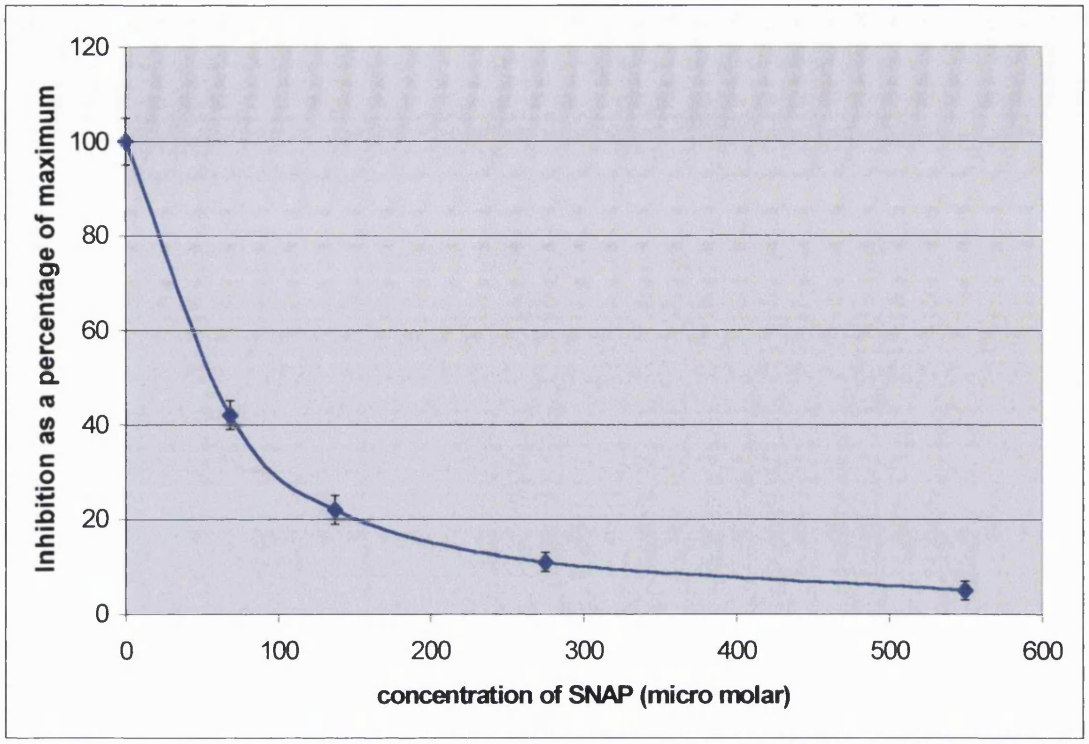


Figure 5.4 Dose-dependent in vitro study of the SNP induced inactivation of brain methionine synthase by SNP
The results are expressed as percentage of remaining enzyme activity. Methionine synthase activity was assayed using a modified method of Weissbach et al. The IC50 for SNAP was found to be 50 μM. Each data point represents 5 separate assays averaged and SD calculated.

The important role of vitamin B_{12} in cell division as well as in development and healthy maintenance of the nervous system, is widely accepted (Metz, 1992). In brain, vitamin B_{12} is an essential cofactor for the transmethylase, methionine synthase (B_{12}-MS), which uses methylcobalamin to produce methionine from homocysteine. It utilises the cofactor methyltetrahydrofolate and has an absolute requirement of 5-adenosylmethionine.

Through its regulation of methionine and hence 5-adenosylmethionine levels, B_{12}-MS is implicated in biological methylation reactions. It also affects the folate cycle and polyamine production via the levels of decarboxylated SAM and the enzyme putrescine and spermidine aminopropyl transferases.

The N_2O inhibition of B_{12}-MS has provided a rationale for N_2O analgesia and cytotoxicity and, by analogy, can also provide a similar mechanism for NO cell

toxicity and analgesia. Thus, B_{12}-MS inactivation inhibits folate-dependent production of dTMP and hence DNA replication (Figure 5). A second cell toxicity mechanism could involve inhibition of SAM-dependent protein, DNA and RNA methylation by reduced SAM levels, since DNA/RNA methylation is critical for gene expression. A third mechanism would involve decrease of polyamine levels due to reduced concentrations of SAM and hence decarboxylated SAM. The correlation of reduced polyamine levels with cell growth and differentiation is well known (Morgan, 1990) and it is therefore not unreasonable to attribute NO toxicity, at least in part, to a B_{12}-dependent mechanism. These three cytotoxic mechanisms need not obviate NO toxicity due to inhibition of Fe containing redox enzymes of mitochondrial enzymes and the glycolytic pathways.

The short time-dependence of the N_2O inhibition of B_{12}-MS and of N_2O analgesia must implicate B_{12}-MS and its pathways in the rapid changes characteristic of cell signalling. Since NO has been proposed as an endogenous brain signalling molecule and is involved in analgesia (Nakamura, 1993), it is possible that the reported rapid dose-dependent NO inhibition of B_{12} MS is also related to the role of NO in cell signalling aspects of analgesia and/or anesthesia.

This leaves the question of the relative importance of (a) the B_{12}-MS mechanisms for the effects of NO and N_2O on general cell signalling; and (b) the more established cGMP signalling mechanism. Additionally, since in analgesic mechanisms, morphine and opioid peptide receptor activation produces changes in intracellular K+, Ca^{2}+, and cAMP, these changes need to be related to the guanylate cyclase and B_{12}-MS mediated events. It would therefore be important to compare N_2O and NO effects on these signalling processes.

Another biochemical analgesic mechanism involves inhibition of the enzyme cyclo-oxygenase and hence eicosanoid formation. Again, these apparently different cellular analgesic mechanisms can be rationalized. Provided the SAM-dependent formation of PC from PE is a source of arachidonic acid and

eicosanoids and morphine receptor-generated signals could regulate B_{12}-MS guanylate cyclase or eicosanoid formation?

Regretfully, time permitted study of only NO/ N_2O effects on cGMP production in this thesis (see later).

Many groups have recently associated B_{12} with neuropathology, specifically Alzheimer's disease (Kirstenen, 1993)(Levitt, 1992)(Ikeda, 1992), Multiple Sclerosis (Sandyk, 1993)(Reynolds, 1992), and Down's syndrome (Hestnes, 1991). This is not surprising in view of the importance of B_{12} in normal cell growth. Since NO has also been implicated in neurological disorders, the observation here that the NO/B_{12}-MS system may be relevant to neurology, neuropathology, and neurotoxicity is not unreasonable.

The vital importance of vitamin B_{12} for the health and proper function of nervous system is widely accepted. B_{12}-dependent methionine synthase is one of only two mammalian B_{12} enzymes. It regulates homocysteine, methionine and 5-adenosylmethionine-dependent pathways and metabolites. NO is a free radical, ubiquitous throughout the brain and affects brain functions through cell signalling and neurotoxicity mechanisms. B_{12}-dependent methionine synthase inactivation by the predominantly anesthetic gas, N_2O, has been shown to decrease SAM and folate levels and generate symptoms synonymous to B_{12} deficiency. The inactivation of brain B_{12}-dependent methionine synthase by gaseous NO and N_2O was shown to occur in both a time and dose-dependent manner, and also by SNP. Since the B_{12}-dependent methionine synthase dependent biochemical pathways include biological methylation, polyamine, folate and sulfur amino acid formation, the NO-induced inactivation may indicate a mode for the NO action that utilises this network. Thus, in addition to the established NO effects on cyclic GMP pathways, NO mediation of the vitamin B_{12} network may be involved in brain cell signalling, growth and development, neurotoxicity and neurological diseases.

4.6 *Inhibition studies on rat liver enzyme using NO*

The assay mixtures were deoxygenated by flushing with nitrogen, and preincubated for 5 min at 37°C. The reaction was initiated by the addition of homocysteine through a gas tight syringe and the reaction mixtures were incubated for 10 min at 37°C unless otherwise stated. The reaction was terminated by the addition of 400μl ice cold water and the resulting solution immediately passed through a 0.5 x 5.0 cm column of AGiX8 resin. [^{14}C]methionine was eluted with 2ml of water, collected and quantified by scintillation counting. Methionine synthase activity was determined at different times and with different concentrations of NO. Aliquots of a 3% (v/v) NO solution (~1.3mM) were used (21). NO solutions of various concentrations were prepared in deoxygenated 50mM potassium phosphate buffer, pH 7.4. The aliquots were injected into the assay vials using a gas tight syringe. All the NO solutions were kept in ice and used within 30 min of their preparation. When authentic NO gas was used, it was directly inserted to the thoroughly deoxygenated assay vial by a gas tight syringe.

The rat liver methionine synthase was completely inactivated when the assay was performed in the presence of authentic NO gas or aliquots of saturated NO solutions. The inactivation occurred almost instantly (<30 sec) after the NO introduction and the enzyme did not regain its initial activity when incubated for 1 hour. A time curve of the enzyme inhibition performed under nitrogen and in air-tight vials, was used as a control for the NO inactivation experiment (Fig 5.5).

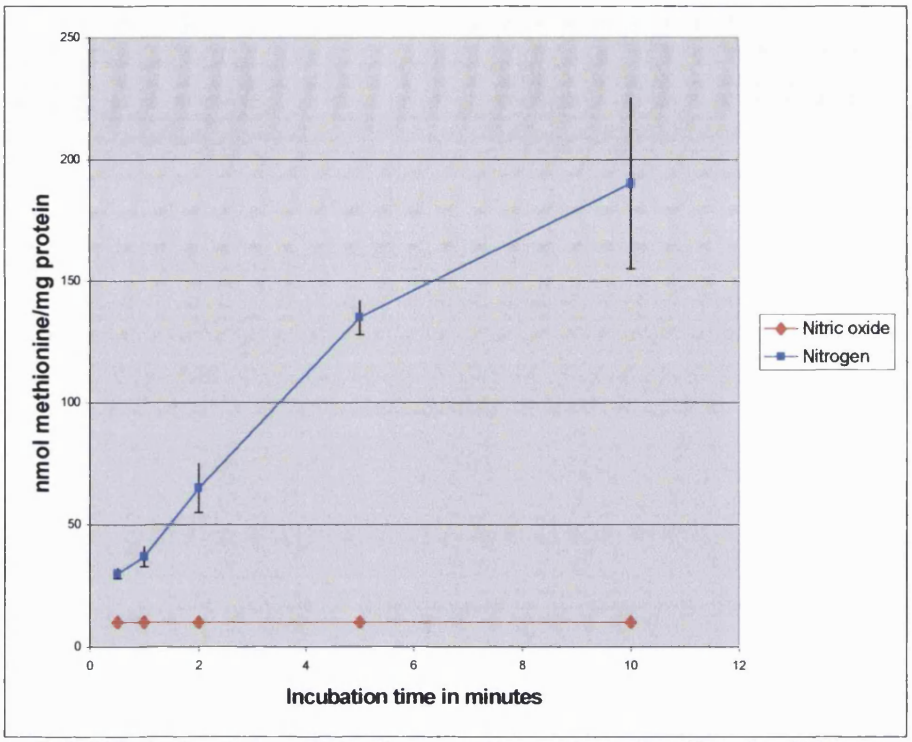


Figure 5.5 NO-induced methionine synthase inactivation by NO
Methionine synthase activity is plotted against time in the presence of nitrogen gas (N_2) and in the presence of 50μl injection of a saturated solution of NO (3% v/v). Enzyme activity was measured using a modified method of Weissbach et al. The 3% saturated solution equates to 3 mM solution of NO. The final concentration of NO in the assay mixture equates to 50 μM. At this concentration we observe a complete inhibition of methionine synthase activity which does not recover with time. Each measurement was done in triplicate.

A dose-dependent study performed with aliquots of serial dilutions of a freshly prepared NO solution gave an apparent IC_{50} value of $3\pm2\mu M$ (Fig 5-7). Given the poor solubility of NO (3% at room temperature) and its high reactivity in the presence of even traces of oxygen, this value represented an upper limit to the effective IC_{50}. In many cases of NO production gives local concentrations of NO approximate by 1-5 mM, implying that the IC_{50} works at physiological concentrations of NO as well as in pathological concentration.

