The effect of normobaric normoxic and hypoxic exercise upon plasma total homocysteine and blood lipid concentrations

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This thesis is dedicated to my parents Joan and Douglas,
without whose helped this thesis would never have
been completed.

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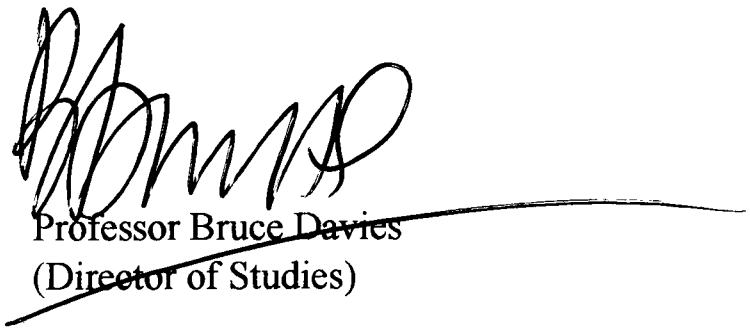
Abstract

The aim of the experiment was to find the effect of intermittent normobaric hypoxic exercise upon a group of young adults. Thirty male subjects aged eighteen to twenty-eight years old were weighed and sum of four skinfolds measured before and after four weeks of chronic exercise. A blood sample was taken at rest pre and post chronic exercise to ascertain their lipid profile, plasma total homocysteine, folate and vitamin B_{12} levels. Each subject completed a seven day food and fluid intake pamphlet pre and post chronic exercise. Aerobic power was tested normoxically and hypoxically using an incremental cycle test to volitional exhaustion before chronic exercise and normoxically after chronic exercise. A blood sample was taken immediately post acute exercise to measure plasma total homocysteine concentration which was adjusted to take account of plasma volume shifts. Fourteen subjects exercised normoxically and sixteen subjects exercised hypoxically three times a week. The intensity and duration of the exercise sessions increased throughout the period from 70 to 85% and 20 to 30 minutes. The Normoxic group's normoxic acute cycle test significantly increased plasma total homocysteine concentration (+0.6±1.3 $\mu mol.dm^{-3}$, p=0.035), whereas the hypoxic group's change was not significant (+0.2±1.2 $\mu mol.dm^{-3}$, p=0.408). Normoxic chronic exercise significantly increased plasma homocysteine concentration (+1.2±1.0 $\mu mol.dm^{-3}$, p=0.002) whereas hypoxia showed no significant change (-0.4±1.7 $\mu mol.dm^{-3}$, p=0.361). The changes were not physiologically significant. Normoxic and hypoxic chronic exercise had no effect upon the subjects total cholesterol concentration (-0.51±0.51 $mmol.dm^{-3}$; -0.32±0.52 $mmol.dm^{-3}$, respectively, 2-way ANOVA p=0.339) nor upon their aerobic power (+3.2±4.8 $cm^3.kg^{-1}.min^{-1}$; +5.1+10.9 $cm^3.kg^{-1}.min^{-1}$ respectively, 2-way ANOVA p=0.568). An advantage of training under hypoxic conditions is that it minimises the fluctuations in plasma total homocysteine concentration that would be obtained when training normoxically.

Declaration

This is to certify that the work described in this thesis is the result of my own work. This research programme was carried out at in collaboration with the Clinical Biochemistry department University of Wales Medical School.

Neither this thesis, nor any part of it, has been presented, or is currently submitted, in candidature for any degree at any other University.



Professor Bruce Davies
(Director of Studies)

Andrew I. Heusch
(Candidate)

Part I

'Life can only be understood backwards;

but it must be lived forwards.'

Kierkegaard, Sören

1813 - 1855

{Concise Dictionary of Quotations

Pocket Reference Library

Wm Collins Sons & Co Ltd. 1985. p.185}

Chapter 1

Risk factors associated with Cardiovascular disease with particular reference to Homocysteine.

1. Overview

This thesis considers cardiovascular risk factors and the effect of acute and chronic exercise upon them. The focus is plasma homocysteine concentration and lipid factors (total cholesterol, high density lipoprotein cholesterol, low density lipoprotein cholesterol, triacylglycerol and non esterified fatty acid concentrations).

Serum total cholesterol refers to the sum of cholesterol in the serum. This is the sum of cholesterol sources including: very low density lipoprotein cholesterol, low density lipoprotein cholesterol, high density lipoprotein cholesterol and intermediate density lipoprotein cholesterol. A high serum concentration of total cholesterol (>4.7 $mmol.dm^{-3}$), has been associated with an increased risk of cardiovascular disease.

Low density lipoprotein cholesterol can become oxidised and get taken up by macrophages. These cells undergo a physical change as a consequence, becoming "foam cells" a reflection of their appearance histologically. White streaks in the intima of the conducting arteries are accumulations of foam cells. Not all cholesterol sources are necessarily bad with respect to cardiovascular system. High density lipoprotein particles contain cholesterol that has come from the tissues and is en route to the liver to be break down. Endurance exercise has been shown to produce a reduction in low density lipoprotein cholesterol and an increase in high density lipoprotein cholesterol and non esterified fatty acids.

Plasma total homocysteine is the sum of all the derivatives of homocysteine. Homocysteine is the single unit whereas homocystine is a molecule of two homocysteines linked by a disulphide bond. Homocysteine can also form a disulphide

bond with cysteine (cysteine-homocysteine) and cysteine amino acids in proteins (homocysteinyl moieties).

Plasma total homocysteine concentration has been associated with an increased risk of arteriosclerosis. However, normoxic exercise causes an acute increase in its concentration. Red blood cell folate and vitamin B_{12} concentrations have been shown to be inversely correlated to plasma total homocysteine concentration. Plasma total homocysteine has been shown to cause hyperplasia of smooth myocytes of the intima, it is also associated with increased oxygen radicals and therefore lipid peroxidation.

Although hypoxic exercise might have a smaller acute effect upon plasma total homocysteine, there have been no studies into this effect. There are two possible reasons to why plasma total homocysteine concentration may be lower under hypoxic than normoxic exercise conditions. The adenosine required for coronary arteries dilation is provided from adenosine monophosphate, whereas in normoxic conditions it is supplied from S-adenosine homocysteine. The second reason is that athletes train at a lower intensity under hypoxic conditions, so therefore probably egress less homocysteine from the muscles into the plasma.

1.1 Cardiovascular disease

Cardiovascular disease is a general term that groups together diseases of the heart and blood vessels of the body, such as weaknesses of the vessel wall (aneurysms), narrowing of the vessels (arteriosclerosis - coronary, cerebral and peripheral), and clot activation (thromboembolism - arterial and venous), (Figure 1.1). It is the highest cause of death in men (39%) and second highest cause of death in women (30%) under the age of 75, in the United Kingdom in 1998, (British Heart Foundation 2000a).

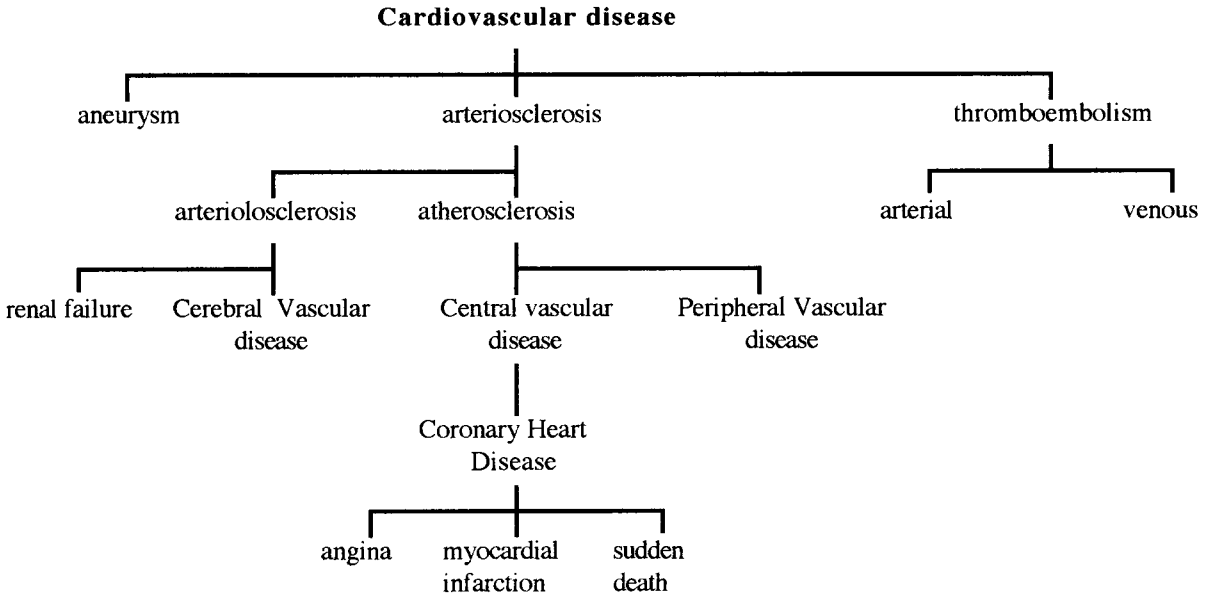


Figure 1.1 Cardiovascular Disease

1.1.1 Coronary Heart Disease

Coronary heart disease is a chronic disease which is seldom found in young men <35 years old and women <45 years old, (British Heart Foundation 2000b). The major clinical manifestations are angina pectoris, myocardial infarction and death, (Levy & Wilson 1998). In men, coronary heart disease accounts for 26% of all deaths making it the number one cause of death, whereas in women, it was the second, (16%), (British Heart Foundation, 2000a, Table 1.1.1). The national death rate from coronary heart disease is still amongst the highest in the western world" (British Heart Foundation 2000c), (Figure 1.1.1).

1.1.2 Peripheral Vascular Disease

Peripheral vascular disease, is also referred to as peripheral artery disease. This can cause intermittent claudication (exercise induced pain caused by localised ischaemia), a major uncorrected peripheral restriction in blood flow which can result in amputation of the limb. This is a particular occurrence in diabetes mellitus sufferers, (amputation rate seven times greater than that in non-diabetes patients with peripheral artery disease) who have a tendency for poor circulation in the feet which result in loss of toes and feet. About 70% of patients show no change or even become less symptomatic after 5 to 10 years, whereas less than 30% will require intervention and less than 10% will require amputation, (Rosenfield & Isner, 1998).

1.2 Cardiovascular risk factors

There are many risk factors for cardiovascular disease related to the range of stresses that the body experiences: diet, smoking, psychological factors and physical activities. Elevated levels of serum total cholesterol, low density lipoprotein cholesterol and plasma total homocysteine with decreased levels of serum high density lipoprotein cholesterol, folate, and vitamin B_{12} are associated with increased risk of coronary

	men	women
Cardiovascular Disease	**39 %**	**30 %**
Coronary heart disease	26 %	16 %
Stroke	6 %	7 %
other cardiovascular disease	7 %	7 %
Cancer	**32 %**	**40 %**
Lung cancer	9 %	8 %
Breast cancer	-	8 %
Colo-rectal cancer	3 %	3 %
other cancer	20 %	21 %
Respiratory disease	**9 %**	**11 %**
Injuries and poisoning	**7 %**	**4 %**
All other causes	**13 %**	**15 %**

Table 1.1.1 Deaths by cause, gender under 75, 1998, United Kingdom. (British Heart Foundation 2000a)

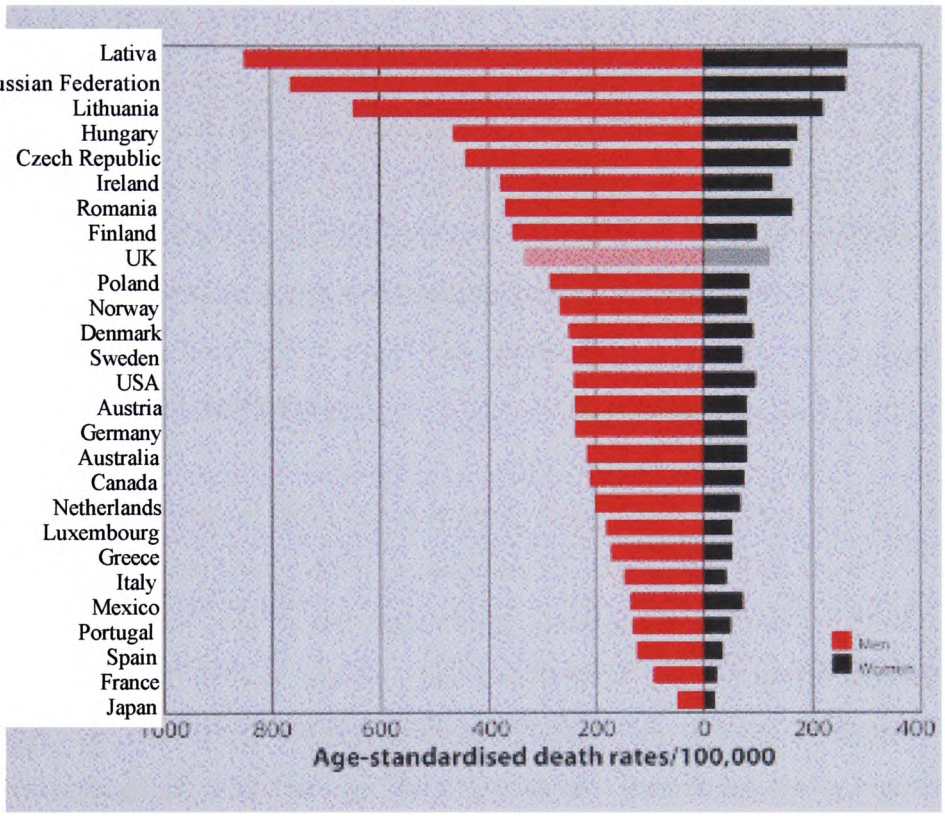


Figure 1.1.1. Death rates from Coronary Heart Disease, men and women aged 35-74, 1994, selected countries. (British Heart Foundation 2000d).

atherosclerosis, thrombosis, cerebral and peripheral vascular diseases, (Ueland & Refsum, 1989, Boers et al., 1985). Due to the relevance of plasma total homocysteine concentration to cardiovascular disease. It will be dealt with in greater detail following a general survey of cardiovascular risk factors, (refer to 1.3).

Apart from plasma total homocysteine, investigations into other nontraditional cardiovascular risk factors are occurring such as: C-reactive protein; lipoprotein (a); plasma insulin levels and markers of insulin resistance; activation of the renin-angiotensin system, (Oparil & Oberman, 1999). The Lewis blood group phenotype - Le(a-b-), had significantly elevated triacylglycerol concentration. It was associated with increased risk of coronary heart disease in the Copenhagen Male Study, (Ellison et al., 1999). The British Heart Foundation (2000e,f) estimated the effect of unhealthy diets and alcohol consumption as risk factors for coronary heart disease.

1.2.1 Cholesterol

Forty-five percent of deaths from coronary heart disease in men, was estimated to be due to a blood cholesterol level greater than 5.2 $mmol.dm^{-3}$, (201 $mg.dL^{-1}$). The mean blood cholesterol level for men aged 16 and above in England was 5.5 $mmol.dm^{-3}$, (213 $mg.dL^{-1}$). About 18% of men have blood cholesterol levels above 6.5 $mmol.dm^{-3}$, (251 $mg.dL^{-1}$), (The British Heart Foundation 2000g). In children of fathers with premature myocardial infarction, 16.7% of them had cholesterol levels above 6.0 $mmol.dm^{-3}$ (232 $mg.dL^{-1}$), compared to 4.7% of children of 'healthy' fathers, ($p = 0.01$), (Hennekens et al., 1976). Patients with homozygous familial hypercholesterolaemia had abnormal blood velocities in the aorta at the valvular area, (which is near the origin of the coronary arteries), whereas healthy controls had normal velocities measured by Continuous-Wave Doppler, (Faccenda et al., 1990). [Continuous-Wave Doppler measures the velocities of blood cell flow towards or away from the transducer along the scan line where there is no spatial resolution].

Adults under the age of thirty years, should be encouraged to achieve a total cholesterol level $\leq 4.7 mmol.dm^{-3}$ (182 $mg.dL^{-1}$), and those above thirty years to have a value $\leq$ 5.2 $mmol.dm^{-3}$ (201 $mg.dL^{-1}$), according to the National Institute of Health Consensus Development Panel and the study group of the European Atherosclerosis Society. There is a seasonal variation for cholesterol, the highest level in autumn, the lowest in late spring and summer, (Durrington, 1990).

1.2.2 Triacylglycerol

Body position and meals prior to the blood sampling, greatly affect serum triacylglycerol concentration. Illness and acute myocardial infarction, angina or coronary artery bypass surgery, produce increases in triacylglycerol, decreases in low density lipoprotein cholesterol, high density lipoprotein cholesterol and apolipoprotein B.

The Prospective Cardiovascular Munster study observed 4,849 middle-aged men (40 to 65 years), and in an eight-year follow-up showed a significant and independent association between serum triacylglycerol concentration and the incidence of major coronary events, (Assmann et al., 1996). Meta-analysis of seventeen studies (46,413 men and 10,864 women) showed, after adjustment for high density lipoprotein cholesterol and other risk factors, the univariate relative risk for triacylglycerol was 1.14 (men) and 1.37 (women), (Hokanson & Austin, 1996). The triad of elevated triacylglycerol, small dense low density lipoprotein particles (LDL subclass phenotype B) and reduced high density lipoprotein cholesterol comprises the atherogenic lipoprotein phenotype (Austin et al., 1990). An Italian study has shown significantly more abnormal arteries between hypercholesterolaemic / hypertriacylglycerolaemic patients than hypercholesterolaemic / normotriacylglycerolaemic patients, (Rubba et al., 1990).

The Adult Treatment Panell I (NCEP 1993), considers levels of triacylglycerol above $4.5 mmol.dm^{-3}$ ($400 mg.dL^{-1}$) as a high risk factor and less than $2.26 mmol.dm^{-3}$ (200

mg.dL^{-1}) as a low risk factor.

1.2.3 High Density Lipoprotein

Women have higher serum high density lipoprotein concentration than age-matched men, (Gustafson et al., 1974; Nazir et al., 1999; male 1.09 mmol.dm^{-3}{42 mg.dL}, female 1.47 mmol.dm^{-3}{57 mg.dL^{-1}}). High density lipoprotein cholesterol is reduced in the summer months like the other lipids, (1.29 to 1.25 mmol.dm^{-3}{50 to 48 mg.dL^{-1}}, Nazir et al., 1999 statistical significant {p=0.010}, but not physiologically significant as the critical difference was less than 21%). High density lipoprotein levels are lower in the obese (Wilson & Lees, 1972), poorly controlled diabetes mellitus (Lopes-Virella et al., 1977), and in those who smoked (Garrison et al., 1978). The Adult Treatment Panel II (NCEP 1993), considers levels of high density lipoprotein cholesterol below 0.9 mmol.dm^{-3} (35 mg.dL^{-1}) as a high risk factor and greater than 1.6 mmol.dm^{-3} (60 mg.dL^{-1}) a low risk factor.

High density lipoprotein removes cholesterol from tissues and deposits it in the liver (Rader, 1998), where it undergoes catabolism.

1.2.4 Low Density Lipoprotein

The Adult Treatment Panel II (NCEP 1993), considers levels of low density lipoprotein cholesterol above 4.1 mmol.dm^{-3} (160 mg.dL^{-1}) as a high risk factor and less than 3.4 mmol.dm^{-3} (130 mg.dL^{-1}) a low risk factor.

Macrophages take up low density lipoprotein via receptor mediation or as oxidized low density lipoprotein by receptor-independent pathways to form foam cells. Foam cells accumulate in the intima to form fatty streaks, (Stevens & Lowe 1995a). Extracellular low density lipoprotein accumulates in the intima forming a lipid plaque. This develops into a fibrolipid plaque as the extracellular low density lipoprotein

calcifies and there is a loss of elastic lamina, collagenous replacement of myocytes, and myocyte proliferation.

1.2.5 Apolipoproteins

There is a wide range of apolipoproteins, the major ones are A_1 & A_2 on high density lipoproteins; A_4, $C_{1\ to\ 3}$, E and (a); and B_{100} and B_{48} on all the lipoproteins except high density lipoprotein.

Maciejko et al. (1983), found that apolipoprotein A_1 was a better marker of angiographically documented coronary artery disease than either high density lipoprotein cholesterol or total cholesterol.

Plasma concentrations of apolipoprotein B_{100}, the protein on low density lipoproteins that bind to the low density lipoprotein receptor, has been found higher among patients with coronary artery disease than control subjects. Apolipoprotein B_{100} is a better indicator of low density lipoprotein atherogenicity, than is cholesterol level. Some patients have hyperapobetalipoproteinemia and normal low density lipoprotein cholesterol levels, (Sniderman et al., 1980).

Naito (1985), showed the importance of both apolipoproteins A_1 and B_{100} over total cholesterol, low and high density lipoprotein cholesterol levels. They concluded that the ratio of apolipoprotein A_1 to B_{100} was a better predictor of risk of coronary artery disease.

1.2.6 Lipoprotein (a)

Lipoprotein (a) was first identified by Berg (1963) and is a family of lipoprotein particles. It contains apolipoprotein (a) bonded to low density lipoprotein by disulphide

bridges (Gaubatz et al., 1983; Fless et al., 1984). It is assumed that apolipoprotein (a) wraps itself around the low density lipoprotein particle and forms disulphide bridges with the apolipoprotein B-100 (hepatic origin, M_r = 550,000 daltons) or interlocks with the low density lipoprotein particle without forming any direct covalent bonds, (Mbewu & Durrington, 1990). Whereas most Lipoprotein (a) contain one copy of apolipoprotein (a), some contain two copies of apolipoprotein (a), (Fless et al., 1989). Scanu and Fless, (1990) stated that apolipoprotein (a) should not be classed as an apolipoprotein because it does not bind directly to lipids and was water soluble, although the $apoB_{100}$-apo(a) complex does bind to lipids via $apoB_{100}$. Tissue cultures of baboon hepatocytes have shown that Lp(a) assembly can occur at the hepatocyte cell surface. Apo(a) binds to the cell surface via its kringle domains and then attaches to LDL $apoB_{100}$, inducing conformational changes and release, (White & Lanford 1994).

Apolipoprotein (a) binds to cholesteryl ester-rich lipoproteins (low density lipoprotein and less effectively to high density lipoprotein), and to triacylglycerol-rich particles (chylomicrons and chylomicron remnants, Bersot et al., 1986).

Plasminogen comprises a signal peptide, a pre-activation peptide, five kringle domains (K1 -K5) and a serine protease domain, whereas Apolipoprotein (a) is a plasminogen homologue (McLean et al., 1987), it contains the signal peptide, 15 to 37 copies of kringle 4 with 75 - 85% identity to that of kringle 4 in plasminogen, one copy of kringle 5, and a serine protease domain (Scanu & Fless, 1990; White & Lanford, 1995). The serine protease domain was an inactive form of the proteolytic site found in plasminogen and therefore could not lyse blood clots. Lipoprotein (a) binds to the endothelial cell surface receptor for plasminogen, inhibiting fibrinolysis and promoting thrombosis and atherosclerosis, (Hajjar et al., 1989, Gonzalez-Gronow et al., 1989).

Lipoprotein (a) does not appear to increase immediately post 30 minutes exercise of either low or high intensity, (Durstine et al., 1996), nor after chronic exercise (Hubinger & MacKinnon, 1996).

Lipoprotein (a) does appear to increase the basal production and release of nitrogen(II)oxide, (Schlaich et al., 1998), and thus Lipoprotein (a) associated with atheroma appears to have a beneficial effect.

1.2.7 Blood Pressure

It is estimated that 14% of deaths in men are due to raised blood pressure (at rest, systolic blood pressure of 140 mmHg or over or a diastolic blood pressure of 90 mmHg or over), (British Heart Foundation, 2000h).

1.2.8 Smoking

Approximately 20% of deaths from coronary heart disease are due to smoking. The habit is more prevalent in Scotland than England and higher in the North (28%) than the South (26.6%) and lowest in East Anglia (23%). It is more prevalent in the manual (36%) than non manual (21%) social groups, (British Heart Foundation, 2000i). Cigarette smoking has been associated with increased reliance upon glycolytic metabolism during exercise, elevated carbon monoxide and chronotropic incompetence, so that smokers fatigue earlier than non smokers who exercise, (McDonough & Moffatt 1999; Srivastava et al., 2000).

1.2.9 Psychosocial Factor

The British Heart Foundation (2000j) states four different types of psychosocial factor, namely: work stress, lack of social support, depression (including anxiety), and personality (particularly hostility), have been consistently associated with increased risk of coronary heart disease.

From 1986 to 1992 the male age-standardised death rates per 100,000 population from coronary heart disease showed that more from the partly skilled/unskilled social

class died when compared to the professional/intermediate class (266 to 160 respectively, ratio manual to non manual 1.58. British Heart Foundation 2000k).

For the male population, there is a strong correlation (0.866, p=0.0006) between the average yearly unemployment figures as a percentage of the workforce from 1988 to 1991 and the age standardised death rates from coronary heart disease per 100,000 population from 1989 to 1998 (refer to Table 1.2.9). The data implies that there is a possible stress factor from unemployment that could be caused by psychological stress (depression), low physical activity status and a financial effect upon diet. Coronary heart disease is a chronic illness and therefore the effect of unemployment any time in their working life period is likely to increase their risk of a coronary event, particularly if they are the type of person that gets easily worried.

1.2.10 Radicals

Radicals are molecules or atoms with an odd number of electrons. This causes them to react quickly with other radicals (termination) or with other molecules producing another radical, (propagation). About 2-5 % of the oxygen used by the mitochondria forms the superoxide anion ($O_2^{-\bullet}$, Reid, 1999). Other reactive oxygen species (ROS), are the hydroxyl radicals ($OH^{\bullet}$), and hydrogen peroxide (H_2O_2). In hypoxic tissue the adenosine produced from adenosine monophosphate is deaminated to inosine. Inosine is converted to xanthine then to uric acid using xanthine oxidase. The by-product of these reactions is hydrogen peroxide, (Blakley, 1989).

Reactive oxygen species have damaging effects upon cells such as: cell membranes (peroxidation of lipids); DNA; thiol groups in proteins, and mitochondrial membranes. The net effect of this damage can be cell death, (Stevens & Lowe, 1995b). The main scavenging systems for removing radicals are: antioxidants (vitamin C, A and E); glutathione peroxidase; superoxide dismutase, and catalase, (Stevens & Lowe, 1995b).