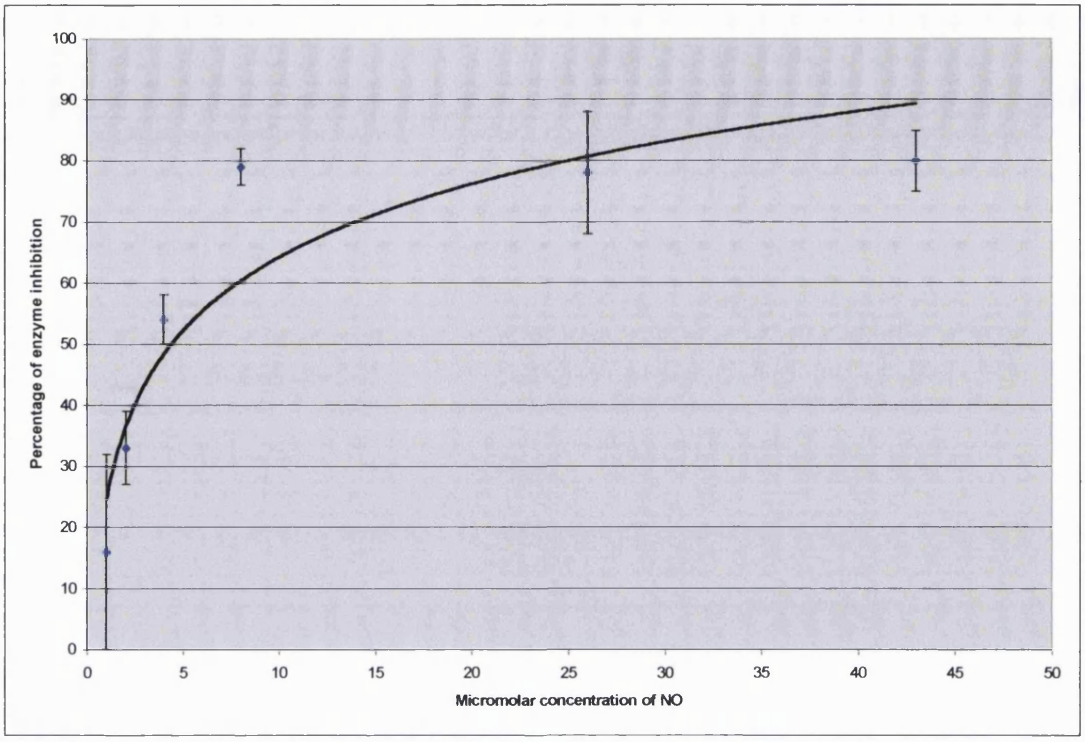


Figure 5.6 Dose response curve for the rat liver methionine synthase inactivation by NO

Serial dilutions of a saturated NO solution (3mM) were used). Enzyme activity was measured using a modified method of Weissbach et al. An IC_{50} value of 3μM was estimated from graphing of the results. Care was taken during the experiment to ensure that all buffers used were degassed to limit oxidation of NO gas. Each measurement was an average of three assays.

Homocysteine, the substrate for methionine synthase, is cytotoxic at high concentrations. Elevated concentrations in plasma are considered as a risk factor for vascular disease and have been correlated with homocystinuria and atherosclerosis .

Homocysteine metabolism is complex and its cell and plasma levels can also be affected by selective inhibition or stimulation of its conversion to cysteine, and a whole family of progressively oxidized sulfur amino acids. Nevertheless the research described here, if confirmed at the cell level, can bring together the fields of NO and homocysteine in cardiovascular physiology since it provides evidence for the inhibition of methionine synthase, and hence elevated homocysteine levels, by nitric oxide at physiologically occurring levels of the latter. By inhibiting methionine synthase NO should lower methionine and SAM levels. These two effects combined with the control methionine synthase exhibits on folate levels, biological transmethylations and

transulfurations, and polyamine pathways, could provide an attractive and simple explanation of the cellular and cytotoxic effects of NO.

Chapter 5 Effects of hyperbaric oxygen on nitric oxide formation

5.1 Reactive oxygen species

Oxygen toxicity was first described by Joseph Priestley in 1774 when he discovered the gas. His assumption was that just as a candle would bum hotter and brighter in pure oxygen, so might human metabolism be increased and bum out (Priestley, 1775). Since then knowledge of oxygen and its effects on the body have increased significantly. However, we still are at a loss to explain what oxygen does to the body to cause the pathological side effects of breathing it at partial pressures greater than 0.5 bar.

Oxygen would be lethal to most organisms at normal atmospheric pressures and concentrations if they had not developed a method of controlling the toxic intermediates or free radicals that are a by-product of aerobic glucose metabolism. The pathways for reactive oxygen species metabolism may be linked to the pathogenesis of oxygen toxicity. The mechanisms by which the body controls superoxide anions, (O_2^-), hydroxyl radicals, (OH^-), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2) is related to oxygen pressure (Fridovich, 1976).

Oxygen is used in a whole range of important metabolic reactions in the body besides the oxidation of glucose. It is also a necessary reactant in the synthesis of several neurotransmitters and lipid products:
synthesis of 5-hydroxytryptophan from L-tryptophan requires molecular oxygen and the enzyme tryptophan hydroxylase
phenylalanine to tyrosine
lipid peroxidation.
Changing the concentration of oxygen available to the reactions will affect the equilibrium of these reactions and possibly cause toxicity at high pressures.

5.2 Oxygen toxicity

Oxygen toxicity can be divided into two separate, but not necessarily unconnected, pathophysiological conditions. Aerobic organisms are adapted to breathing air at approximately 1 bar or atmospheric pressure. The

concentration of gases and their partial pressures at atmospheric pressure are 21% oxygen (pp 0.21 O_2) and 79% nitrogen (pp 0.79 N_2). Small changes can occur in these partial pressures without having any significant effect on respiration or metabolism in the organism. However if an organism is placed in an atmosphere containing pure oxygen for extended periods (over 24 hours) then we begin to see the chronic effects of hyperoxia poisoning (Dasta 1992).

The first symptoms of this condition are seen after the initiation phase of the injury. The beginning of the acute phase is marked by oedema, alveolar haemorrhage and inflammation, necrosis of the pulmonary endothelium, and type I pneumocytes. This is followed by a proliferative phase, marked by an increase in number of type II pneumocytes and resorption of pulmonary exudates in the pulmonary tissue. During this phase there is an increase in fibrous tissue causing potential long term scarring of pulmonary tissue. After extended exposure to partial pressures in excess of 0.6 bar, death will occur. (Crapo, 1980)

The conditions where a person would be likely to be exposed to oxygen at a raised concentration or a raised pressure occur only in hospitals where it is used therapeutically to treat a number of conditions and in the SCUBA/commercial diving industry. It has been thoroughly studied due to the use of oxygen therapy in cases of gas poisoning and the treatment of cardio-pulmonary disease (Hart, 1986). Here the benefits oxygen might give the patient must be weighed against the effects oxygen will have on the lung's ability carry out gas exchange and the long-term damage the lungs will sustain.

The conditions outlined above are found mainly in hospitals where oxygen treatment is carried out. The effects oxygen can have when breathed at increased atmospheric pressure occur much sooner. This is most commonly seen in the diving world. A great deal of research was conducted by the British Admiralty before W.W.II. However, hyperbaric oxygen is commonly employed not only in the treatment of diving related injuries (Van Genderin, 1968) but also in the case of gas poisoning and in the treatment of gas gangrene.

Studies were first carried out on the hyperbaric effects of oxygen on humans in 1910 when two engineers breathed 95% pure oxygen while working in a caisson at 3 ATA for thirty minutes, with no effect. In the 1930s the Admiralty in Britain carried out studies on divers at 2 ATA for 1 hour, and 3 ATA for 30 minutes, without ill effect. When, in 1933, two British officers breathed oxygen in a dry chamber at 4 ATA, convulsions occurred between 13-16 minutes. When trials were repeated in a wet pressure chamber, the results were dramatically different. Here volunteers were not able to withstand the same pressures or time that they were able to withstand in the dry atmosphere. Most of the volunteers showed symptoms after 10 minutes at 90 feet sea water (fsw) in the wet, but were able to last four times longer in the dry. One question this raises is what the difference is between breathing oxygen in the wet and breathing it in a dry chamber.

Also studied was the effect of exercise on the time to convulsions. The Admiralty, in 1937, showed that increasing activity had a significant effect on decreasing the time to convulsion (Donald, 1945)(Donald, 1992). The studies on the effects of oxygen on the body were concerned with what happens when humans are exposed to increased concentrations of oxygen but were not able to explain the mechanisms behind these effects. It is only now, with our greater knowledge of neurotransmission in the brain and the physiology and pharmacology of humans that we are able to begin to explain what happens at a cellular and system level.

5.3 Effects of oxygen on the body

To better understand how oxygen can affect an organism pathologically, we need to first look at its role physiologically. Oxygen is taken up by the lungs, where it diffuses across the lipid membranes to be taken up by the haemoglobin in red blood cells. The concentration of oxygen is measured physiologically as partial pressure of the gas in mm of mercury (mm Hg). In the atmosphere we find oxygen at approximately 160 mm Hg (atmospheric pressure is 740 mm Hg), and this decreases to 105 mm Hg in the alveoli. Oxygenated blood has about 105 mm Hg oxygen and at the cells we find 40 mm Hg at normal physiological conditions. At atmospheric pressure, oxygen is

carried bound to haemoglobin that is 99% saturated at atmospheric pressure. The other factor that affects oxygen carrying capacity in the blood is the solubility of oxygen in the plasma, and the pressure. Oxygen is poorly soluble in plasma, with a solubility coefficient of 0.024 as compared to that of CO_2 of 0.57. Only about 2% of the oxygen carried by the blood is dissolved in the plasma. Henry's law states that one can increase the concentration of dissolved gas by increasing the pressure according to the equation:

concentration of dissolved gas = pressure x solubility coefficient

When a subject is placed under pressure, breathing pure oxygen, not only is the concentration of the gas, but there is also an increase in the amount dissolved due to the pressure.

Thus, at atmospheric pressure, by breathing 100% oxygen, the alveolar oxygen concentration is increased from 105 mm Hg to 680 mm Hg. When breathing oxygen at pressure, this will increase proportionately with the increase in pressure. Therefore, if the concentration of oxygen in the breathing mixture is increased, there is not a large effect on the oxygen carrying capacity of haemoglobin, since this is already saturated with oxygen at 105 mm Hg, but the concentration dissolved in the plasma will increase. This will increase enormously the amount of oxygen reaching the tissues and so affect the equilibrium of any reactions in the body involving oxygen.