Area	Male Unemployment 1988 - 91	Male coronary heart disease mortality 1989 - 1998 per 100,000 population
Scotland	11.95	426
North	13.85	401
North West	11.78	389
Yorkshire & Humberside	10.58	363
Wales	10.53	362
West Midlands	9.50	346
East Midlands	7.98	325
East Anglia	5.60	277
South West	6.88	283
South East	6.35	280

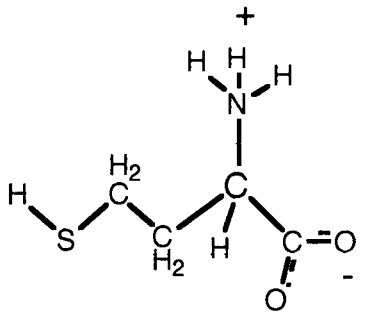
Table. 1.2.9: Comparison of unemployment and mortality rates for coronary heart disease.

{The unemployment figures are averaged from the yearly statistics -*Employment Gazette* June 1992 **100**: S18 - S20. The coronary heart disease figures are averaged from the Coronary Heart Disease Statistics 2000 Chpt 1 p. 25}

A wide range of fruit and vegetables are desirable to provide the antioxidants necessary to protect against oxidative damage and disease, (Jacob and Burri ,1996; Cao et al., 1998). The daily reference nutrient intakes for vitamins A, C and E are in adult males 1.8 μmol (0.50 mg), 0.142 mmol (25 mg) and 9.3 μmol (4 mg) respectively (RHSS41 1999). It is difficult to state a daily individual antioxidant intake quantity as the level of stress and the person's reaction to it are not constant, nor absorption across the enterocytes. Some athletes believe that their intake is low and supplement with multivitamin pills containing vitamins A, C, E, and mineral selenium. Alessio and Blasi (1997) concluded in their review that most studies showed that acute and chronic exercise increases antioxidant levels.

1.3 Homocysteine

Homocysteine is not found in the normal diet, but is obtained solely from methionine.



Homocysteine $\{HSCH_2CH_2CH(N^+H_3)CO_2^-\}$ is a thiolated amino acid with a relative molecular mass of 135 daltons. Homocysteine binds to proteins and its highest concentration is in the liver with lower levels in the kidney, brain, heart, lung and spleen (Svardal et al., 1986). The plasma level depends upon the age, gender, vitamin status (folate, vitamins B_{12}, B_6 and to a limited extent B_2), protein intake (methionine), the genetic makeup of the subject, renal function and the subject's history of smoking. Even after multivariate adjustment, homocysteine was independently associated with

gender, race, serum folate, vitamin B_{12} levels and systolic blood pressure (Table 1.3).

Intracellular utilization of homocysteine:

1. It can be methylated to methionine. The methyl group could be transferred either from N^5-methyltetrahydrofolate involving cobalamin (vitamin B_{12}){Universal} or from Betaine {Liver}.
2. It can be adenosylated to S-adenosylhomocysteine.
3. It can under go trans-sulphuration producing cystathionine and cysteine, (requiring vitamin B_6), and glutathione, {Liver}.
4. It is hypothesised that it can be oxidized to Homocysteic acid.
5. It may be bound to proteins via the thiol moiety.
6. Converted to homocysteine thiolactone.
7. Egresses from the cell.

(modified from McCully 1993, Do et al. 1988, refer to Figure 1.3)

If relatively large amounts of homocysteine are fed to young rats (seven weeks old), it inhibits growth either directly or by affecting absorption across the jejunum. It also causes anatomical alterations in many organs, particularly in pancreatic acinar cells and testes. There was also damage to salivary glands, liver, gastric mucosa, Brunner's glands, kidneys, seminal vesicles, prostate, adrenals, thymus, spleen, lymph nodes, haemopoietic system, epiphyseal plates and Herder's glands, (Klavins, 1963, refer to section 1.3.1i Klavins et al., 1963). The rats diet included vitamins $B_{1\text{-}6}$, it did not include folate, nor vitamins $B_{12,}$ C & E. The homocysteine could undergo trans-sulphuration, but might not be able to become methylated from N^5-methyltetrahydrofolate. Some methionine could be produced from the choline in their diet. As their diet was deficient in antioxidants, some damage may have been caused by radicals. Brown and Strain (1990) found that homocysteine in the diet of young rats caused a reduction in copper status, this affects ceruloplasmin, superoxide dismutase and cytochrome c oxidase. There were increased levels of superoxide dismutase and iron in

geometric means of total homocysteine ($\mu mol.dm^{-3}$)
males (5.22) vs females (4.84)
blacks(5.51) vs whites(4.96) vs Hispanics(4.93)
nonusers(5.09) vs users of multivitamins(4.82)
smokers(5.19) vs nonsmokers(5.00)

Table 1.3 Serum homocysteine levels in Teenagers

(Osganian et al., 1999).

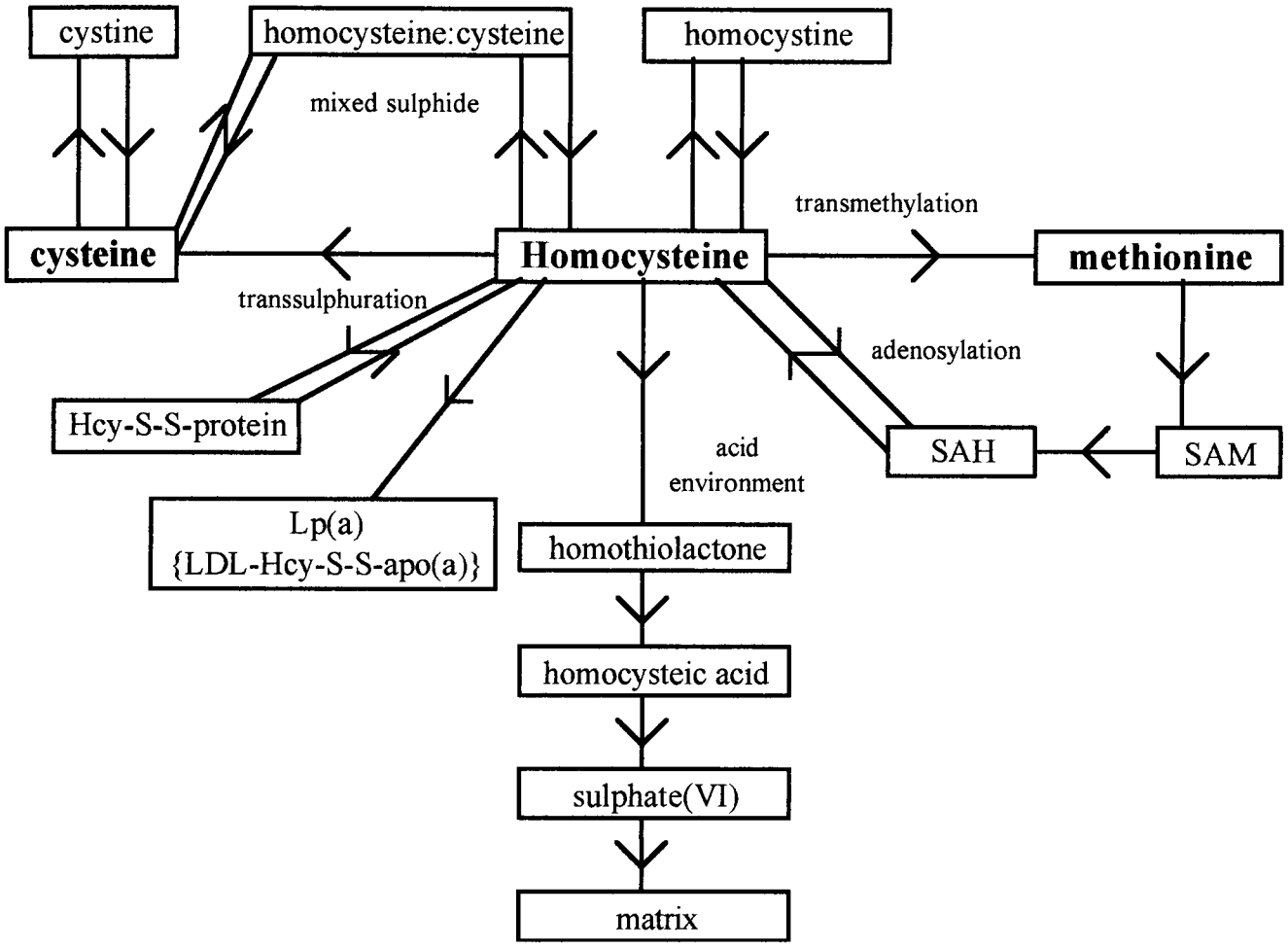


Figure 1.3 Utilization of Cellular Homocysteine

Key

Lp(a)	- lipoprotein (a)
SAM	- S-adenosylmethionine
SAH	- S-adenosylhomocysteine
Hcy-S-S-protein	- homocysteine linked to a protein molecule via the disulphide bridge

the liver and increased levels of thiobarbituric acid reactive substances. Low dose oral administration of homocysteine thiolactone to rats for six weeks was found to cause an increase in plasma total homocysteine and triacylglycerol. There was also an associated decrease in beta-hydroxybutrate/3-oxobutanoate ratio, which showed inhibited fatty acid oxidation and therefore triacylglycerol increased, (Frauscher et al., 1995).

1.3.1 Cellular Homocysteine metabolism

Homocysteine is only produced via the methionine cycle and its intracellular concentration appears to be cell dependent. Non-hepatic cells appear to have a constant intracellular concentration, excess being exported from the cell. Hepatocytes have increased homocysteine under conditions of low protein diets, (Finkelstein & Martin, 1984). Homocysteine is one of two loci in Thiol biochemical pathways, the other is Methionine. Homocysteine regulates re-methylation and trans-sulphuration, whereas Methionine regulates protein synthesis and transmethylation, (Storch et al., 1990). An overview of the Thiol chemistry is shown in Figure 1.3.1.

1.3.1i Methionine

Methionine is an essential amino acid. All translated polypeptides start with methionine and in certain polypeptides, it is removed post-translationally, (Bodley, 1989). Methionine can be obtained from three main sources: autolysis of proteins, diet or re-methylation of homocysteine. It can also be synthesized from methylthioribose. The requirement of the cell for methionine depends upon its rate of protein anabolism and transmethylation. The recommended dietary allowance for methionine in the United States for adults is 0.9 $g.day^{-1}$, (Munro & Crim, 1988). Yet if there is excess methionine in the diet, it has been shown to cause inhibition of growth in young rats (7 weeks old) and anatomical alterations in organs (pancreas, gastrointestinal tract, salivary glands, kidneys, spleen, thymus, thyroid and adrenal glands, Klavins et al., 1963). They were unable to measure plasma methionine or homocysteine and were therefore unable to say

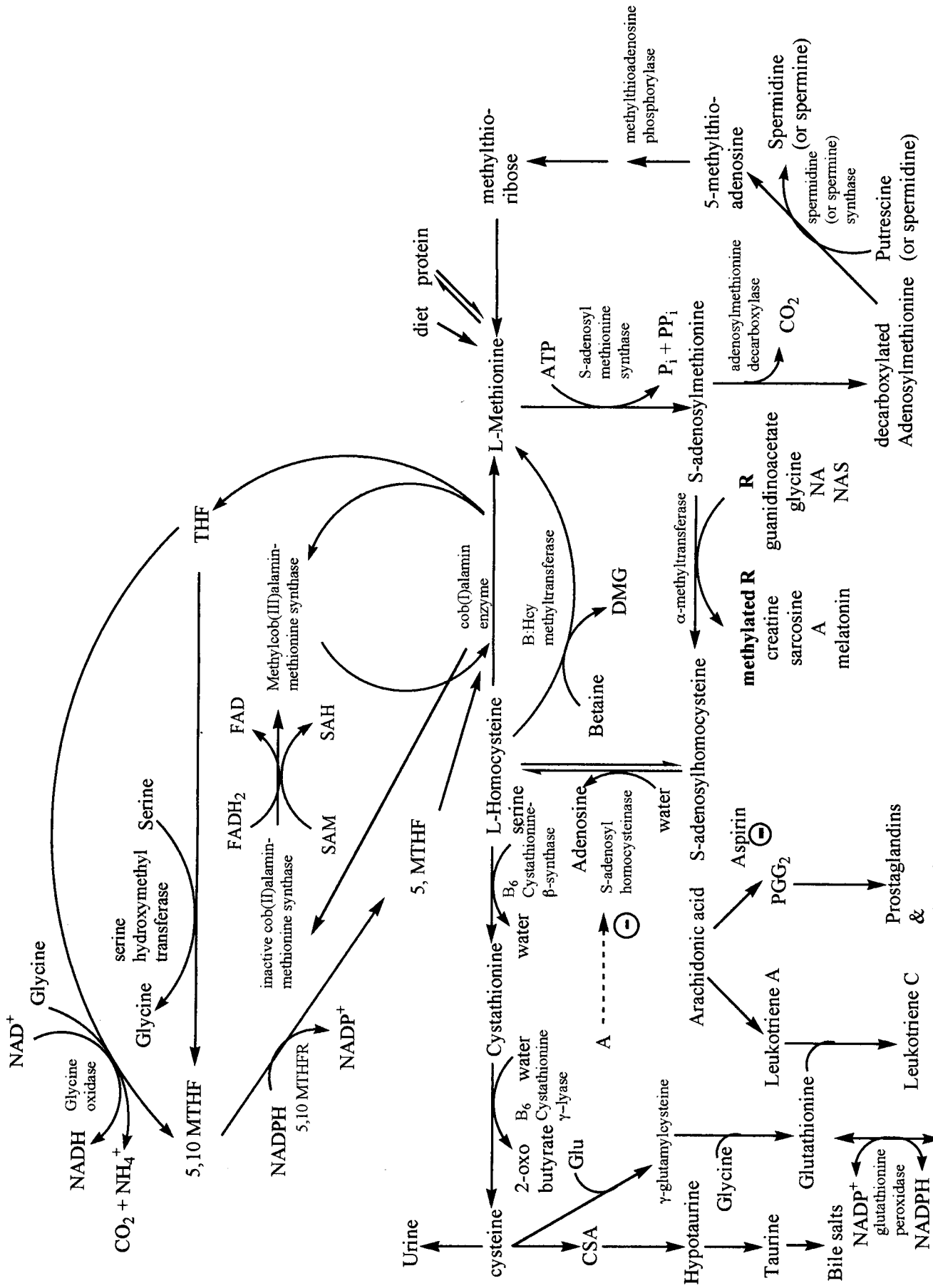


Figure 1.3.1 Biochemistry of Thiol groups (modified from Finkelstein 1997)