5.4 Reactive oxygen species and oxygen toxicity

The role that reactive oxygen species (ROS) play in the genesis of oxygen-induced seizures has been investigated by several groups. The idea that ROS are important in oxygen seizures has been around for some time. However, investigation of ROS is difficult because of the very short half-life of these compounds. Torbati (Torbati et al, 1992) used "spin trapping" by which ROS are bound to organic compounds which are much more stable. These adducts between the spin traps and ROS can then be detected by electron spin resonance spectroscopy. They found that in rats exposed to 5 ATA of oxygen, ROS were generated in the brain and the appearance of the ROS preceded

the onset of an overt seizure in the animal. This suggests that ROS are generated in brain during hyperoxic exposure, but does not directly link the ROS to the seizure. Several other groups of investigators have used glutathione (a thiol based ROS scavenger) and demonstrated that treatment of animals with glutathione extended the time to oxygen-induced seizure and also extended survival time (Jenkinson 1989,Peacock 1994,Sanders 1972). This suggests that generation of ROS may be important in the genesis of the seizure. Glutathione can react with the enzyme glutathione peroxidase and hydrogen peroxide (a ROS but not a radical) to form a nontoxic compound, glutathione disulfide (Jenkinson 1989). Glutathione peroxidase is a selenium-dependent enzyme, and rats deficient in selenium are not protected by glutathione against oxygen-induced seizures (presumably because their glutathione peroxidase cannot function properly (Jenkinson 1989). Rats fed supplemental beta-carotene (a natural antioxidant) are significantly protected from oxygen-induced seizures (time to seizure was doubled) (Bitterman 1994). This study is very interesting because, to be effective, the beta-carotene had to be natural, as a 1:1 mixture of cis/trans isomers. Synthetic beta-carotene, which was the all-trans isomer was not effective. Beta-carotene is a precursor of vitamin A, and in this study some data suggested that the protection from seizures was related to increased levels of vitamin A. (It is also interesting to note that these rats were fed enough beta-carotene to tum their fur yellow.) This data strengthens the argument that ROS are important in the genesis of oxygen seizures. In a more definitive study, intravenous administration of liposome- encapsulated superoxide dismutase (SOD) and catalase were used to increase the levels of these enzymes in rat brain (Yusa 1984). Treatment with SOD/catalase extended the time to seizure about three-fold. Both SOD and catalase are enzymes with highly specific actions that play important roles in oxygen metabolism. An increase in the tolerance to hyperbaric oxygen by SOD/catalase was therefore relatively good proof that ROS were involved in the genesis of oxygen seizures. That catalase was more protective than SOD suggests that hydrogen peroxide may be more important in seizures than superoxide (which SOD converts to hydrogen peroxide). One single study with vitamin E demonstrated that mice deprived of vitamin E were more likely to have a hyperoxic seizure than mice fed a normal diet (Kann 1964).

5.5 Anti-oxidants

As stated earlier, all aerobic organisms have evolved a mechanism for dealing with the reactive intermediates produced in aerobic metabolism. Oxygen itself can occur in many different states depending on the number, spin and placement of the electrons in its outer shell. Free radicals, such as the superoxide anion O_2^-, are produced by the addition of one electron to O_2, as a by-product of aerobic glucose metabolism, and need to be dealt with as they are highly reactive and can cause damage to other metabolites and pathways. At the terminal end of the electron transport chain, oxygen usually accepts two electrons and two protons to form water. However, occasionally the product is O_2^-.

To deal with superoxide produced during respiration as well as that occurring in the atmosphere, respired aerobic organisms have an antioxidant system of enzymes which have evolved to rid the body of these reactive oxygen species. The enzymes and systems include superoxide dismutase (SOD), glutathione redox mechanism, and catalase. The body has other antioxidants that may play a significant role in controlling free radicals, including vitamins A (retinal), C (ascorbic acid), E (alpha-tocopherol) and uric acid. These enzymes and systems work in tandem to rid the body of free radicals and dangerous redox species which might otherwise react and cause damage (Pryor 1980).

Superoxide dismutase is one of the most studied enzymes in both prokaryotes and in higher mammals. SOD catalyses the reaction of two molecules of superoxide anion to produce hydrogen peroxide and oxygen. It has been purified and studied to a great extent with different iso forms of the enzyme present in different tissues. It catalyses the reaction converting O_2^- to H_2O_2 and O_2.

$$2\ O_2^- + 2\ H^+ \xrightarrow{\text{SOD}} O_2 + H_2O_2$$

The H_2O_2 produced can be removed by catalase which catalyses the reaction of two hydrogen peroxide molecules to produce two water and one oxygen.

catalase

$$2\,H_2O_2 \text{ ----------> } 2\,H_2O + O_2$$

These two enzymes help rid the body of two highly reactive molecules which are potentially toxic. They have also been shown to effect the synthesis of nitric oxide.

Superoxide dismutase was shown to directly affect the formation of nitric oxide from arginine. The authors used formation of L-citrulline to measure changes in NO metabolism. They concluded that SOD was stimulating the formation of NO (Hobb 1994).

5.6 Superoxide dismutase and nitric oxide

Researchers at Duke University in the United States looked at SOD in mice to see if they could alleviate some of the symptoms of oxygen toxicity. To do this they developed a strain of transgenic mice with over-expressed SOD (Oury 1992). Their assumption was that the damaging effect of oxygen was due to excessive concentrations of reactive oxygen species. However, when they exposed the mice, which over-expressed SOD to 6 bar hyperbaric oxygen, they found that not only did the animals have a higher morbidity (83%) than the control animals (33%), but they also began to have seizures at a lower pressure and sooner into the compression time. Following these results the researchers tried an SOD inhibitor, diethyldithiocarbamate. Both the transgenic and the control mice were protected against seizures by the diethyldithiocarbamate. This indicated that the superoxide anion is not solely responsible for hyperbaric oxygen toxicity.

If O_2^- is not responsible, then what is? At the same time as these experiments were being done, the EDRF molecule nitric oxide (NO) was being studied for its effects on the body as a neurotransmitter along with its role in vasodilatation. NO reacts quickly with O_2^- to form the peroxynitrite adduct, $NO.O_2^-$. By over-expressing the SOD enzyme and reducing the concentrations of O_2^-, the half-

life for NO increases rather than reacting with O_2^- to form peroxynitrite ($ONOO^-$). Oury et al decided to try a specific NO synthase inhibitor, mono-methyl-arginine (MMA). By repeating the experiments with the transgenic animals now dosed with MMA one hour before compression, they found that both the control and the transgenic mice were protected from seizures. This would indicate that NO is playing an important part in the pathogenesis of hyperbaric oxygen seizures?

The synthetic pathway for nitric oxide is synthesised from arginine and in the brain is dependent on calcium and an electron donor.

$$\text{Arginine} + O_2 \xrightarrow{\text{NO synthase}} \text{citrulline} + NO$$

NO is readily diffusible and so cannot be stored in the body. It therefore must be synthesised immediately before its use. The main factor in the control of NO synthesis appears to be intracellular Ca^{2+} concentration. NO synthase has been shown to be calcium-dependent, requiring calmodulin. By limiting Ca^{2+} in the neurons we can therefore inhibit NO synthesis. As oxygen is necessary for the synthesis of NO we can hypothesise that its synthesis will be affected by changes in oxygen concentrations. (Rengasamy 1991) showed that NO synthesis was affected by the oxygen partial pressure. They showed that by reducing oxygen concentrations below atmospheric levels (hypoxic) they could inhibit NO synthesis and that by increasing the oxygen partial pressure above atmospheric (hyperoxic) they could increase NO synthesis under the correct conditions.

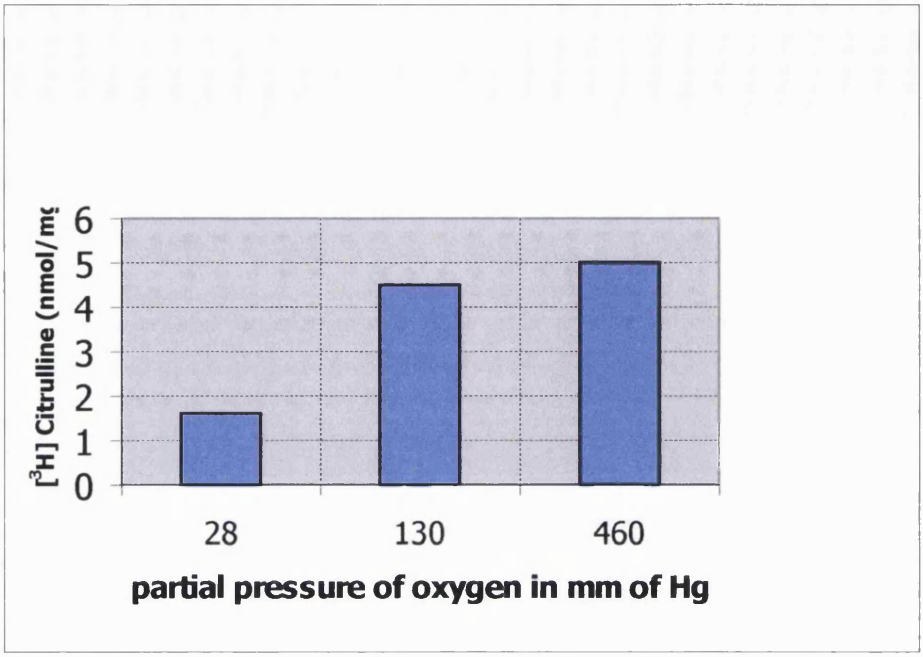


Figure 5.1 Effects of oxygen tension on nitric oxide synthesis
This chart represent the data taken from Rengasamy et al. The graph clearly demonstrates the relationship between oxygen partial pressure and nitric oxide synthesis a measured by the formation of radioactive citrulline. In the paper it was stated that 1300 mm Hg was normoxic, with 28 mm Hg being hypoxic and 460 mmHg taken as hyperoxic. The change in formation of citrulline between 130 and 460 is minimal however between 28 and 130 mm Hg it nearly triples.

The assay was performed on bovine cerebellum cytosol. What the researchers failed to take into consideration was the actual real and effective physiological oxygen concentrations! They assumed atmospheric concentrations as their physiological control whereas they should have used the cellular oxygen concentration? The following table shows that oxygen tension at the cellular level is approximately 40 mm Hg at normal physiological pressure and breathing mixtures. The only tissues in the body that are exposed to high partial pressures at atmospheric pressure are the lungs and arteries.

Location	Atmospheric pressure and oxygen concentration	100% oxygen	2.8 Bar oxygen
Lungs	105 mm Hg	705 mm Hg	2015
Arteries	90 mm Hg	690	2000
Tissue	40 mm Hg	640	1950

Table 4.1 Comparison of partial pressure of oxygen in different tissues at different pressures

Now we can see that oxygen has a much greater effect on NO synthesis at increasing partial pressures than might have been assumed from the conclusions drawn in the paper of Rengasamy.

Other studies on NO synthesis have shown that SOD has an effect on the synthesis of NO. Initially the conclusions from these papers were misleading until the role of oxygen in the synthesis of NO was closely examined. Zembowicz (1993) demonstrated that H_2O_2 could cause dilation of rabbit aorta in both endothelium denuded and the intact endothelium. They also showed that this effect was blocked by methylene blue, an inhibitor of guanylate cyclase, and also by the application of a nitric oxide inhibitor, L-NAME. They concluded that H_2O_2 had a stimulatory effect on NO synthesis. Hobbs (1993) showed that superoxide dismutase caused a direct enhancement of NO synthesis as measured by the production of NO gas. They attributed this to SOD removing O_2^- from the assay mixture and so allowing the detection a greater increase in NO production without concomitant increase production of citrulline. A final link between the effects of SOD and NO production was explained in a paper by Johns and Rengasamy (1994), who examined the effects of catalase on NO synthesis. They showed that catalase alone had no effect on NO synthesis. However, in the presence of H_2O_2, it had a stimulatory effect. They concluded that this was due to an increased availability of oxygen for the reaction to occur.

We are now in a position to draw some conclusions from this previous work. Nitric oxide synthesis is dependent on oxygen concentration at the physiological level. Nitric oxide synthesis is stimulated by SOD whose product is H_2O_2 and O_2. Superoxide dismutase removes O_2^- from the reaction and allows the NO which is formed to last longer in the extracellular space. Catalase stimulates NO production only in the presence of H_2O_2. All the above enzymes that stimulate NO production rely on the removal of reactive oxygen species and the production of oxygen. They have also all been shown to occur at physiological oxygen concentrations

NO has been shown to cause relaxation of smooth muscle in vascular tissue. Research into NO began after it was discovered to be the endothelial derived relaxation factor (EDRF). NO synthesised by the endothelium diffuses into the extracellular space and into the smooth muscle cell where it activates soluble guanylate cyclase in the cytosol to form cGMP. NO can be inactivated by several means. It has a short half life due to its reactivity with oxygen, (O_2). It also reacts with superoxide (O_2^-) to form peroxynitrite ($ONOO^-$). It reacts with haemoglobin to form NO_2^-, and it also reacts with thiols, and organometallo compounds. NO's main effect is to cause vasodilatation. It cannot be stored since it is diffusible and so must be synthesised as it is released. The control of NO synthesis is by intracellular calcium.