Key:

Substrate/Product

CO_2	carbon dioxide
NH_4^+	ammonium ion
5,10 MTHF	5,10-methylenetetrahydrofolate
5 MTHF	5-methyltetrahydrofolate
THF	Tetrahydrofolate
SAM	S-adenosylmethionine
SAH	S-adenosylhomocysteine
Glu	Glutamic acid
CSA	Cysteine sulphinic acid
GSSG	Glutathione disulphide
DMG	Dimethyl glycine
PGG_2	Prostaglandins G_2
R	alkyl group
NA	noradrenaline
NAS	N-acetyl Serotonin
methylated R	methylated alkyl group
A	adrenaline
ATP	adenosine triphosphate(V)
P_i	inorganic phosphate(V)
PP_i	pyrophosphate(V)
B_6	vitamin B_6

Enzyme

5,10 MTHFR	5,10-methylenetetrahydrofolate reductase	(EC 1.7.99.5)
B:Hcy methyltransferase	Betaine:Homocysteine methyltransferase	(EC 2.1.1.5)

Coenzyme

NAD^+	oxidized nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
$NADP^+$	oxidized nicotinamide adenine dinucleotide phosphate(V)
NADPH	reduced nicotinamide adenine dinucleotide phosphate(V)
FAD	oxidized flavin adenine dinucleotide
$FADH_2$	reduced Flavin adenine dinucleotide

Inhibitor

A	Adrenaline

whether excess methionine itself caused these changes, but the changes were similar to homocysteine fed in excess to rats (Klavins, 1963). In the older generation (64 years old) receiving multivitamins, there appears to be a significant inverse dose-response relationship between dietary protein and the napierian logarithm of total homocysteine, (Stolzenberg-Solomon et al., 1999). Thus implying that a high protein diet could reduce total homocysteine levels, if there were sufficient vitamins, particularly vitamin B_6.

Homocysteine is converted to methionine via two different reactions. One reaction uses N^5-methyltetrahydrofolate, as the methyl donor and methionine synthase (coenzyme B_{12}) as the catalyst. This reaction is ubiquitous, occurring in every mammalian cell. Methionine and S-adenosylhomocysteine inhibit methionine synthase (EC 2.1.1.13), whereas S-adenosylmethionine acts as a co-substrate. The other reaction uses betaine as the methyl donor with betaine:methionine methyltransferase as the catalyst and occurs only in the Liver and kidneys in primates. Methionine, S-adenosylmethionine and S-adenosylhomocysteine inhibit Betaine:homocysteine methyltransferase (EC 2.1.1.5). An *in vitro* system which mimics a rat's liver demonstrated that the enzymes that remove homocysteine may be affected by the amount of protein in the diet, most likely the quantity of methionine, Table 1.3.1i, (Finkelstein & Martin, 1984).

Mudd & Poole, (1975) showed that in humans on normal diets, the homocysteine moiety was converted to methionine on average, 1.9 times in males and 1.5 times in females before undergoing trans-sulphuration. When the diet was restricted in methionine and choline the average number of cycles increased to 3.9 for males and 3.0 for females. The work by Finkelstein & Martin, (1984) implies that this increased cycling could be partly accounted for by the increase in Betaine:Homocysteine methyltransferase activity in the Liver, thereby increasing plasma methionine levels.

enzymes	normal diet	low protein 3.5% casein	high protein 55% casein
MS	26.6%	5.1%	2.9%
B:HMT	27.3%	61.4%	8.0%
CBS	46.1%	33.5%	89.1%

Table 1.3.1i The percentage of Homocysteine consumed by each enzyme. [MS - methionine synthase; B:HMT - betaine: homocysteine methyltransferase; CBS cystathionine β-synthase]

1.3.1ii S-Adenosylmethionine

Methionine is adenosylated to S-Adenosylmethionine using adenosine triphosphate(V) and the enzyme S-Adenosylmethionine synthase (EC 2.5.1.6). S-Adenosylmethionine does not appear to be able to leave the cell in which it was synthesized. Less than 10% adenyoslated methionine is decarboxylated and the propylamine moiety used in the synthesis of spermine. Both spermidine and spermine bind tightly to nucleic acid and are therefore abundant in rapidly proliferating cells. Most of S-Adenosylmethionine undergoes transmethylation with the formation of S-Adenosylhomocysteine, which acts as an inhibitor upon the α-methyltransferase.

Transmethylation reactions are universal throughout the body: creatine synthesis, (Figure 1.3.1ii a); methylation of noradrenaline to adrenaline, (Fonlupt et al., 1979) and metabolism of adrenaline and noradrenaline, (Figure 1.3.1ii b); methylation of N-acetylserotonin to syntheses melatonin. A lack of transmethylation results in demyelination, this causes subacute combined degeneration, (Scott, et al., 1981). Subacute combined degeneration is also associated with a vitamin B_{12} deficiency and thus a S-Adenosylmethionine deficiency, (section 1.6.2).

1.3.1iii S-Adenosylhomocysteine

Cytosolic S-Adenosylhomocysteine inhibits transmethylation reactions and is produced by all transmethylation reactions using S-Adenosylmethionine as one of its substrates. There are three routes to remove S-Adenosylhomocysteine, which are: hydrolysis, binding to specific proteins and transportation out of the cell. In rats' liver 30 - 50% of S-Adenosylhomocysteine can be found bound to proteins in the plasma membrane and microsomes with most of the free S-Adenosylhomocysteine in the cytosol fraction (Svardal et al., 1986 and 1987). The kidney is the major tissue shown to absorb extracellular S-Adenosylhomocysteine, (Duerre et al., 1969).

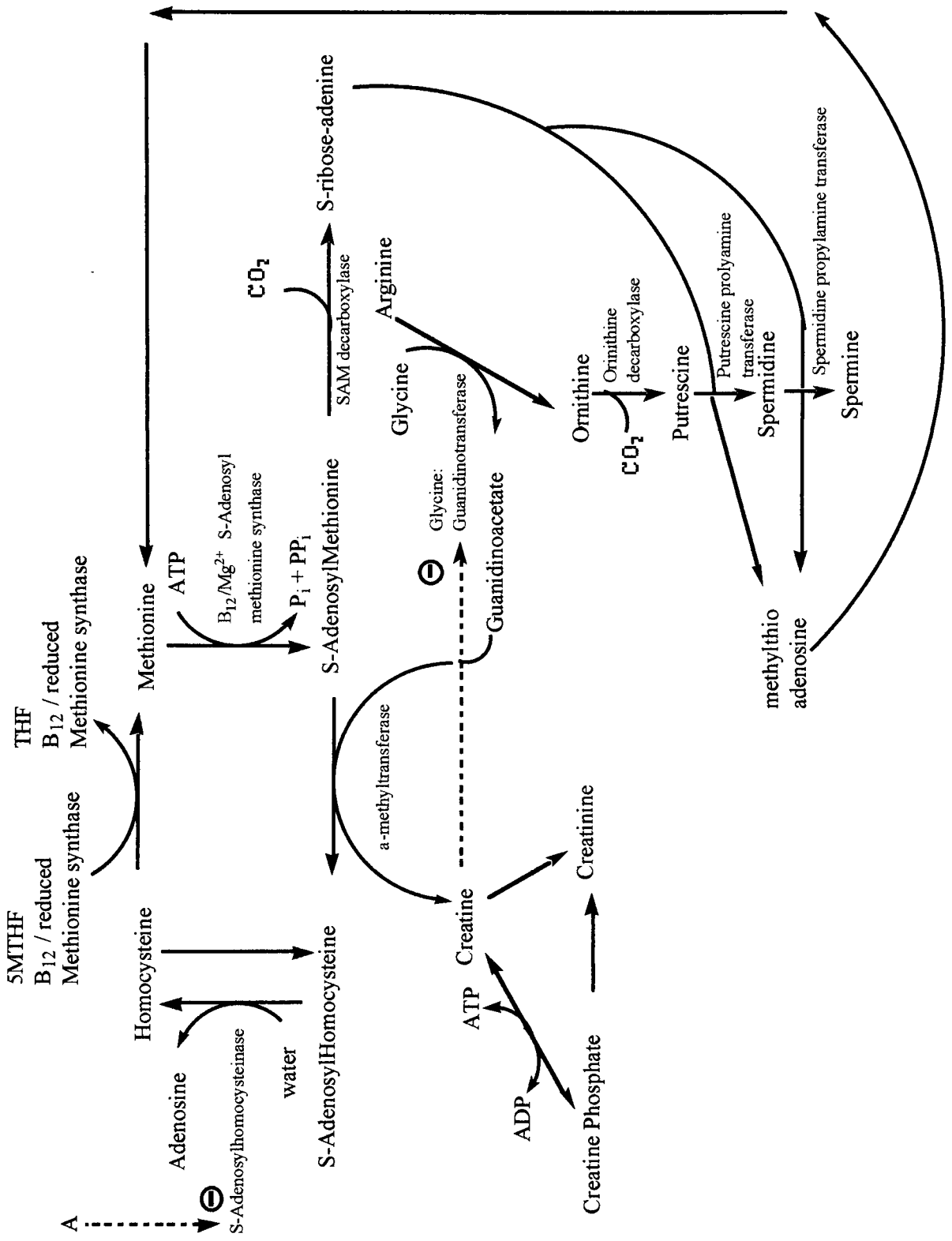


Figure 1.3.1ii(a) Creatine Synthesis

Key

5MTHF	5 methyltetrahydrofolate	THF	Tetrahydrofolate
P_i	inorganic phosphate	PP_i	inorganic pyrophosphate
A	Adrenaline		

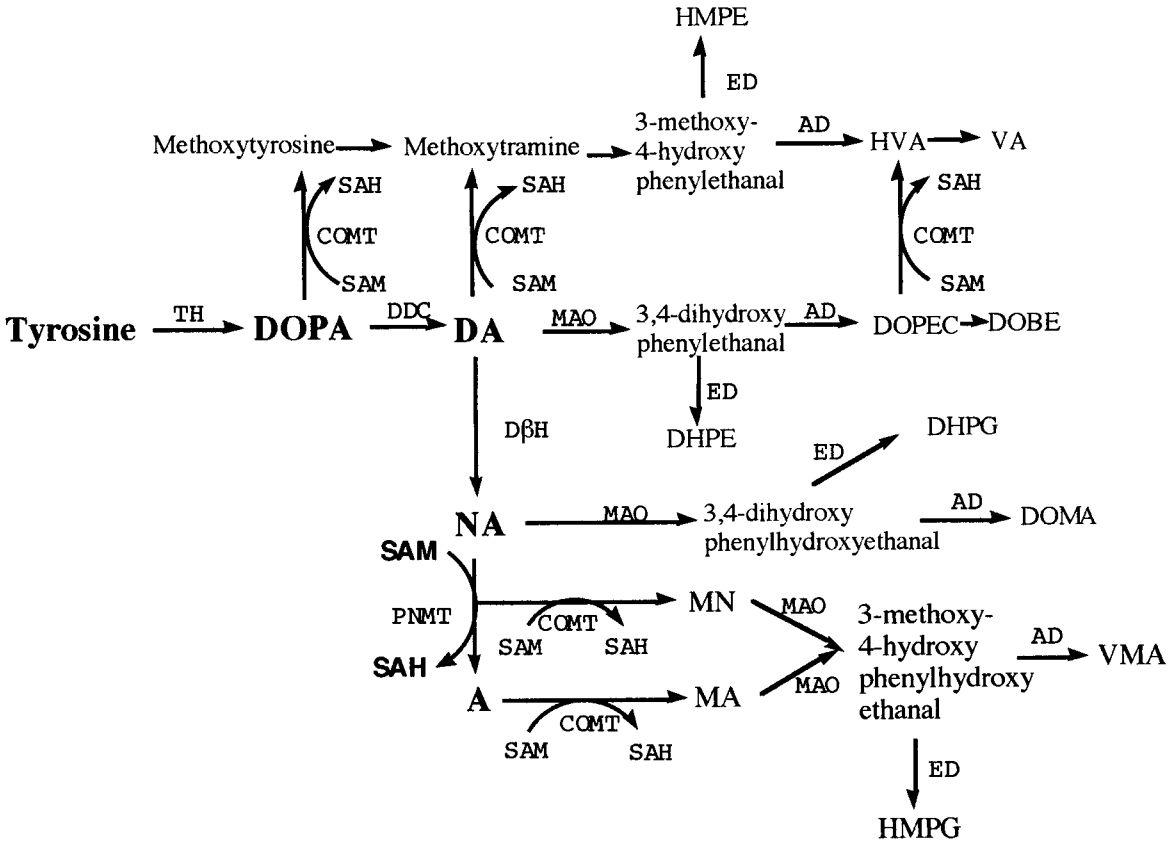


Figure 1.3.1ii b **The biosynthesis and metabolism of the catecholamines from the amino acid tyrosine** (modified from Riederer et al., 1989).