The role of NO in the central nervous system is much more complex. It has been linked with anaesthesia, long term potentiation and learning, cell signalling, excitotoxicity and cell death. It has also been linked to hyperbaric oxygen toxicity. The question is how does NO cause this toxicity?

NO in the central nervous system has been shown to cause release of a range of neurotransmitters including the excitatory neurotransmitters glutamate and aspartate (Prast 1992). Nitric oxide causes the release of acetylcholine from the dorsomedial medulla oblongata, as measured by *in vivo* microdialysis. The research done by Lawrence et al (1993) used microdialysis to demonstrate release of glutamate in the medulla of male Wistar rats. The experiment clearly showed the release of glutamate and aspartate following the application of exogenous NO or sodium nitroprusside. Another study by Guevara-Guzman et al, (1994) on striatal neurons, again using microdialysis techniques, showed increases in glutamate (Glu), aspartate (Asp), serotonin (5-HT), γ-aminobutyric acid(GABA), taurine (Tau), and acetylcholine (Ach) while levels of dopamine (DA) were decreased upon application of exogenous NO or nitric oxide donors.

These experiments show that NO can cause neurotransmitter release in neuronal tissue upon application. Previous research by Meldrum (1988) showed global release of neurotransmitter when animals were compressed

breathing a helium oxygen mixture. Research by Wardley-Smith et al demonstrated gross changes in neurotranmitter release during oxygen exposure. (Wardley-Smith 1989)

NO at low concentrations has been shown to have physiological effects. NO at increased high concentrations has been shown to be toxic. Gross (1991) demonstrated that injections of NO into the ventricles of the brain caused seizures at concentrations 1000-fold lower than that needed to affect the systemic circulation.

5.7 Results and discussion

The aim of the experiment described here was to show that NO synthesis and release could be demonstrated at increased oxygen tensions and pressure in human volunteers. To do this, the work by Rengasamy was repeated to prove that oxygen would increase the synthesis of NO at pressure. Following this, synthesis and release of NO in mast cells exposed to hyperbaric oxygen were examined. And finally, we investigated whether or not human volunteers breathing hyperbaric oxygen would have increased concentrations of NO in the blood following exposure.

With the increased use of oxygen-enriched breathing mixtures in the sport diving community for both decompression and as a bottom mix, divers will increase their chances of encountering oxygen toxicity. Previously divers would only risk this type of oxygen exposure when exceeding safe diving depth limits or when undergoing hyperbaric therapy for a diving disorder. Because of the use of oxygen-enriched breathing mixtures, it will become increasingly important to understand the causes of CNS oxygen toxicity.

Nitric oxide is toxic in high concentrations causing cellular damage. It is involved in the immune system and may mediate some immune responses. Research has identified that NO synthase, the enzyme that produces NO in the body, is present in the brain and its release has been demonstrated. One study showed that concentrations of NO in the ventricles of the brain 1000x lower than that found in the systemic circulation would cause seizures. Another study

had already shown that NO synthase was dependent on the concentration of oxygen. [NO synthase incorporates molecular oxygen in the synthesis of NO from arginine.] The research showed *in vitro* that when oxygen concentrations were increased from 30 mm Hg to 130 mm Hg the level of NO synthesis trebled and continued to increase as oxygen concentration increased further. When NO synthesis is inhibited by mono-methyl arginine (a nitric oxide synthase inhibitor) then hyperbaric CNS oxygen toxicity can be alleviated in mice.

These previous studies have all implicated NO in acute oxygen toxicity. What is left to show is that humans breathing hyperbaric oxygen will have an increase in the levels of NO synthesis.

Nitric oxide will react with superoxide anions to produce peroxynitrite. It will also react in the presence of haemoglobin to produce the nitrate anion NO^-_3. It has been demonstrated previously that raised NO concentrations in the body will result in an increase in circulating nitrate. Any change in plasma nitrate was measured by capillary electrophoresis, which also yielded the concentration of the nitrite anion NO^-_2.

5.8 Effects of hyperbaric oxygen on NO synthesis in vitro

The following diagram Fig 4.2 shows the increase in production of NO as measured by the increased production of radioactive citrulline. As described earlier in Methods and Materials, radioactive arginine is added to a cytosol preparation containing NADPH, calcium, and calmodulin. The reactants and products are separated using an anion exchange resin and the formation of radioactive citrulline used as an indicator for NO production. One molecule of NO is produced for every molecule of citrulline formed. Assay vials were placed in a pressure vessel and pressurised to 1 bar or 5 bar with oxygen or nitrogen as a control.

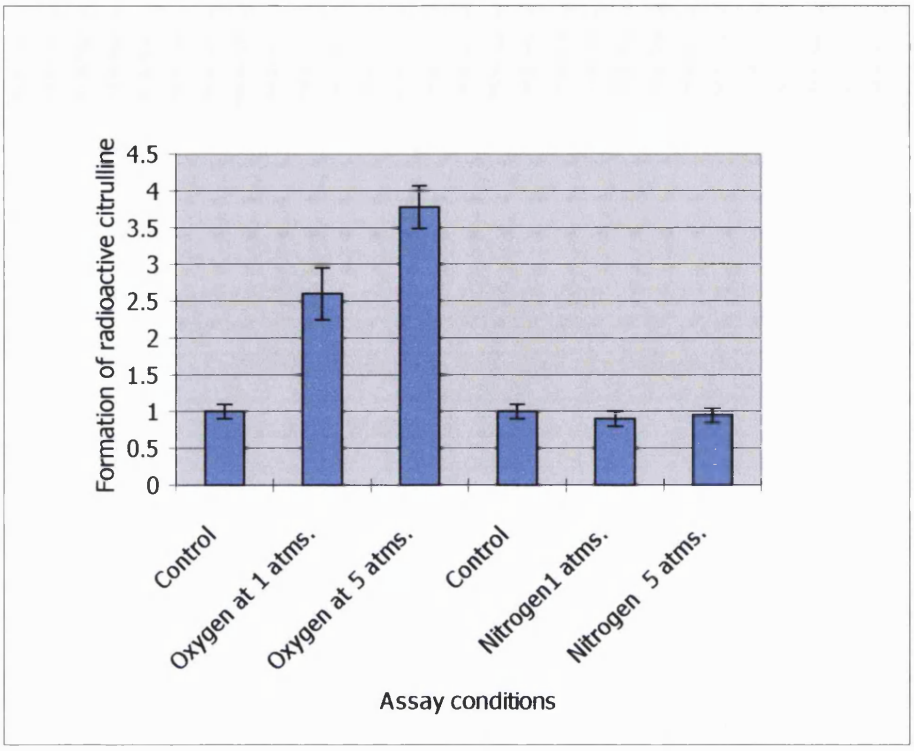


Figure 5.2 Effects of oxygen on NO synthase activity.
The chart shows the increase in production of radioactive citrulline as compared to the control values. Each experiment was performed 5 times. The assay used to measure citrulline formation is described in the method. The chart shows an increase in NO formation to 2.5 x control at 1 bar oxygen, and to 3.5 x at 5 bar oxygen. The level of citrulline formation in a nitrogen atmosphere was reduced slightly possibly due to decreased availability of oxygen. Some enzyme activity is still seen due to dissolved oxygen present in the assay solution.

The data confirms the finding of Rengasamy which also showed an increase in formation of NO in the presence of hyperbaric oxygen, consistent with molecular oxygen being essential for the formation of NO from arginine and that the NO formation is dependent upon O_2 concentration.

5.9 Effects of hyperbaric oxygen on NO release from cultured mast cells

Mast cell cultures were used to measure the stimulation of release from inducible NO synthase. The individual cell culture dishes were then placed in a pressure vessel and pressurized to 5 bar in air and 5 bar in oxygen. The results show that there was an increase in release of NO from the mast cells of 35% in oxygen as compared with no increase when pressurised in air.

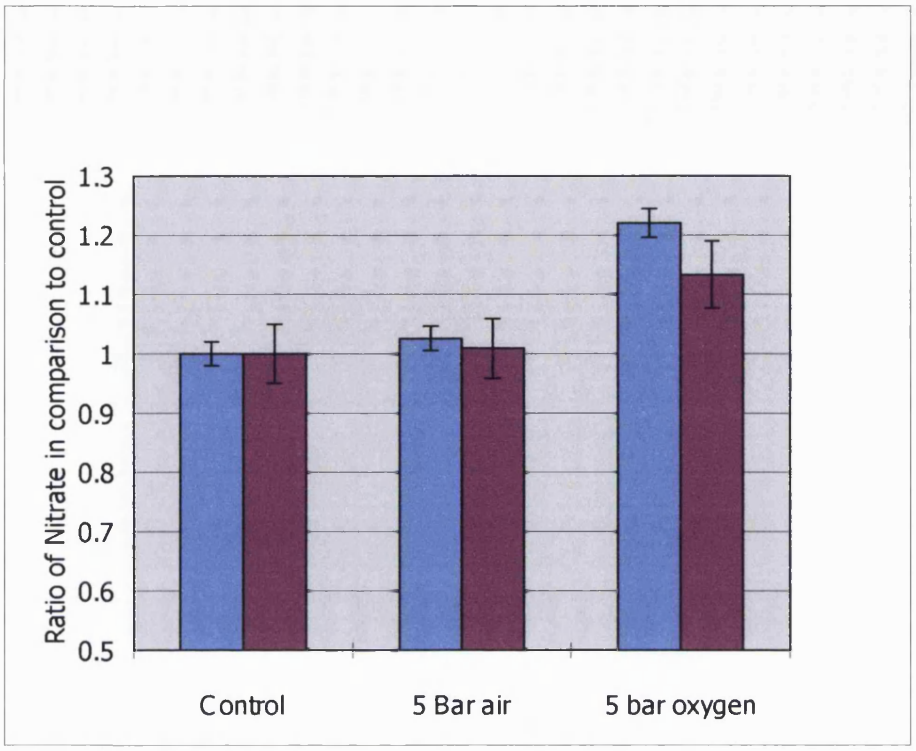


Figure 5.3 The effects of 5 bar air and oxygen on nitrate formation in cultured mast cells.
The chart shows the increase in release of NO as measured by production of nitrate and nitrite. Each experiment was performed 5 times. The nitrate was measured by use of the Giess reagent and measured by UV absorption. No increase in NO release was measured when pressurisation occurred in air while a 35% increase was detected in oxygen.

Nitric oxide has also been linked to some rheumatic disorders with increased production and formation of peroxynitrate detected in synovial fluid (Farrel 1992). The increase in NO production was induced possibly by cytokines that mediate inflammatory disease.

5.10 Effects of hyperbaric oxygen exposure on NO formation in man

The previous two studies showed a possible link between oxygen and NO formation that could explain the pathogenesis of hyperbaric oxygen seizures. In order to measure any increase in NO formation a method had to be developed to monitor the formation of nitrate and nitrite in plasma. The use of capillary electrophoresis simplified the analysis of blood samples as it allowed resolution of the two peaks of nitrate and nitrite which would not be possible using HPLC.

Haemaglobin facilates the conversion of NO to nitrate by acting as a single electron donor. Experiments performed on arterial versus venous blood in the formation of nitrate showed that arterial blood, having a 92% saturation with oxygen, increased nitrate formation to 200 mm in 2 minutes from 120 mm nitrate in venous blood with 50% haemoglobin saturation.

To measure the formation of nitrate and nitrite, capillary zone electrophoresis was used. Due to the negative charge on the anions the silica walls of the capillary had to be coated with an electro-osmotic flow regulator. This reversed the negative charge on the silica wall making it positive. Instead of running the capillary with the anode at the terminal end, the poles were reversed.

This method was optimised before the analysis of the plasma samples taken during exposure to hyperbaric conditions. Leone et al (1994) published a method using a 75 μm I.D. capillary 72 cm in length. The capillary was run with 25mM sodium sulfate containing 5% NICE-PAK OFM Anion BT, a proprietary osmotic flow regulator produced by Waters. The capillary was run at 21,600 volts and detected at 214 nm.