Key:

Substrates and Products:

DA = Dopamine
NA = Noradrenaline;
A = Adrenaline
MN = Metanoradrenaline;
MA = Metadrenaline
DOMA = 3,4-dihydroxymandelic acid
VMA = vanillylmandelic acid (3-methoxy-4-hydroxymandelic acid)
DOPEC = 3,4-dihydroxyphenylethanoic acid
DOBE = 3,4-dihydroxybenzoic acid
DHPE = 3,4-dihydroxyphenylethanol
HMPE = 4-hydroxy-3-metoxyphenylethanol;
DHPG = 3,4-dihydroxyphenylethan-1,2-diol
HVA = homovanillic acid
HMPG = 4-hydroxy-3-methoxyphenylethan-1,2-diol
VA = vanillic acid
SAM = S-adenosylmethionine
SAH S-adenosylhomocysteine

Enzymes

TH = tyrosine hydroxylase
DDC = Dopa decarboxylase
PNMT = Phenylethanolamine-N-methyltransferase
DßH = Dopamine ß-hydroxylase
MAO = monoamine oxidase (found in mitochondria in both neuronal and non-neuronal tissue)
COMT = Catechol-O-methyltransferase (found in the cytoplasm of most cells, including neurons)
AD = Aldehyde dehydrogenase (oxidation)
ED = Alcohol dehydroxygenase (reduction)

Exercise causes an elevation in plasma adrenaline of 1200-1800% following 5-10 minutes intense exercise at 90-100% $\dot{V}O_{2max}$ and an increase of 500-800% following 60-90 minutes moderate exercise - 60-70% $\dot{V}O_{2max}$ (Kjaer & Dela, 1996). Adrenaline has been shown to inhibit S-Adenosylhomocysteine hydrolase in excised guinea-pigs papillary muscles in a concentration dependent fashion. It uses β-adrenergic receptors that stimulate an influx of calcium, (Suárez & Chagoya de Sánchez, 1997). This would imply, that during exercise there would be a transient increase in plasma S-Adenosylhomocysteine and a decrease in L-homocysteine which is not what has been found to date (refer to section 1.7.3). Intravenous injection of tritiated S-Adenosylhomocysteine into rats, produced less than 15% of the radioactivity incorporated into protein methionine or excreted in the urine as 2-oxobutyrate. The remainder is excreted as S-Adenosyl-γ-thio-2-oxobutyrate, (Duerre et al., 1969). The compound is not found in 'normal' rat urine. This implies an enzyme in the kidneys for catalysing S-Adenosylhomocysteine, possibly directly to S-Adenosyl-γ-thio-2-oxobutyrate. So that, during exercise or times of stress, when adrenaline levels are elevated, this shunt pathway removes S-Adenosylhomocysteine that would cause a decrease in cytosolic S-Adenosylhomocysteine and thereby remove the transmethylation inhibitor; allowing the transmethylation of noradrenaline to adrenaline and therefore continued inhibition of S-Adenosylhomocysteine hydrolase during prolonged exercise.

Adenosine released into the coronary system is increased during hypoxia and is affected by L-homocysteine concentration, (Schrader et al., 1981). In an isolated guinea-pig heart normoxically perfused 33% of the adenosine produced came from S-Adenosylhomocysteine, via the transmethylation pathway and most of the produced adenosine is reincorporated into the adenosine triphosphate(V) pool, whereas in the hypoxically perfused heart the increased adenosine comes from 5'-adenosine monophosphate(V) hydrolysis, (Lloyd et al., 1988; Deussen et al., 1989; Lloyd & Schrader, 1993).

Adenosine is believed only to play a paracrine role in coronary vasodilation during sustained hypoxia, when adrenergic receptors are intact, (Herrmann & Feigl, 1992). Myocardial effects of catecholamines were inhibited by adenosine, (Schrader et al., 1977). Adenosine reduces the atrioventricular conduction velocity during hypoxia, (Belardinelli et al., 1980).

1.3.1iv Trans-sulphuration

Homocysteine can enter the trans-sulphuration pathway, where it is converted to cystathionine using cystathionine β-synthase (coenzyme B_6) and then to cysteine using cystathionine γ-lyase (coenzyme B_6, Figure 1.3.1).

Intracellular homocysteine forms a dynamic pool between free and bound homocysteine. The turnover rates in rat livers were calculated as 1.2s for free homocysteine and 1.8s for bound homocysteine, (3s for total homocysteine, Svardal et al., 1986). In tissues that do not have the enzymes for trans-sulphuration (all except liver, kidney and pancreas), excess free homocysteine is transported out of the cell into plasma. This limits intracellular thiol toxicity, but exposes the vascular tissue to the mild reducing agent.

1.3.2 Plasma Homocysteine

The plasma homocysteine level is the summation of the export of excess homocysteine from cells. *In vitro*, homocysteine export post-methionine loading was dependent upon cell type and in relation to lymphocytes whether they are activated, (Christensen B., et al., 1991; Iizasa & Carson 1985; German et al., 1983). The highest homocysteine export occurred during early exponential growth at low cell density in all proliferating cells. Christensen B., et al. (1991), hypothesised that the possible reason for increased egress of homocysteine in proliferating cells that are actively synthesising protein and transmethylation reactions, could be due to a localised folate deficiency as it

was used for purine and pyrimidine synthesis. This would increase cell density and decrease amino acid transport. It appears that by three days post methionine loading, the excess methionine had been converted to homocysteine, exported and removed from the plasma, with the greatest plasma homocysteine level about one and half days post methionine loading.

There are several different proteins in the plasma membrane that transport amino acids. One such transport system is called ASC because it has a high affinity for alanine, serine and cysteine, (Christensen H., et al., 1967). System ASC allows sodium movement in both directions and the associated amino acid movement symport with the sodium. System ACS is present in almost all cell types. It is possible that homocysteine could use other systems, such as system y^+ that transports cationic and zwitter ionic amino acids with sodium, (Palacín et al., 1998).

There are several interactions that plasma homocysteine could undertake:

- Less than 0.05% of plasma total homocysteine is excreted unchanged in the urine, (Mudd & Poole, 1975).
- translocation from the cells of the body going mainly to the liver, but also to the small intestine, pancreas and kidneys. It is within these organs that the homocysteine undergoes trans-sulphuration, those organs also have a rapid turnover of glutathione (Meister, 1983).
- Reaction with endothelial nitrogen(II)oxide (refer to section 1.4.2).
- Stimulates hypertrophy of smooth myocytes (refer to section 1.4.2).
- Reacts with lipoprotein to form lipoprotein(a) (refer to section 1.5.4).
- Bound to proteins via the thiol moiety. (eg albumin).

Since 1985, homocysteine levels are quoted as plasma total homocysteine and include the concentrations of free reduced form of homocysteine (≈1%), disulphide homocystine, mixed disulphide homocysteine-cysteine and protein-bound homocysteine (≈80%, principally to albumin). The acceptable range of fasting plasma total

homocysteine is 5 to 15μmol.dm^{-3}. The upper end of the range has been associated with certain vascular diseases and is dependent upon the patient's genotype. There is a gender difference, males have higher homocysteine levels than females. Plasma total homocysteine has been shown to increase with age, males aged 40 to 42 years recorded a mean plasma total homocysteine level of 10.8μmol.dm^{-3} and those aged 65 to 67 years 12.3 μmol.dm^{-3}.

Plasma total homocysteine concentration of between 16 and 30 μmol.dm^{-3} is taken as moderate hyperhomocysteine, 31 and 100 as intermediate hyperhomocysteine and >100 as severe hyperhomocysteine. Some argue that moderate hyperhomocysteine lowest value should be greater than 10 μmol.dm^{-3} (Omenn et al., 1998).

1.3.3 Homocysteine thiolactone

Under acidic conditions homocysteine becomes homocysteine thiolactone, (McCully, 1994). Acidic conditions are more likely post anaerobic exercise. Homocysteine and adenosine triphosphate(V) with the catalyst Lysyl-transfer ribonucleic acid synthetase passes through the intermediate *Lysyl-transfer ribonucleic acid synthetase.homocysteine.adenosine monophosphate(V)* before yielding Lysyl-transfer ribonucleic acid synthetase, adenosine monophosphate(V) and homocysteine thiolactone (Jakubowski, 1997). Homocysteine thiolactone is procarcinogenic and proneoplastic, and in normal cells, it is metabolised to sulphate(VI) via a series of reactions involving vitamin A and C (McCully 1971, 1994, refer to Figure 1.3.3). The oxidation of homocysteine thiolactone to sulphate(VI) and its incorporation in cartilage is under the control of thyroxine and growth hormone, (Clopath et al., 1976; McCully 1971).

Baboons were infused with homocysteine thiolactone for three months at the end of which certain arteries had endothelial desquamation and early proliferative lesions and a shortening of platelet survival, (Harker et al., 1976). 0.1 to 1.0 mmol.dm^{-3} of

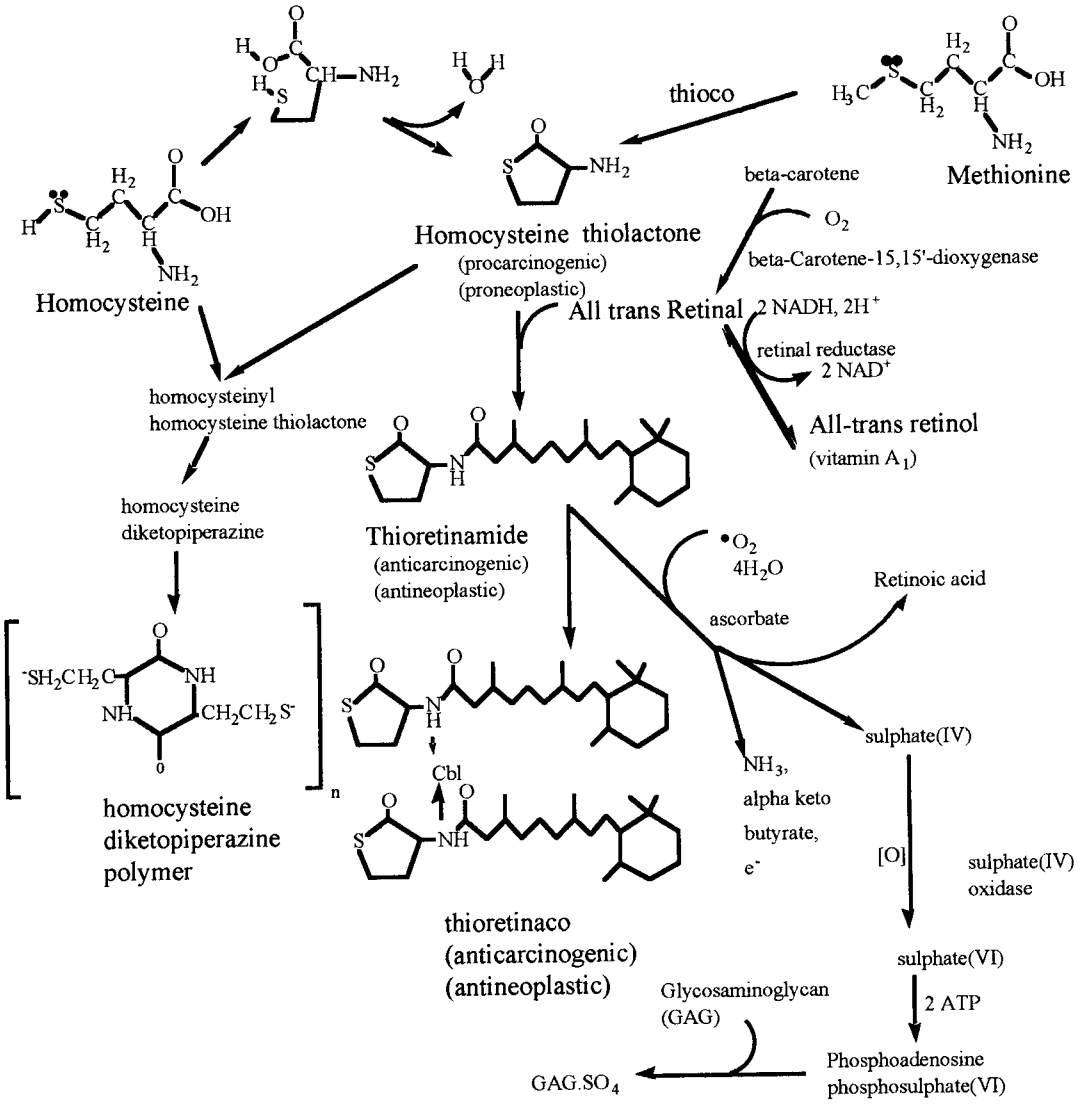


Figure 1.3.3 Homocysteine thiolactone
(modified McCully 1994)

dL-homocysteine thiolactone was found by Wall et al. (1980), to induce endothelial cell detachment in direct proportion to its concentration

1.3.4 Gender

De Laet et al. (1999) found no significant difference between boys and girls under fifteen years old, whereas when most boys and girls were post-pubescent, then girls tended to have a significantly lower plasma total homocysteine level than boys (refer to Table 1.3.6). De Laet's group did not categorise the subjects according to their puberty status (Tanner's scale {Marshall & Tanner, 1969 & 1970) which could mask the plasma total homocysteine differences. Women have lower levels of homocysteine even after other factors such as diets are accounted for, than males, (Nygård et al., 1995; Andersson et al., 1992).