Following the development of this method, further work analysing blood products gave blood values for nitrite and nitrate of 0.15 mg/L and 3.2 mg/L respectively (Ueda 1995). The retention time for nitrite and nitrate were 14.1 and 14.9 minutes respectively giving good peak resolution.

The method was tested using a diode array detector scanning from 195-297 nm using known concentrations of nitrate, nitrite, and bromide. A three dimensional plot of the spectra is shown in figure 4-4. Analysis of the peak shape determined the ideal wavelength for detection was indeed 214 nm that

would allow for the greatest sensitivity in detection.

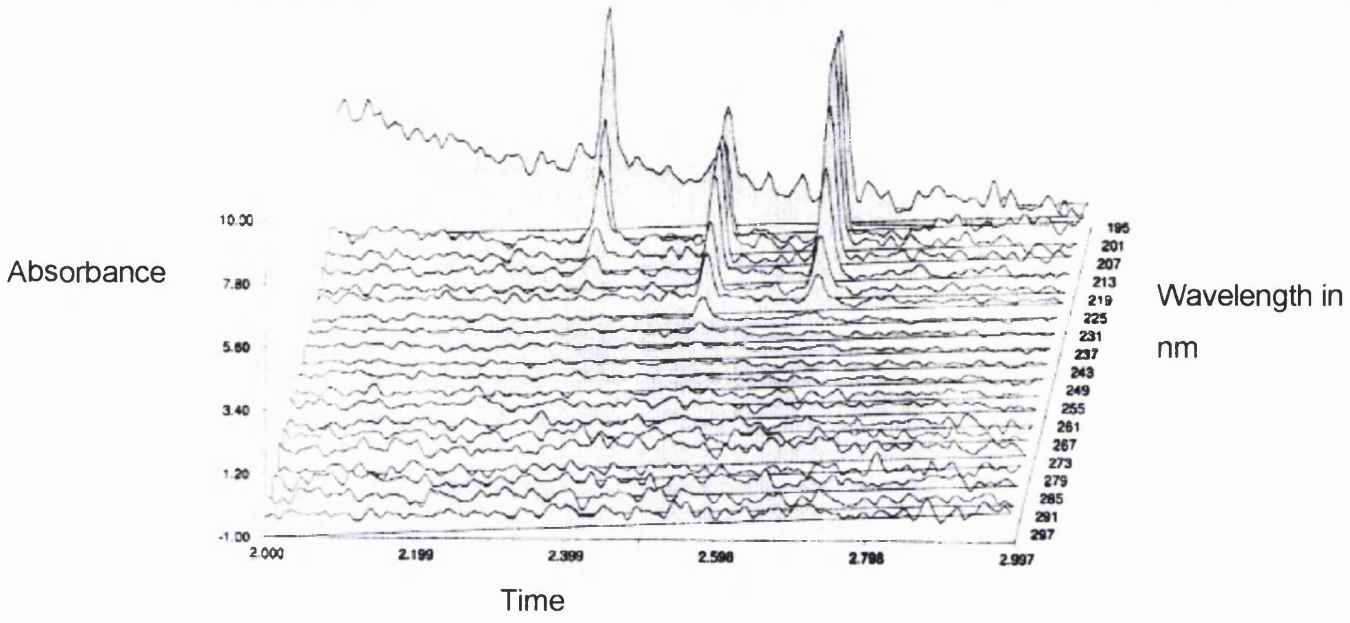


Figure 5.4 Three dimensional spectral analysis of nitrate and nitrite separation using capillary zone electrophoresis
Standard concentrations of nitrate and nitrite were run through a 50 cm 50 mm I.D. silica column at 10kV. The running buffer consisted of 25 mM sodium tetraborate, 150 mM NaCl and 1 mM TTAB at pH 9.6. The separation was achieved in less than 3 minutes with adequate resolution of peaks.

The choice of running buffer for the separation was determined by experimentation. The lack of availability of NICE-PAK led to the use of TTAB as an osmotic flow modifier. Initially the separation was carried out using 750 mM NaCl and 2 mM TTAB. The time needed for the separation was 15 minutes. A reduction in NaCl concentration caused a reduction in the time for separation. The choice of addition of sodium tetraborate and 150 mM NaCl was driven by a need for buffering of the samples, using a biological value for the NaCl concentration and a reduction in the time required for each sample injection. The samples were loaded electrophoretically at a ramped voltage for 2 seconds at 5 kV. Temperature was maintained at 22°C via internal cooling of the capillary.

Figure 4-5 shows the separation of a mixture of standards run on the capillary electrophoresis. The separation was carried out in under 4 minutes. The first

peak to 3.01 mms elute was the nitrite at 3.01 minutes followed by nitrate at 3.55 minutes. The concentration of standards was 0.8 mg/l nitrite and 1.0mg/l nitrate (4.34×10^{-6} and 6.45×10^{-6} molar).

Run time for dive in minutes	Time	Sample
-30	Pre dive breathing air	Blood sample 1
0	Breathing Oxygen at 1 Bar	Blood sample 2
10	10 minutes at 2.8 bar on air	Blood sample 3
30	20 minutes at 2.8 bar on oxygen	Blood sample 4
35	5 minutes air break at 2.8 bar	
55	20 minutes at 2.8 bar on oxygen	
60	5 minutes air break at 2.8 bar	
80	20 minutes at 2.8 bar on oxygen	Blood sample5
85	5 minutes decompression on air	
115	30 minutes post dive	Blood sample 6

Table 4.2 Schedule for a) time on oxygen and b) time for taking blood samples

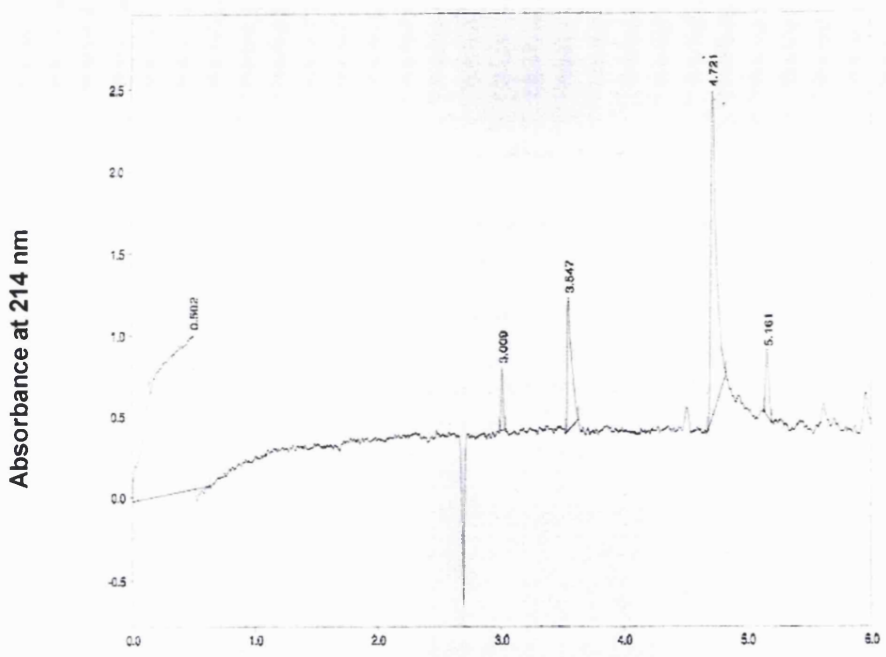


Figure 5.5 2D spectra showing the separation of nitrate and nitrite standards by capillary electrophoresis.
Standard concentrations of nitrate and nitrite were run through a 50 cm 50 mm I.D. silica column at 10kV. The running buffer consisted of 25 mM sodium tetraborate, 150 mM NaCl and 1 mM TTAB at pH 9.6. The separation was achieved in less than 3 minutes with adequate resolution of peaks.

5.11 Nitrate analysis in plasma

Volunteers were compressed in a hyperbaric recompression chamber as described in the method. Blood samples were taken at set intervals and removed from the chamber via the medical lockout hatch. The samples were immediately centrifuged to remove the red blood cell fraction leaving the plasma, which was immediately frozen at -70°C.

While at pressure the volunteers breathed pure oxygen at 2.8 bar via a built in breathing system (BIDS). The schedule for time on oxygen and time of blood samples taken is shown in table 4.2.

Absorbance at 218 nm

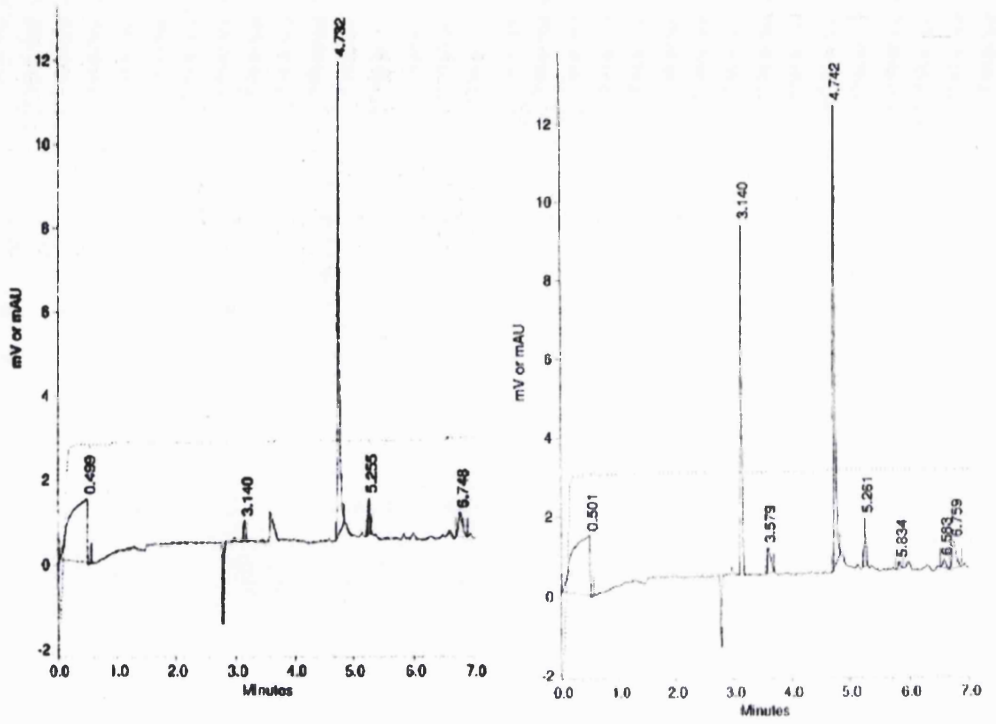


Figure 4.6 Spectra showing the increase in detection of nitrate in sample taken during compression at 2.8 bar oxygen.

Figure 4-6 shows the control values before compression of nitrate. Integration of the peak corresponded to a concentration of 1.4mg/L of nitrate. In figure 4-7 we see the increase height and area of the peak corresponding to 12.3mg/L nitrate. The sample for figure 4-7 was taken after breathing oxygen at 2.8 bar for 40 minutes

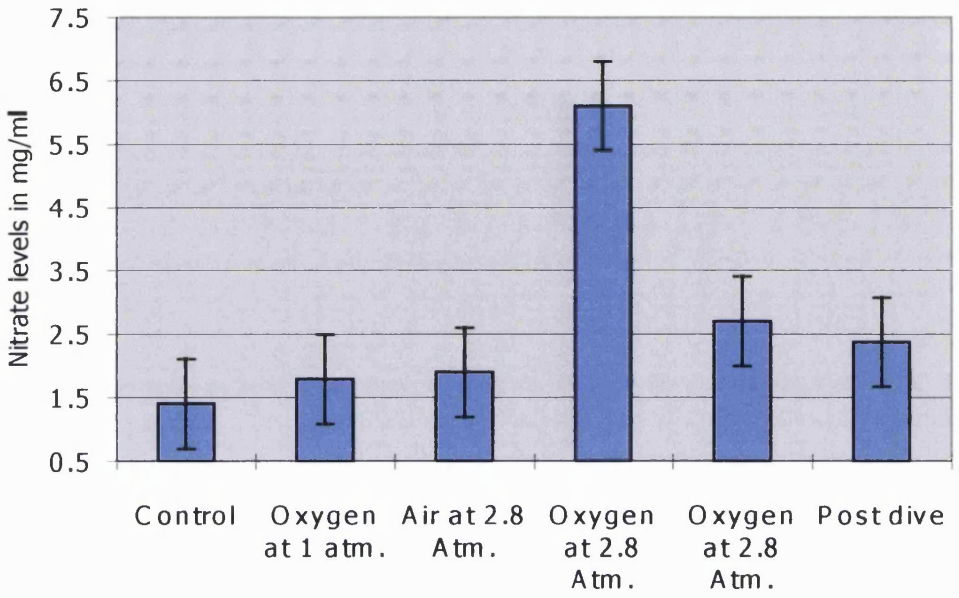


Figure 4.7 Effects of 2.8 bar oxygen on plasma nitrate levels
This chart shows the results of nitrate analysis done on blood plasma. The samples were analysed using capillary zone electrophoresis and an electro-osmotic flow regulator. The last two blood samples were taken once the volunteers were returned to normal atmospheric pressure. Levels of nitrate were raised when breathing oxygen at 1 bar. A significant increase in the level of nitrate was measured when breathing oxygen at 2.8 bar. The data is the average of 5 subjects. All the subjects showed an increase in nitrate during the time on oxygen but two subjects had an increase in plasma nitrate of 7x and 14 x levels of control. Overall the average increase in nitrate 2.8 bar was 4.3x increase. Nitrate is excreted by the kidneys and lower levels were observed post dive.