1.3.5 Heredity

Higher levels of plasma total homocysteine were found in children with a powerful history of coronary heart disease (father, grandfather, or uncle), when compared to the offspring of healthy controls, (Tonstad et al., 1996; Greenlund et al., 1999, refer to Table 1.3.5). Whereas de Jong et al. (1997), found weak evidence for a relationship between hyperhomocysteinemia and arterial disease in the siblings of young patients with vascular disease and hyperhomocysteinemia. In childhood, moderate hyperhomocysteinemia may result from genetical, nutritional (Ueland et al., 1993), and iatrogenic factors (Ueland & Refsum, 1989), renal failure (Soria et al., 1990), human immunodeficiency virus (Müller et al., 1996), or cancer (Ueland & Refsum, 1989). De Laet et al. (1999), found that the frequency of reported cardiovascular disease in the family was higher in the child that had a total homocysteine concentration above the 95th percentile. Indicating a possible behavioural/dietary problem, namely low physical activity, high saturated fatty diet and limited fresh vegetables and fruit.

	Experimental group- Homocysteine geometric mean ($\mu mol.dm^{-3}$)	Control group- Homocysteine geometric mean ($\mu mol.dm^{-3}$)
white boys	6.8	5.7
white girls	6.9	5.6
black boys	6.4	5.5
black girls	6.6	5.4

Table 1.3.5 Comparison of Children aged 5 to 17 years old with (experimental) and without (control) parental coronary artery disease.

(Greenlund et al., 1999).

1.3.5i Methylenetetrahydrofolate Reductase

Methylenetetrahydrofolate Reductase is the enzyme complex that reduces N^5,N^{10} methylenetetrahydrofolate to N^5methylhydrofolate. A number of mutations have been identified that are associated with hyperhomocysteinemia (Goyatta et al., 1994; Frosst et al., 1995).

Heredity deficiency of methylenetetrahydrofolate reductase is the most widely studied inborn error of folate metabolism (Rosenblatt, 1995). Phenotypes of patients with severe deficiency of methylenetetrahydrofolate reductase (0-20% activity in fibroblasts) covers the gambit of symptoms from asymptomatic (Haworth et al., 1993) to developmental delay, motor and gait abnormalities, seizures and psychiatric disturbance (Rosenblatt, 1995). The homozygous mutation Threonine227Methionine produces 0-3% enzyme activity, another mutation is Arginine157Glutamine and a third mutation causes termination of the synthesizing protein. The alanine/valine gene polymorphism is significantly correlated to strokes with an odds ratio of 1.51 (95%CI 1.02 to 2.23) in the heterozygote and 3.35 in the homozygote. A milder deficiency of methylenetetrahydrofolate reductase (50% activity) has been described in patients with coronary artery disease (Kang et al., 1991).

Homozygosity for 677Cysteine-->Thymine was also associated with higher total homocysteine levels and tended to be more common in children whose parents had a history of cardiovascular disease than those who did not, (Tonstad et al., 1997). The change of 677Cysteine to Thymine alters the activity of methylenetetrahydrofolate reductase and therefore the production of methyltetrahydrofolate. The association between the homozygous mutation in the methylenetetrahydrofolate reductase gene (677Cysteine-->Thymine) and premature cardiovascular disease, produced an odds ratio of 3.1 (95%CI 1.0-9.2), (Kluijtmans et al., 1996).

In a European study, 35.5 % of working men, with total homocysteine levels in

the top decile had the ThymineThymine genotype, whereas in the population as a whole it was only 11.5%, (Harmon et al., 1996), whereas in the Hordaland study, practically everyone with total homocysteine levels of >20 $\mu mol.dm^{-3}$ had the ThymineThymine genotype, (Guttormsen et al., 1996). Supplementation of 1000 μg of folic acid produced the following reductions in total homocysteine levels: ThymineThymine genotypes - 21%; heterozygous genotype - 13%; CysteineCysteine genotype - 7%, (Harmon et al., 1996; Malinow et al., 1997). A possible explanation for the greater reduction in ThymineThymine homozygotes is that patients with that allele tend to have higher baseline values of total homocysteine. Woodside et al. (1998), found using a double blind randomised factorial-design controlled trial, that supplementation of 1000 μg folate, 7.2 mg B_6 and 20 μg B_{12} produced a reduction of approximately 27% in homocysteine levels that was dependent upon the methylenetetrahydrofolate reductase genotype. This implies that the individuals underlying pathophysiology of vascular disease should be assessed to see if there is a genetic variant of one of the enzymes or a nutritional shortage either due to a deficient diet or poor absorption. Further research is required to find the particular quantity of each vitamin (folate, B_{12} and B_6), choline and serine to be supplemented to produce the greatest reduction in total homocysteine level with the least potential risk for each particular pathophysiological variant. The homozygous mutation appears to have a protective effect against colorectal cancer, (Ma et al., 1997). Men with homozygous mutation that drank little or no alcohol had an eightfold decrease in risk of colorectal cancer in comparison to homozygous normal, (Ma et al., 1997).

1.3.5ii Cystathionine β-synthase

Fifteen Dutch homozygous cystathionine β-synthase deficient patients had the 833Thymine -->Cysteine (isoleucine278threonine) mutation in 50% of their alleles, however sixty cardiovascular patients did not test positively for the mutation and only one out of 111 controls showed heterozygosity for the mutation, (Kluijtmans et al., 1996). This implies that the heterozygosity for the mutation was not a necessity for premature cardiovascular disease.

1.3.6 Age

Plasma total cholesterol and homocysteine concentrations tend to increase with age, (Mann et al., 1988; Silberberg et al., 1997; de Laet et al., 1999; refer to Table 1.3.6). The diet changes with age, partly due to financial capacities, a lack of mobility and a lack of motivation to prepare, cook a meal for one and then wash up. There is a tendency for some elderly people to develop severe atrophy of the gastric mucosa (autoimmune chronic gastritis). They develop antibodies against gastric parietal cells (90%) and intrinsic factor (60%) which causes vitamin B_{12} deficiency, (Stevens & Lowe, 1995b) and therefore mild hyperhomocysteinemia.

1.4 Homocysteine and Cardiovascular Disease

1.4.1 The Homocysteine Hypothesis

Children and adults suffering from cystathionine β-synthase deficiency had very high levels of homocysteine (~100 $\mu mol.dm^{-3}$) and atheromas (McCully, 1969; Harker et al., 1974). This led to the development of the homocysteine hypothesis that there was an association between hyperhomocysteinemia and the pathogenesis of atherosclerosis, (McCully, 1983; McCully & Wilson 1975).

Many trials have shown that the ratio of plasma total homocysteine concentrations of patients suffering from a particular cardiovascular disease to controls was fairly constant, (Table 1.4.1). It is possible that the range of control plasma total homocysteine concentrations was due to sub-optimal folate levels. They did not have cardiovascular disease because the sum of their risk factors was below the threshold.

From the summarised data of 750 vascular patients and 200 controls up to 1994, 21% of patients with coronary artery disease, 24% of patients with cerebrovascular

plasma tHcy ($\mu mol.dm^{-3}$) Age group	Boys geometric mean(±1SD)	Girls geometric mean(±1SD)
5 - 9 years old	6.30 (5.18, 7.66)	6.11 (5.11, 7.30)
10 - 14 years old	7.12 (5.58, 9.01)	7.07 (5.81, 8.60)
15 - 19 years old	9.78 (6.70, 14.30)	8.33 (6.29, 11.02)

Table 1.3.6 Plasma total homocysteine concentrations in Belgian children, (de Laet et al., 1999).

Disease	control	Patients	Ratio	Reference
Coronary artery disease				
	8.50±2.80	10.96±3.44	1.3	Kang et al., 1986
	10.9±4.9	13.7±6.4	1.3	Genest et al., 1990
	13.7±4.8	18.1±7.5	1.3	Ubbink et al., 1991a
male			1.3	Graham et al., 1997
female			1.4	Graham et al., 1997
Familial coronary artery disease				
male	11.1±2.6	14.3±7.1	1.3	Williams et al., 1990
Coronary heart disease-				
male	11.3±3.7	13.1±4.3	1.2	Malinow et al., 1990
female	10.1±5.0	13.0±7.4	1.3	Malinow et al., 1990
(male%)	9.73(71.3)	11.16(84.1)	1.1	Graham et al., 1997
Myocardial infarction				
	13.5±3.6	16.4±6.9	1.2	Israelsson et al., 1988
Cerebral thrombosis				
	11.0±3.4	12.5±7.8	1.1	Brattström et al., 1988a
Cardiovascular disease				
(male%)	9.73(71.3)	11.11(53.1)	1.1	Graham et al., 1997
Cerebral infarction				
	7.3±2.9	13.1±5.6	1.8	Araki et al., 1989
	10.7±3.2	15.8±5.4	1.5	Coull et al., 1990
Peripheral occlusive artery				
	9.80±3.44	16.6±6.94	1.7	Malinow et al., 1989
Peripheral vascular disease				
(male%)	9.73(71.3)	11.67(70.5)	1.2	Graham et al., 1997
Aortoiliac disease				
	11.0±3.4	18.7±14.9	1.7	Brattström et al., 1988a

Table 1.4.1: Mean plasma total homocysteine levels (μmol.dm^{-3}) from people suffering from different types of cardiovascular disease, (Kang et al., 1992).

disease, 32% of patients with peripheral artery disease, and 2% of the control subjects had mild hyperhomocysteinemia post methionine load test, (Boers, 1994). The European Concerted Action Project tested 750 patients with documented vascular disease and 800 control subjects matched for age and sex, and found that their fasting homocysteine concentrations greater than 12.1μmol.dm^{-3} were associated with an elevated risk of vascular disease independent of all traditional risk factors, (Robinson et al., 1998).

1.4.2 Atherosclerosis

Athero is taken from the Greek word *athere* meaning soft, fatty or gruel-like, whereas scler- (skleros) means hard. Atherosclerosis involves an atheroma, which is a plaque of fatty deposits in the endothelial lining of the arteries (Tunica intima) that can impinge upon the media. Atheroma formation in the intima can encroach upon the lumen and interfere with blood flow. The later stages of atheroma formation, the liquid plaque disrupts the internal elastic lamina, (Stevens & Lowe, 1995a).

The Committee on Vascular Lesions of the Council on Arteriosclerosis (American Heart Association), have proposed three stages that are clinically silent and occur before the advance lesions (atheroma): initial, fatty streaks and intermediate lesions. The initial lesion (type I), is characterized by an increase in intimal macrophages and those filled with lipid droplets (foam cells). The fatty streak (type II), the first grossly visible lesion. It contains layers of foam cells and lipid droplets within the intimal smooth myocytes, also a few coarse-grained particles and heterogeneous droplets of extracellular lipid. The intermediate lesion (type III), are morphologically and biochemically between the fatty streak and advanced lesion, (Stary et al., 1994). Atheromas occur in high pressure large and medium sized arteries (aorta, coronary, carotid, mesenteric, iliac and femoral arteries and the cerebral arteries derived from both the vertebrobasilar and internal carotid arteries), rarely in arteries under 2mm diameters, (Stevens & Lowe, 1995a).

Investigations using autopsies have found fatty streaks in the aortas of foetuses and young children. There could be a decrease in lesion area before a steady increase with age in aortic arch and abdominal aorta (Napoli et al., 1999). The Bogalusa Heart Study looked at autopsies of 204 young people between the ages of two and thirty-nine. They found that the extent of fatty streaks and fibrous plaques in the aorta and coronary arteries increased with age, (Berenson et al., 1998). Napoli et al. (1997), found that in spontaneously aborted foetuses less than six months old, the foetal plasma cholesterol levels positively correlated with maternal levels. Foetal aortas from hypercholesterolemic or temporary hypercholesterolemic mothers had significantly more and larger lesions than from normocholesterolemic mothers. Further study (Napoli et al., 1999), revealed that the progression of the lesions was significantly faster in children from hypercholesterolemic mothers than children from normocholesterolemic mothers, ($p<0.0001$), ie the slope of the regression line drawn through the points of the area of the largest lesion divided by the section area plotted against age was steeper for children of hypercholesterolemic mothers. In the Pathobiological Determinants of Atherosclerosis in Youth study (1993), all the aortas and about half of the right coronary arteries in the 15 to 19 age group had atherosclerotic lesions, (left anterior descending and circumflex branches were used for different investigations). Steinberg (1988), postulated the linkage between the Lipid-Infiltration hypothesis and the endothelial-injury hypothesis, (Figure 1.4.2a).