Before analysis the samples were centrifuged using Microcon 10 ultrafiltrators. A 0.5 ml sample of plasma was added to the Microcon and spun at 5,000g for 10 minutes. The resulting deproteinated sample was then ready for analysis.

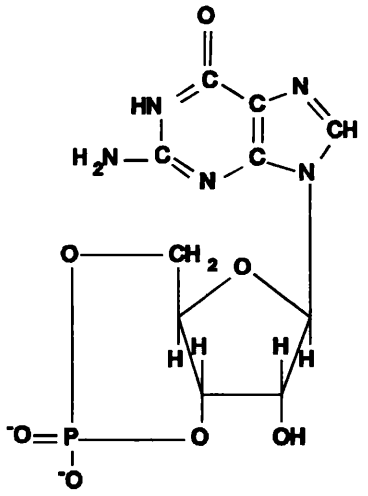
The results of the nitrate analysis can be seen in figure 4-5. Little variation in nitrate levels can be seen in the samples taken before compression. Pre-compression values for nitrate were 1.4 mg/L. This value can be highly dependent on the subject and where they live, especially since nitrate can be found in local water supplies and is generally higher in agricultural areas due to run of from farmland entering the water table. All subjects were from the Greater London area so giving a low variance (3.6%)

Chapter 6 A comparison of the effects of N_2O and NO on guanylate cyclase

6.1 Cyclic GMP

Since its discovery in 1963 by Ashman et al, cyclic guanosine mono-phosphate (cGMP) has been studied for its role as a second messenger and has been found in a variety of animal tissues. It is synthesised by the enzyme guanylate cyclase and has been shown to be sensitive to calcium ion concentrations. By challenging receptor systems with ligands it has been shown that changes in cellular cGMP play a role in the actions of a variety of drugs and neurotransmitters such as acetylcholine, serotonin and insulin namebut a few. Most exciting of all was the demonstration that increases in the level of cGMP can been found with sodium nitroprusside, nitroglycerine and sodium nitrite. These potent vasodilators are used in cardiac care to control angina.

Figure 6.1 Chemical structure of cyclic guanosyl monophosphate



(cGMP)

The role of cGMP has been widely studied in the transduction system of the retina where it acts as a second messenger. Stimulation of the photosensitive cells of the retina causes a decrease in cGMP levels. This decrease in cGMP levels causes a closure of sodium channels thus hyperpolarizing the cell. Levels of cGMP in the cell are controlled by two enzymes: guanylate cyclase catalyses the conversion of guanosine triphosphate (GTP) to cGMP; while cGMP phosphodiesterase hydrolyses the cGMP to 5^1-GMP. In the retina, levels of cGMP are regulated by phosphodiesterase in light. In the dark, the enzyme is inhibited allowing an increase in cGMP levels. The increase in cGMP causes opening of sodium channels, so depolarising the cell and the

release of neurotransmitter. Through this well studied system one can see how the photosensitive cells of the retina have evolved to control increases and decreases in levels of cGMP by controlling the activity of guanyl cyclase and phosphodiesterase.

6.2 Nitric oxide stimulation of guanylate cyclase

The interest in cGMP increased when, during the search for the endothelial derived relaxation factor (EDRF), it was found that EDRF induced changes in cGMP were linked to relaxation of smooth muscle and that stimulation of bronchial smooth muscle cells by sodium nitroprusside caused increase in cGMP and cGMP-dependent protein kinases. Atrial naturetic peptide, ANF, also caused relaxation of smooth muscle that is linked to a 50-fold increase in levels of cGMP. The interest and excitement in EDRF and cGMP culminated in the discovery that EDRF was, in fact, nitric oxide (NO), a highly reactive soluble gas with a short half-life able to diffuse out of the cell in which it is synthesised to act on cells in the surounding area. Since NO itself cannot be stored, it must be synthesised de novo each time. The mechanism by which NO synthesis is controlled was also later shown to be calcium dependent and NO was shown to increase levels of cGMP along with sodium nitroprusside, and nitroglycerine.

Garthwaite et al (1988, 1994, 1995) showed that nitric oxide (NO) is present in the brain as an important neurotransmitter. They showed that stimulation of neurons with the neurotransmitter glutamate was dependent upon a short-lived unstable soluble factor to cause an increase in intracellular cGMP. This factor was shown to be identical to the newly-discovered endothelial derived relaxation factor, NO. Garthwaite was able to show that stimulation with glutamate caused an increase in calcium levels and release of NO. The synthesis and release of NO was found to be dependent on calcium in the neurons of the brain.

The excitement about NO continues to this day. NO has been looked at in most systems of the body as a neurotransmitter, neuromodulator, second messenger, and a cytotoxic agent. NO has been found to act in two separate pathways. The one outlined above, where glutamate stimulates the synthesis

of NO by the influx of Ca^{2+} ions via the N-methyl-d-aspartate receptor (NMDA). The NO, once synthesised, can not be stored and is diffusible in and out of the cell's lipid membranes. NO diffuses out of the cell into the extracellular fluid where it may enter presynaptic neurones to affect cGMP synthesis, which can then diffuse out of the cell to act presynaptically reinforcing those pathways via long term potentiation (LTP). NO may also be released presynaptically due to the influx of Ca^{2+} ions caused when an action potential reaches the terminal endbud and opens voltage-gated calcium channels.

The synthesis of NO and cGMP are linked to levels of Ca^{2+} in the cell. The synthesis of NO requires the presence of Ca^{2+}. Moncada et al (1991, 1993, 1995) showed that nitric oxide synthase catalyses the reaction of L-arginine to citrulline with the production of NO. The enzyme is dependent on physiological concentrations of Ca^{2+}. The levels of Ca^{2+} needed for the production of NO are within the range shown by Marchbanks et al (1993) Half maximal stimulation of NO synthase was shown at 160 nM whereas, when the Ca^{2+} levels were controlled in the guanylate cyclase assay, it was shown that inhibition was demonstrated at 80 nM with complete inhibition at 1.5 □m. What these experiments showed was that the NO synthesised in one neuron was having an affect on the cGMP levels of other neurons since the Ca^{2+} levels required within the neuron to stimulate NO synthesis would inhibit guanylate cyclase and so cGMP synthesis. This system of inhibition/stimulation of NO/cGMP allows NO to work either pre- or post-synaptically, depending on the site of synthesis.

6.3 Excitotoxicity

Problems can occur in this system when the cells involved become depolarised in large numbers and remain so. The opening of Ca^{2+} channels and synthesis of NO can cause excitotoxicity. Whereas NO in low concentrations can act as a neurotransmitter, in large concentrations it can act as a cytotoxic agent.

Duarte et al (1991) showed that part of the analgesic properties of morphine and acetylcholine were mediated guanylate cyclase NO. Nakamura (1983,

1993) had proposed a role for NO in CNS anaesthesia involving cGMP and guanylate cyclase.

From the previous work we were able to conclude that NO was involved with signalling in the CNS as well as in the circulatory vascular system. NO's role in neuromodulation could act pre- or post-synaptically depending on its release. NO would also cause an increase in cGMP in cells. What we were now interested in was whether some other gas might also have an effect on cGMP levels.

Nitrous oxide has long been used as an anaesthetic and analgesic. The method by which it elicits its analgesia, its major function has yet to be fully explained at the cellular and physiological levels. To explore further the relationship between analgesia and nitric oxide we sought to find out if nitrous oxide would have an effect on cGMP levels, similar to that of NO.

6.4 Results and discussion

The method used to measure guanylate cyclase activity was by competitive binding between labelled [^{3}H]cGMP and unlabelled cGMP, the theory being that known amount of labelled product will undergo competitive binding with the unknown concentration of cGMP of the assay and the cGMP specific antibody – the higher the levels of unlabelled cGMP, the lower the amount of radioactivity that will be detectable. The level of radioactivity in the precipitated sample can be measured once the antibody is precipitated with ammonium sulfate. By producing a standard curve with known concentrations of cGMP it was possible to calculate the unknown levels present in the cell. The cGMP RIA assay allowed concentrations of cGMP to be measured between 0.5 - 8 picomoles.

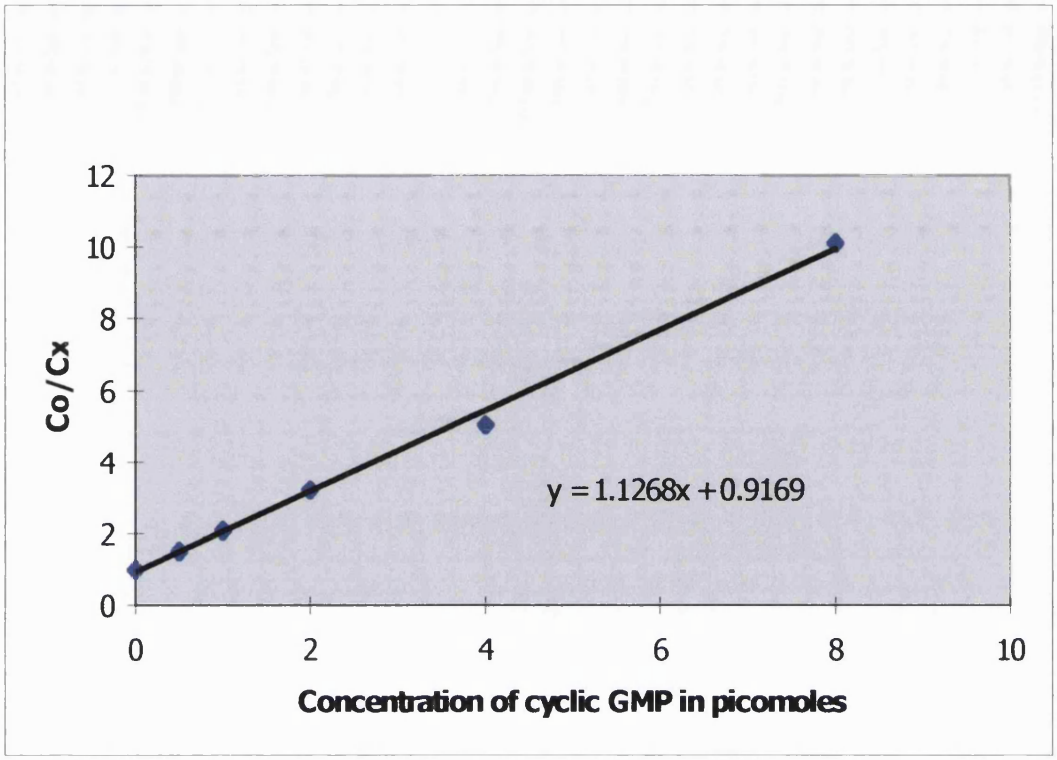


Figure 6-2 Standard curve for cGMP radio-immuno assay
The standard curve for the cGMP radio-immunoassay was derived using known concentrations of cGMP. The assay uses competitive binding of labelled cGMP to antibodies. The antibody-cGMP complex is then acid precipitated to determine the amount of cGMP present in the cell. The assay gives detection of values down to 0.5 picomoles.

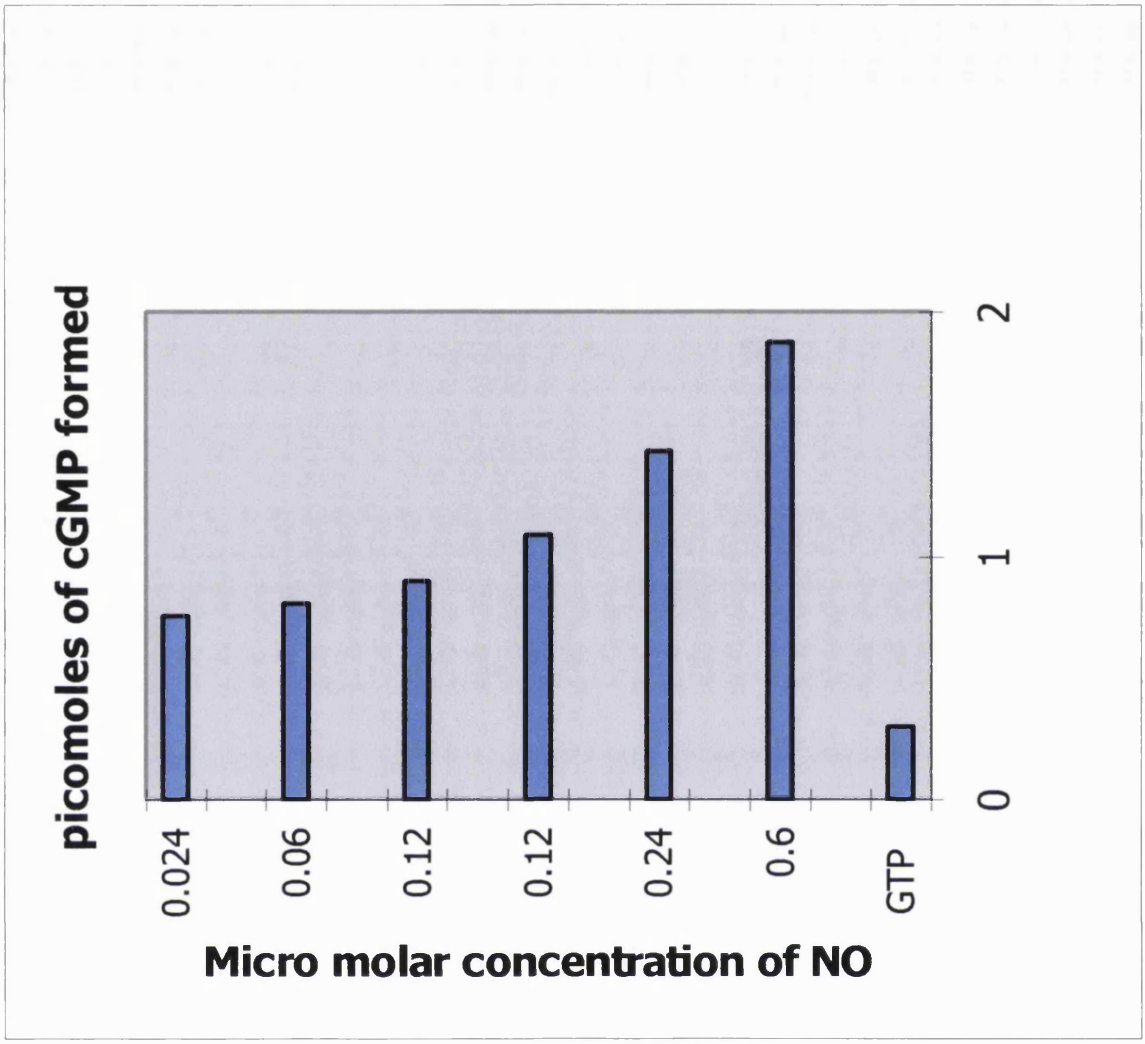


Figure 6.3 Effects of NO on cGMP formation in brain cytosol
The chart shows formation of cGMP in response to stimulation of guanylate cyclase by nitric oxide. The levels of cGMP were determined using an antibody specific radioimmunoassay. Stimulation of guanyl cyclase occurred at 0.12 mM nitric oxide.

The experiments performed by Garthwaite were repeated to confirm the sensitivity of the assay and gain the required techniques. Stimulation of guanyl cyclase by a saturated solution of nitric oxide in buffer (3.0 mM). Serial dilutions were made of this solution and injected into the assay mixture to achieve stimulation. Stimulation of the guanyl cyclase was measured down to 0.12 mM as a final concentration of NO in the assay (fig. 6-3). The formation of cGMP was determined using the radio-immunoassay technique and compared to a standard curve of concentrations.

Stimulation of guanyl cyclase was also measured using sodium nitroprusside as a NO donor (fig. 6-4). The stimulation was measured using the same assay as previously described.

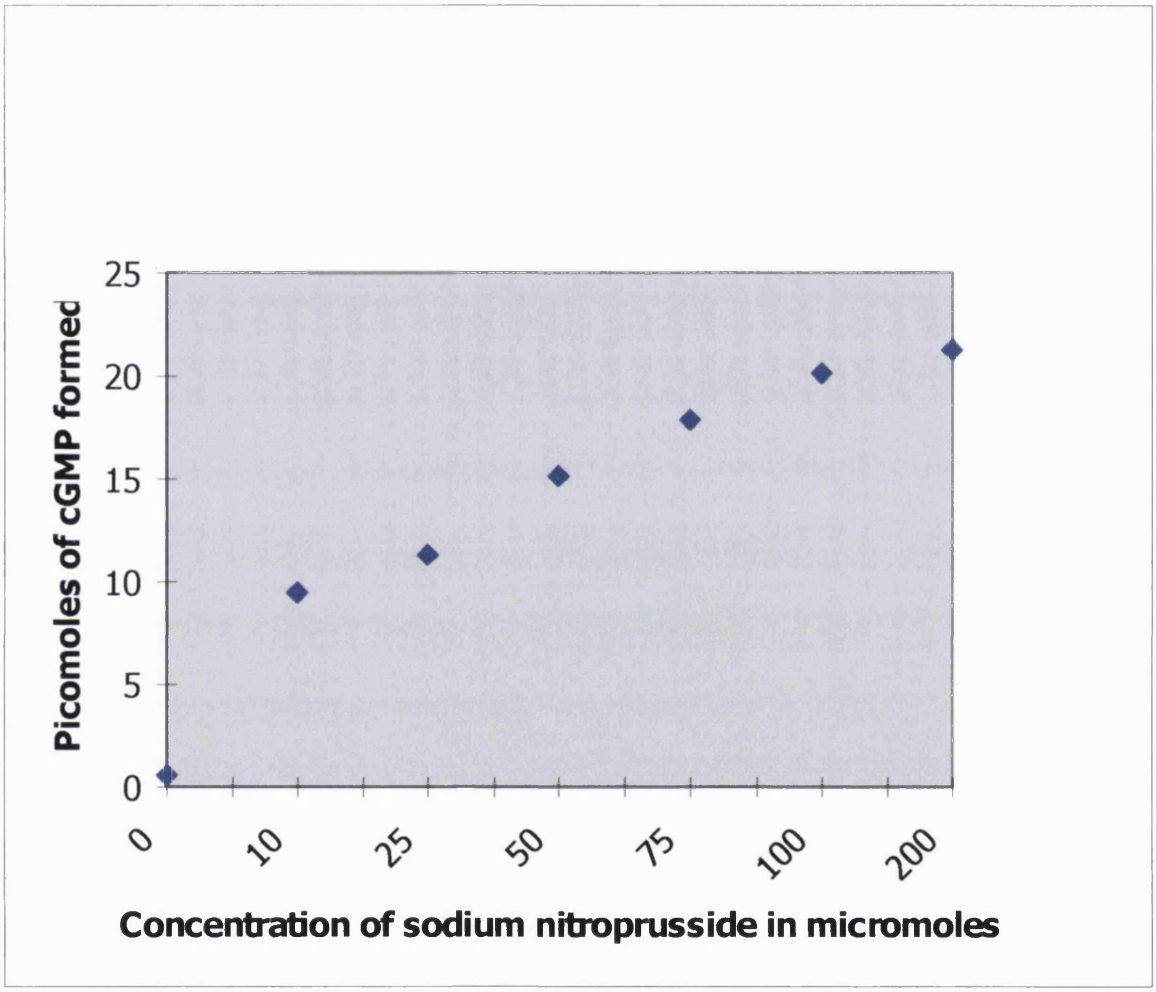


Figure 6.4 Stimulation of guanyl cyclase by sodium nitroprusside

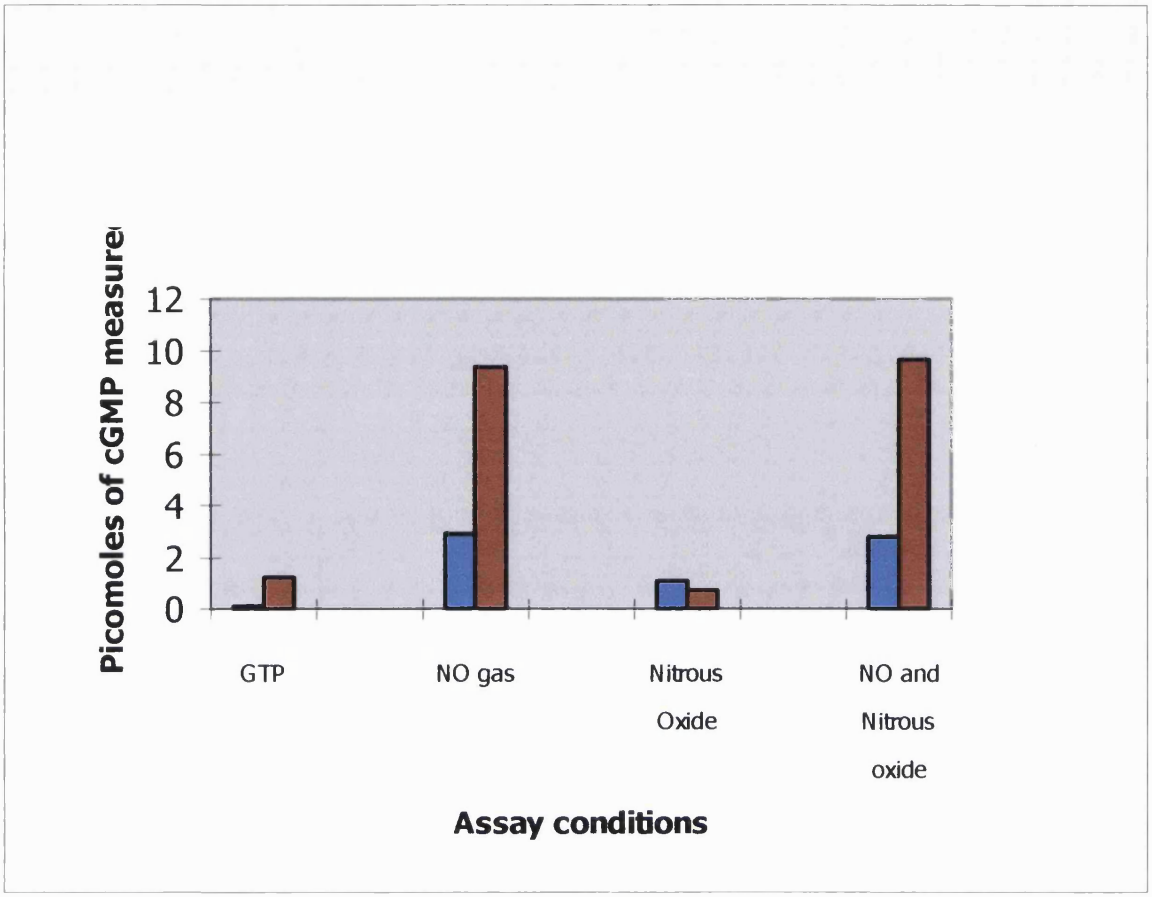


Figure 6.5 Effects of nitric oxide and nitrous oxide gas on cGMP formation in brain cytosol

These results clearly confirm that NO stimulates guanyl cyclase to form cGMP whereas N2O had no effect on cGMP formation within experimental. The final experiment of Figure 6-5 in which NO and N2O where co-administered confirmed both these conclusions.