Endothelial detachment induced by homocysteine was shown to be prevented by catalase, but not superoxide dismutase. This implied hydrogen peroxide was involved in the detachment, (Panganamala et al., 1986; Saez et al., 1982) because catalase catalyses the dismutation of hydrogen peroxide. Pathophysiological concentrations of homocysteine on human umbilical vein endothelial cells *in vitro* increased neutrophil-endothelial cell adhesion. The neutrophils were then able to migrate across the endothelial layer (Dudman et al., 1999), and activated neutrophils produce hydrogen peroxide that can be involved in desquamatization. The idea that homocysteine is involved with radicals to initiate atherosclerosis is high lighted by the use of vitamin C to

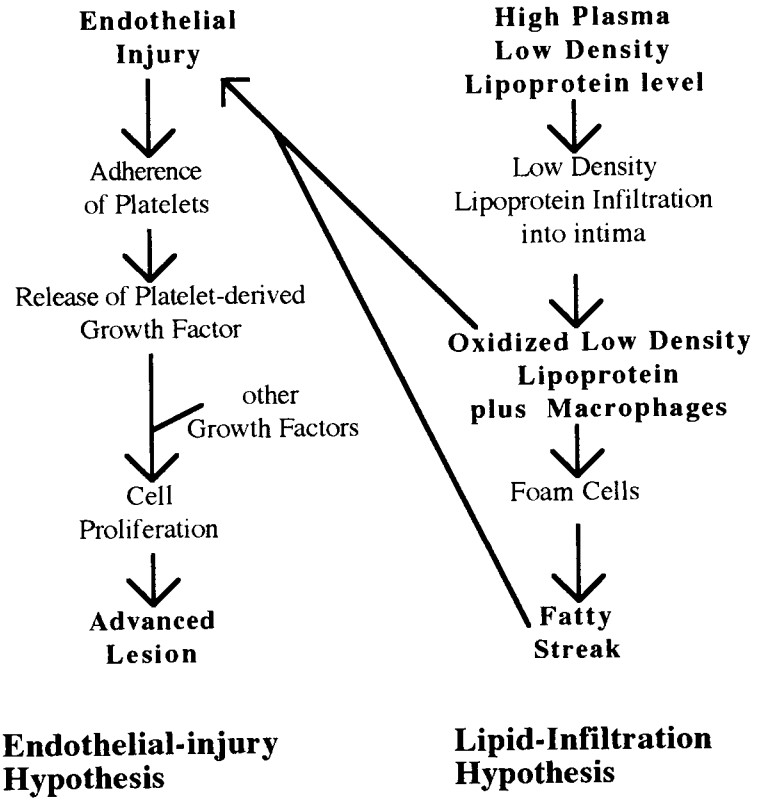


Figure 1.4.2a Postulated Atherosclerotic mechanism

(Steinberg, 1988)

reduce the effects of hyperhomocysteinemia upon impaired vascular response (Bonham et al., 1998; Chambers et al., 1999). Both groups showed that the homocysteine concentration was not affected by the addition of vitamin C, implying that homocysteine was not converted to thiolactone and reacting with vitamins A and C to produce sulphate(IV) (refer to Figure 1.3.3), but was reacting with radicals.

Glueck et al. (1995), found that 3.7% hyperlipidaemic patients had moderate Hyperhomocysteinemia and had a tendency for elevated cystathionine, methylmalonic acid and decreased levels of high density lipoprotein cholesterol. They had a higher incidence of atherosclerotic vascular events compared to those with normal homocysteine and cystathionine (72% & 44% respectively). They were more likely to have had at least 1 first-degree relative with an atherosclerotic event before the age of 65, implying a genetic or learnt risk factor, (78% & 50% respectively). Sutton-Tyrrell et al. (1997), showed that in a normotensive group (systolic blood pressure less than 160 mm of mercury) the higher homocysteine quintile was associated with a higher incidence of atherosclerosis. There was also an association between homocysteine and hypertension, but no association with atherosclerosis within their group.

Squamous endothelial cells release nitrogen(II)oxide and prostacyclin, which causes vasodilation and inhibits platelet aggregation, (Bunting et al., 1976; Moncada & Vane, 1979). Nitrogen(II)oxide nitrosates homocysteine (S-nitrosohomocysteine) rendering it nontoxic to the endothelium, but still inducing vasodilation and platelet inhibition, (Stamler & Loscalzo, 1992). Pathological concentrations of homocysteine (100 $\mu mol.dm^{-3}$) upon aortic rings *in vitro* with copper, caused a copper concentration-dependent inhibition of nitrogen(II)oxide-mediated endothelium-dependent relaxation, which was reduced in the presence of either or both, superoxide dismutase and catalase, (Emsley et al., 1999). Homocysteine increased the inducible nitrogen(II)oxide synthase III, which interleukin-1β had stimulated in vascular smooth myocytes, (Ikeda et al., 1999). Endothelium appears to have a mechanism for coping with moderately elevated levels of homocysteine by interleukin-1β stimulated/homocysteine enhanced

nitrogen(II)oxide synthesis. Increasing nitrogen(II)oxide production in the short term, so that the nitrogen(II)oxide can react with the increased homocysteine levels. In the presence of copper ions and low antioxidants, homocysteine reacts with the copper ions to produce the radicals, superoxide and hydrogen peroxide which could damage endothelium. The loss of homocysteine and nitrogen(II)oxide reduces their ability to cause vasodilation.

Homocysteine appears directly to injure endothelial cells *in vitro,* (Wall et al., 1981). Serum from familial hypercholesterolemia patients inhibited both endothelial and arterial smooth muscle cells migration, (Wall et al., 1981). Homocysteine has been shown on several occasions to cause desquamatization (deendothelialization), (baboons-Harker et al., 1976; rats-Hladovec, 1979). Homocysteine increases the uptake of low density lipoprotein by macrophages and monocytes, which get trapped in the intimal layer. Homocysteine (10-50$\mu mol.dm^{-3}$) inhibits deoxyribonucleic acid synthesis in vascular endothelial cells and has no effect upon vascular smooth muscle cells (Wang et al., 1997). This is because homocysteine increases S-adenosylhomocysteine in vascular endothelial cells, but not in vascular smooth muscle cells. Homocysteine (10-50 $\mu mol.dm^{-3}$) decreases carboxyl methylation of $p21^{ras}$ in vascular endothelial cells and there is an associated reduction in extracellular signal-regulated kinase 1/2 which modify's vascular endothelial cell growth, (Wang et al., 1997; Figure 1.4.2 b).

Two studies have shown no association between homocysteine and atherosclerosis, (Donahue et al., 1974; Smolin et al., 1983). Donahue's group tried to repeat McCully and Ragsdale (1970), work of inducing arteriosclerotic lesions by giving homocysteine with rabbit chow. They used weanling New Zealand white rabbits and found gross fibrous thickening in the ascending and arch of the aorta in three of the four experimental animals and two of the four control animals, whereas in McCully and Ragsdale (1970), all their experimental rabbits had aortic fibrous plaques (4/4) and just one of their controls (1/3). With such small numbers, there is little confidence in the result as the power of the test is likely to be low.

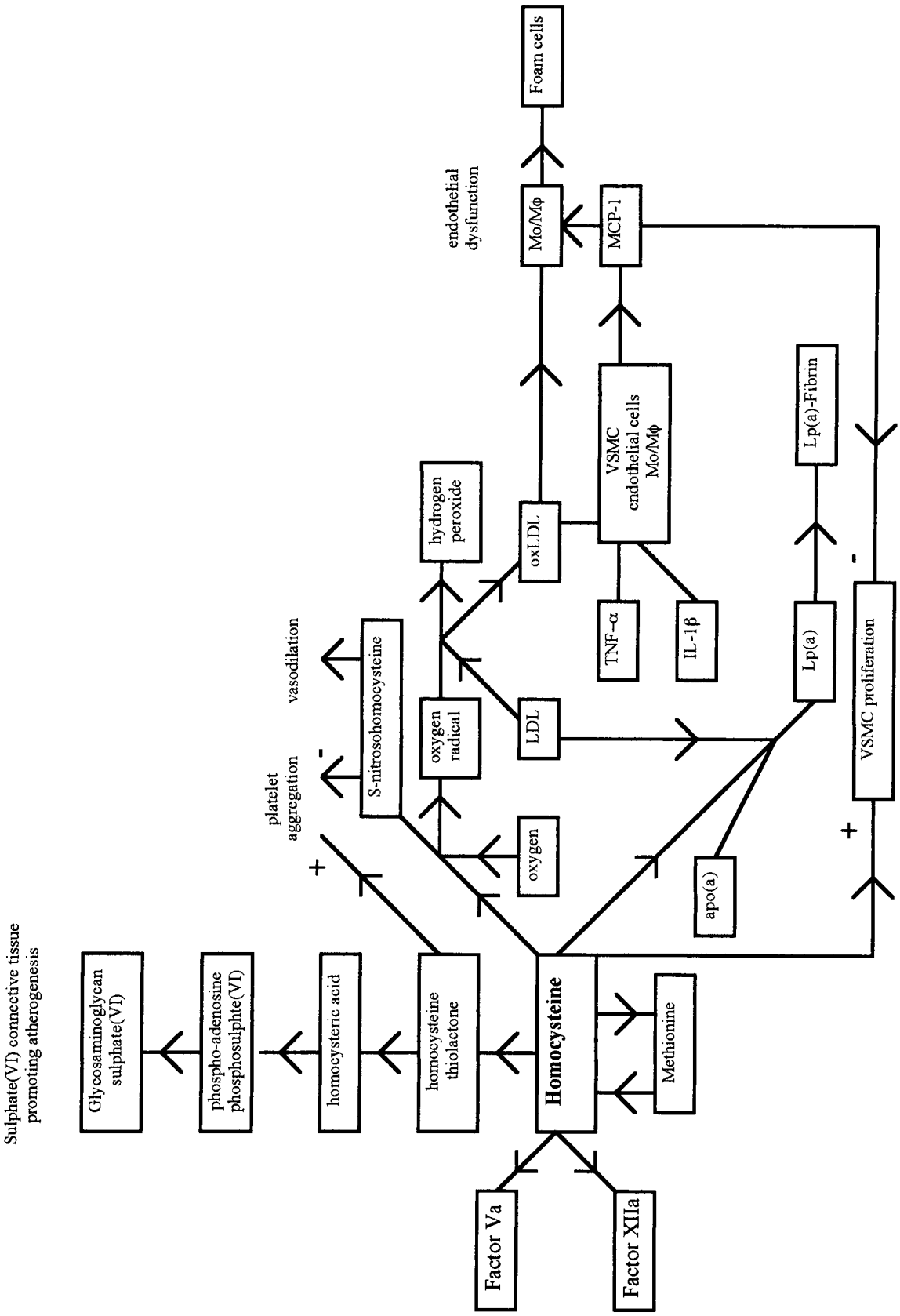


Figure 1.4.2b **Atherogenic pathways of homocysteine**
(modified Stamler & Slivka, 1997)

Key
LDL, low-density lipoprotein; oxLDL, oxidized LDL; Mo, monocytes; Mϕ, macrophages; apo(a), apolipoprotein (a); Lp(a), lipoprotein(a); MCP-1, monocyte chemoattractant protein-1; VSMC, vascular smooth muscle cells; TNF-α, Tumour necrosis factor-α; IL-1β, interleukin-1β.

1.4.3 Coronary Heart Disease

Malinow (1997), concluded from a review of the literature that most of the studies support the hypothesis that hyperhomocyst(e)inemia is a risk factor for coronary artery disease. Higher levels of mean plasma homocysteine levels were found associated with a greater degree of atherosclerosis, (Table 1.4.3). The patients and controls were age and gender matched, and they found no association between the presently accepted risk factors for coronary artery disease: smoking, hypercholesterolemia, diabetes and obesity, and higher levels of plasma homocysteine.

A prospective investigation was conducted into the relationship between plasma total homocysteine levels and mortality among 587 patients with angiographically confirmed coronary artery disease (at least 50% stenosis of any coronary vessel diameter). After four years, 3.8% of patients with homocysteine levels < 9μmol.dm^{-3} had died (overall mortality), compared with 24.7% of those with levels of ≥15μmol.dm^{-3}. 78% of the deaths were classified as due to cardiovascular causes, (Nygård et al., 1997). Ueland et al. (1992), summated the data from 1500 vascular patients and 1400 controls up to 1992. 10 to 24% of the patients with coronary artery disease, and 23 to 47% of patients with cerebrovascular or peripheral artery disease had fasting hyperhomocysteinemia, whereas only 7% of the control subjects had it. Landgren et al., (1995) found that plasma homocysteine levels post acute myocardial infarction increased over a period of six weeks from 13.1 ± 4.6 to 14.8 ± 4.8μmol.dm^{-3}. Folate was then administered for a further six weeks and this produced a decrease of 4.4μmol.dm^{-3} in the homocysteine level. These results show that the low homocysteine is associated with lower mortality risk post acute myocardial infarction and if folate is administered the homocysteine level will is reduced. There is no threshold, just a continuum of risk dependent upon homocysteine concentration, (Robinson & Loscalzo, 1998).