Chapter 7 Overview

The primary objectives of this thesis were:
To isolate and purify B_{12}-dependent methionine synthase from porcine brain and characterize its active forms.
To characterise and compare the mass, pI, Km and Vmax of the brain B_{12}MS with other B_{12}MS enzymes.

This led to the further experiments to:
To compare the inhibition of brain B_{12}MS by nitrous and nitric oxides and therefore to correlate B_{12}MS with common signalling molecules and systems, especially the NO/cyclic GMP system.
Compare N_2O and NO as possible inhibitors of cyclic GMP.

Finally, since diving physiology and diving are major significant personal interests, I felt it important to investigate the role of NO in human volunteer divers.
To investigate the possible role of NO in situations related to change in oxygen tension such as SCUBA diving.

7.1 Purification

The purification of pig brain methionine synthase was difficult due to the instability of the enzyme as well as the range of molecular weights already reported in the literature for mammalian enzymes. At the beginning of the purification it was found that there were two molecular weights at which pig brain methionine synthase was active. These were determined from gel chromatography using two different resin. The Superose 12 gave molecular weights of 160 and 101, while the S-200 gave molecular weights of 162 and 97 kD.

This corresponds with the previous reported molecular weight of the mammalian enzyme. Utley (1985) purified methionine synthase using human placenta as a source for the enzyme and got a 160 kD enzyme, which upon analysis had subunits composed of 90, 45 and 35 kD. Chen (1994) purified a

single protein of 155 kD from pig liver. Work carried out in our lab by Dr T. Ast and Dr S Kenyon on the rat liver enzyme showed an active enzyme with a molecular weight of 160 kD and subunits of 95 and 60 kD. These results were determined by a combination of calibrated gel filtration chromatography and SDS-PAGE analysis.

The results determined by SDS-PAGE for the pig brain methionine synthase enzyme back up the results from the gel filtration and show a progressive breakdown of the enzyme into two sub-units, one at 36 kD and the other approximately 94 kD. Loss of enzyme at the HTP chromatographic step may be partly due to breakdown of the enzyme and separation of its subunits. Loss of a 45 kD subunit would explain differences in the reported molecular weights and those determined for the pig brain enzyme.

The pI determination was not carried out for any of the mammalian enzymes and only values for the bacterial enzymes have been reported. Dr Ast did report a pI of 5.1 for the rat liver enzyme (unpublished data T. Ast 1994) in agreement with these data. The bacterial enzymes had several reported pIs between 5.2 and 4.8 (Frasca et al 1988)

The degree of purification and yields are summarised in Table 3-3. The large quantity of starting material allowed for analysis of the enzyme and determination of molecular weight. Pilot studies made it clear that the ability to test for the enzyme activity was in no means linked to its detectability on SDS-PAGE even with silver staining.

Now that the brain B_{12}-MS or at least an enzyme fragment has been purified and the molecular weight and pI determined, the next logical step would be for tryptic digestion and sequencing. Following this a partial sequence can be determined and compared with existing published sequences to confirm that some degree of homology exists between species. Since completion of the purification of the pig brain methionine synthase enzyme a full sequence has been determined for the pig liver enzyme (Chen 1997) and a DNA sequence determined. Using this sequence and the DNA sequence located on

chromosome 1 the purification sequence and steps should be much simpler. Production of a monoclonal affinity column and the use of elisa techniques for identification will speed purification and remove the need for such large volumes of starting material.

7.2 Inhibition

Unlike other vitamins, B_{12} is used as a cofactor for only two mammalian enzymes. Despite this apparent simplicity, B_{12} is an absolute requirement for growth, differentiation, and proper functioning in the CNS. The corollary, that neurotropic and neurodegenerative diseases may involve B_{12} dependent enzymes, is rapidly becoming accepted. The absence of a gene sequence and protein structure for mammalian B_{12}dependent methionine synthase has so far meant that direct cause and effect relationships between B_{12}-dependent cell enzymes and pathways and these pathological conditions has not been possible. (This became available after this research was complete and the thesis written). Equally, although the structure of B_{12}-dependent methyl/malonyl/CoA mutase (the second B_{12} enzyme) is known, its cellular role in the brain also remains to be elucidated. This absence of cause and effect relationships, however, has not inhibited the search for correlations between vitamin B_{12} levels, homocysteine or methionine levels, and diseases. The latter includes Multiple Sclerosis, Neural Tube Disease, Down's Syndrome, Huntingdon's and Parkinson's diseases, AIDS, Dementia and others. B_{12} has, however, been directly associated with anaemias, haematoietic cell growth and differentiation and their associated neuropathies.

It is known that methionine synthase (a) converts homocysteine to methionine and is the only source of the latter amino acid in brain other than diet, (b) that it plays an initiating role in folate and polyamine biosynthesis plus DNA and RNA methylation. As such, it is an obvious candidate for a key role in cell division. Since B_{12}-methionine synthase also controls phospholipid methylation and hence choline lipid production (via S-adenosyl methionine), this B_{12} enzyme would play a role in cell signalling through coupling to phospholipases A_1, A_2, C and D? The relevance and importance of these phospholipases can be seen through their initiation of the arachidonic acid cascade, to form eicosanoids,

and through the fact that SAM (or AdoMet) is the only source, in the brain, of endogenous choline. The fact that polyamines have also been shown to be involved as intracellular ligands for K^+ and Ca^{2+} channels, for the NMDA receptor and for a variety of signalling kinases, confirms the putative role of B_{12}-methionine synthase (through SAM) in cell signalling. A final signalling role for B_{12}-methionine synthase is implied from the fact that it's substrate (homocysteine) is also the starting point for biosynthesis of several sulfur amino acids which have been proposed as neurotransmitters or neuro-modulators, namely, taurine, homocysteic and cysteic acids. Glutathionine is also formed from homocysteine via cystathionine.

Two other products of B_{12}-dependent methionine synthase pathways have now been shown to affect neurotransmission. Both S-adenosyl homocysteine (SAH) and methylthioadenosine (MTA) bind to the GABA receptor benzodiazepine site with IC_{50}=2 micromolar, a value lower than that of any so-far-proposed endogenous benzodiazepine. These molecules also potentiate the opening of the GABA-chloride channel in iontophoresis experiments and are proven and potent agonists of adenosine Al receptors (Tsvetnitsky, 1994).

By further study of methionine synthase and its control and regulation a better understanding of its role in disease will unfold. Current research has now provided us with the protein sequence and its cDNA sequence and location.

7.3 NO production and Nitrate

From the literature and the research carried out in this project nitric oxide can be considered a factor in the pathogenesis of acute and chronic oxygen toxicity. From the initial work carried out by Rengasamy on the involvement of oxygen in NO formation later work on the NO inhibitor NMMA, NO does have an affect.

When Oury et al (1992) over-expressed SOD in an attempt to alleviate the acute oxygen toxicity they were clearly surprised that it did not have the desired effect. The work carried out by Oury did however show that SOD enhanced the production of NO, the involvement of catalase in combination with SOD also

heightened the effect of stimulation of NO synthesis. Inhibition of NO synthesis in rats did however alleviate the onset of oxygen seizures and might in future be used in their prevention.

Hydrogen peroxide seems to be important in the genesis of the seizure (Jenkinson 1989, Peligrino 1993, Zhang 1996, Yusa 1984). It is not surprising that mice over-expressing SOD were more sensitive to HBO (Oury 1992), H_2O_2 is a vasodilator (Wei 1985,1990), and increased brain blood flow may increase O_2 delivery and enhance toxicity (Bean 1972). H_2O_2 can increase the activity of isolated NOS, this appears to be due to increased O_2 available to the enzyme (Rengasamy 1994). H_2O_2 appears to be derived from metabolism of catecholamines by MAO (Zhang 1996). Other investigators have demonstrated that catecholamine depletion with reserpine will also block HBO seizures. Treatment with NOS inhibitors reduces HBO seizures (Oury 1992, Zhang, 1993) Unfortunately, this does not directly implicate NO as the cause of the seizure. When NOS inhibitors are administered, many systems are affected which make assignment of a cause difficult. For example, there are some alternative explanations for the effect of NOS inhibitors. When NOS inhibitors are given, cerebral blood flow (especially in rats) is reduced. If blood flow is reduced, then oxygen delivery is reduced, tissue oxygen levels are reduced and perhaps this is the explanation for the protective effect of NOS inhibitors? This, however, seemed unlikely as a later study reported that tissue oxygen remains unchanged (Zhang 1995). A second explanation is that H_2O_2 can be produced by NOS, and perhaps the inhibitor of NOS was reducing H_2O_2 production and not NO production (Mayer 1993, Katusic 1994, Mayer 1994) A third explanation could be due to changes in CO_2 responsivity following NOS inhibition (Niwa 1993, Pellegrino 1993). CO_2 is though to enhance O_2 toxicity by increasing tissue oxygen levels (Bean 1972). Thus, by reducing the response of the vasculature to CO_2, you might limit tissue O_2 increase. A fourth explanation is that by inhibiting NO-dependent neurotransmission, some how the seizure cycle was interrupted.

Whether NO can directly cause seizures is not clear. In some studies, it appears that NO can suppress seizures (Buisson 1993, Rigaud-Monnet 1994).

NO also appears to be neurotoxic in high concentrations. However, neurotoxicity does not appear to play a role in oxygen seizures, as brains appear normal after hyperoxic seizures (Jerret 1973).

This research has demonstrated an oxygen-induced increase in production of NO in vitro, an increase in release of NO in vitro, and an increase in nitrate a marker for NO production in vivo. Although many questions remain to be answered as to the direct mechanism of NO in the seizures some tentative hypotheses can be put forward.

NO has been shown to cause seizures when injected into the ventricles at levels far lower than those found in the systemic circulation, and also causes the release of excitatory neurotransmitters in the brain. If production were increased or its inactivation and metabolism interrupted this could lead to seizures.

7.4 cGMP/NO/N_2O

It seems clear from these experiments, and those of chapter 4, that any search for a mechanism of N_2O does not involve its effect on the cyclic GMP cell signalling system and that the more common explanation must hold – N_2O inhibition of B_{12} methionine synthase and the consequent effects of this on the B_{12}-dependent intracellular network. It is also of interest that NO affects cGMP formation at a much lower concentration than it inhibits B_{12}-MS. This, however, does not eliminate the latter as one (additional) mode of action of NO that parallels that of N_2O. Indeed, NO is produced at high cellular concentrations and it diffuses rapidly from its cellular source to affect neighboring cells. By axiom, both in its cellular source, and in neighboring cells, NO is at sufficiently high concentrations to directly, or indirectly, affect metallo enzyzmes. B_{12}-MS is one such enzyme.

7.5 Summary

A great deal of future work is needed including: finally sequencing the gene for the brain enzyme; elucidating the nature of the various cellular forms of B_{12}-MS; establishing a role for B_{12}-dependent enzymes and metabolites in intracellular

signalling; and, studying the control and regulation of B_{12}-dependent enzymes and pathways and their role in normal and abnormal growth and pathology.

It is hoped that the experiments in this thesis have constituted a beginning towards more detailed studies of B_{12}, B_{12}-MS, homocysteine, and SAM in health, disease, and in therapy.

At the same time as this I hope this work can begin to elucidate the complex and multifactoral effect of placing man under pressure and allow him to adapt to this harsh and unforgiving environment.

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