In the Framingham Study, elderly United State women (58.9%) and men (41.1%) with a mean age of 70±7 years had their nonfasting plasma total homocysteine levels

	Coronary patients (Coronary artery disease only) n=50	control	probability (p)
Mean plasma total Homocysteine ($\mu mol.dm^{-3}$)	11.7±0.7	9.9±0.5	0.03
	Coronary artery disease and symptomatic atherosclerosis in 2 or 3 arterial sites n=25		
	15.7±1.5	10.8±0.7	0.007
p (between the coronary patient	0.01		

Table 1.4.3 Mean plasma homocysteine concentration and level of cardiovascular disease. (Montalescot et al., 1997).

compared to mortality. The upper quartile ($\geq$14.26μmol.dm^{-3}) vs the lower three quartiles (<14.26μmol.dm^{-3}) were associated with relative risk estimates, (adjusted for several other risk factors): 1.54 for all-cause mortality; 1.52 for cardiovascular disease mortality, (Bostom et al., 1999). There are several studies that have not shown any association between homocysteine and cardiovascular disease. After adjustment for other risk factors the Multiple Risk Factor Intervention Trial showed no association between total homocysteine and cardiovascular disease (relative risk 0.94; confidence interval, 0.56 to 1.56, Evans et al., 1997). The Atherosclerosis Risk in Communities Study also showed no association between total homocysteine and the incidence of cardiovascular disease, (Folsom et al., 1998). Therefore, the association between homocysteine and cardiovascular disease has not been shown unequivocally. In my opinion this shows the problem of trying to adjust for a multitude of risks that in some case are synergistic.

In patients that had unstable angina or myocardial infarction there was a threshold for total plasma homocysteine. Patients with total plasma homocysteine concentration greater than 12.2μmol.dm^{-3} had a 2.6-fold increase in risk of a cardiac event, (Stubbs et al., 2000). Robinson et al. (1998) found that fasting total plasma homocysteine concentrations greater than 12.1μmol.dm^{-3} was associated with an elevated risk of vascular disease. Graham et al. (1997) found that the geometric mean of total plasma homocysteine concentration for cardiovascular disease was 11.25μmol.dm^{-3}. Omenn et al. (1998) believe that total plasma homocysteine concentration should be down to the range of 9 to 10μmol.dm^{-3}.

1.4.4 Peripheral Vascular Disease

Mölgaard et al. (1992) showed that patients with intermittent claudication only had significantly higher levels of total plasma homocysteine than the controls when their serum folate levels were less than 11.0nmol.dm^{-3}, (normal serum folate level 7.0 to 28.1 nmol.dm^{-3}). Serum folate levels above 11.0 nmol.dm^{-3}, there was no significant

difference between the patients' and controls' total plasma homocysteine. Boers et al., (1985) investigated 75 patients, that were under 50 and had clinical signs of ischemic disease. They found that 28% (7/25) of patients with occlusive cerebrovascular disease and 28% (7/25) with occlusive peripheral artery disease and none (0/25) that had myocardial infarction had post methionine load hyperhomocysteinemia (serum homocysteine concentration above the mean+2 standard deviations of the control group) and had low cystathionine synthase activity (they were heterozygous for cystathionine synthase). This implies that 28% of patients with premature vascular disease are heterozygous for cystathionine β-synthase.

1.4.5 Thrombosis

Thrombosis is the process of blood clot formation (thrombus) in the body's circulatory system. Endothelial cell damage can activate the extrinsic and intrinsic coagulation pathways, (Aoki et al., 1998, Figure 1.4.5). Hydrogen peroxide has been indicated as the mediator of homocysteine-induced endothelial damage in vitro because of the effect of catalase, (Wall et al., 1980; Starkebaum & Harlan, 1986). The exposure of tissue factor in the plasma membranes of subendothelial cells like fibroblasts and other cells in the walls of blood vessels, allows the binding of plasma protein factor VII triggering the extrinsic coagulation pathway, (Higashi & Iwanaga, 1998).

The plasma total homocysteine concentrations found in homocystinuria patients (100 to 300μmol.dm^{-3}) were sufficient to cause an increase in tissue factor messenger ribonucleic acid in endothelial cells, (Fryer et al., 1993). Homocysteine has been shown to activate the Hageman factor (factor XII - Ratnoff, 1968) and factor V in arterial endothelial cells, (Rodgers & Kane, 1986).

Elevated levels of plasma activated protein C and the ratio of activated protein C/protein C were found to be significantly higher in venous thromboembolism patients (VTE) and they also tended to have hyperhomocysteinemia (HHcy), (VTE-HHcy

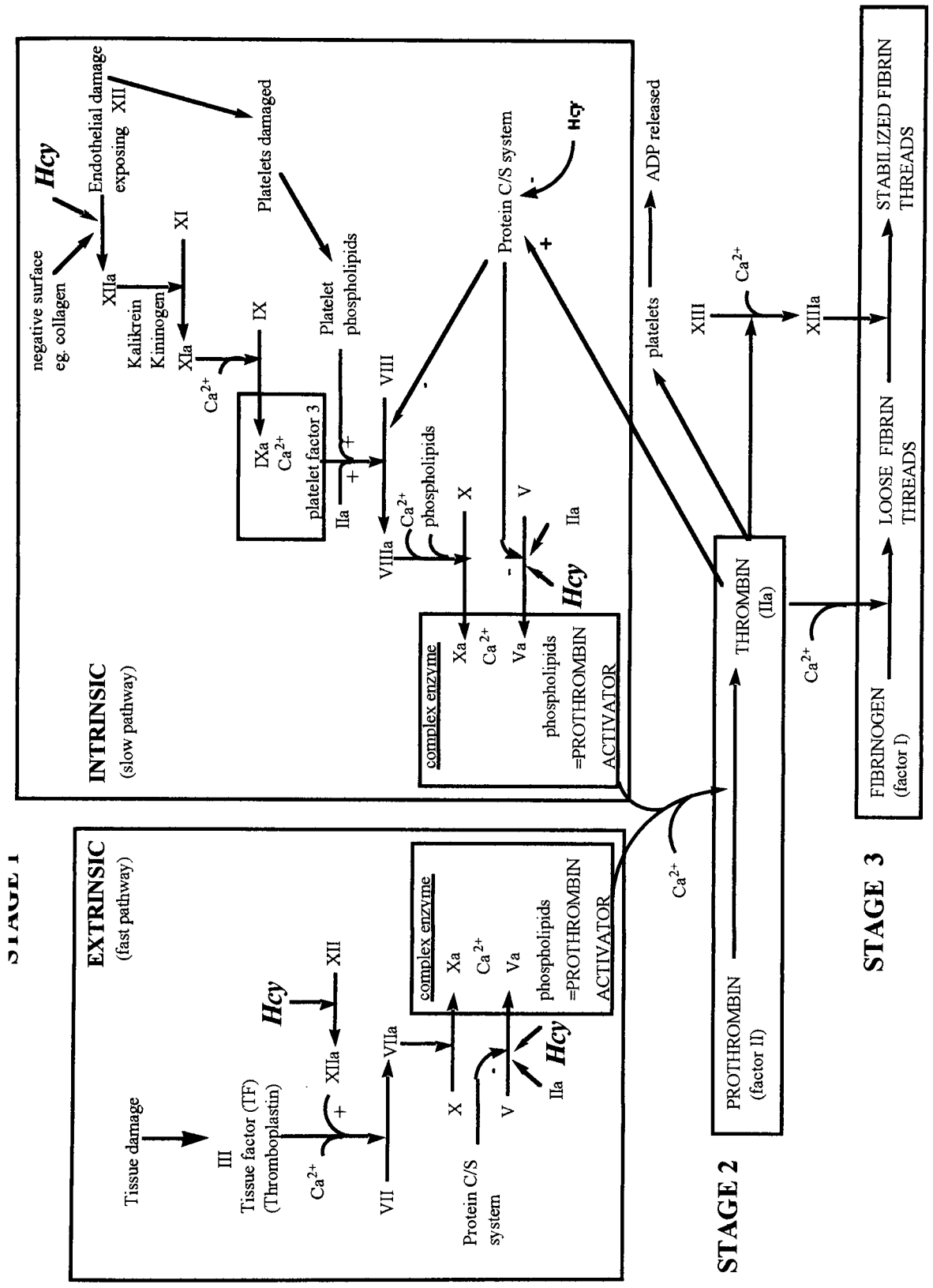


Figure 1.4.5 The Coagulation pathways
(modified from Tortora & Anagnostakos 1990)

Key:
Hcy - homocysteine;
Ia - XIIIa - activated blood factors;
I - XIII - blood factors;
Ca^{2+} - calcium.

28.8±19.5μm.dm-3; VTE-NormalHcy 12.0±5.2μm.dm^{-3} & control 11.0±5.3μm.dm^{-3} of homocysteine. Cattaneo et al., 1998). Yet, there was no correlation between fasting plasma levels of activated protein C and the ratio of activated protein C/protein C with total homocyst(e)ine. Homocysteine has been shown to down-regulate protein C activation, (Rodgers & Conn, 1990). In my opinion this shows that plasma total homocysteine is not the only factor that affects activation of protein C

Olszewski & McCully, (1993) suggests that lipoprotein (a) is synthesized in the liver by the action of homocysteine on low density lipoprotein (LDL) and the formation of homocysteinyl low density lipoprotein which forms disulphide bridges with apolipoprotein (a) (apo(a)) (high concentrations) to form lipoprotein (a) [LDL-$NHCOCH(NH_2)CH_2CH_2SS$-apo(a)]. Harpel et al., (1992) found that homocysteine concentrations as low as 8μmol.dm^{-3} significantly increases the affinity of lipoprotein (a) for fibrin.

1.4.6 Fibrinolysis

The fibrinolytic system (Figure 1.4.6), digests the clot and there is normally a balance between the fibrinolytic and coagulating systems. The effect of homocysteine upon tissue-plasminogen activator is equivocal. Bienvenu et al. (1993), found that plasma total homocysteine concentrations were significantly correlated to tissue-plasminogen activator concentrations in vivo ($p<0.001$) in patients with deep-venous or arterial thrombosis, whereas Kottke-Merchant et al. (1990) found that homocysteine or homocysteine thiolactone, had no effect on tissue-plasminogen activator production *in vitro.*

1.4.7 Venous thromboembolism

Venous thromboembolism appears to account for 50% of vascular complications of homocystinuria (total plasma homocysteine > 0.1mmol.dm^{-3}, Hirsh et al., 1994).

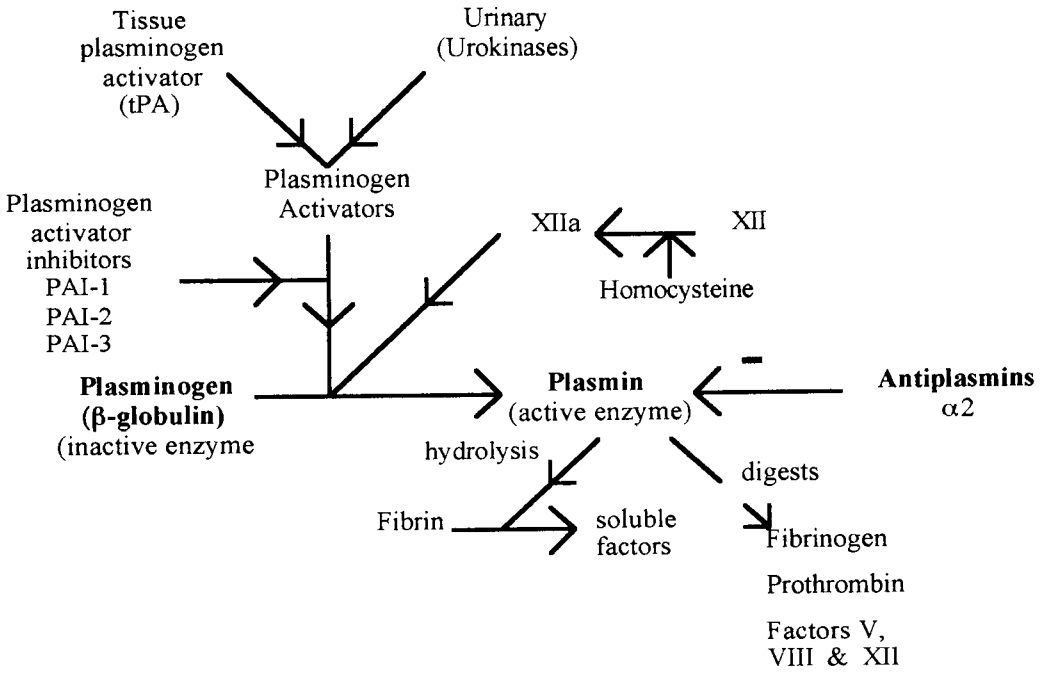


Figure 1.4.6 **Fibrinolysis**
(modified from Verstraete & Vermylen 1984)

Although, there appears to be no relationship between mean homocysteine and thromboembolism, at least twice as many patients compared to controls have hyperhomocysteinemia (Table 1.4.7). The variability illustrates the multifactorial nature of the disease, but also the non standardization of hyperhomocysteinemia.

1.5 Homocysteine and its interaction with cardiovascular risk factors

1.5.1 Cholesterol

A review by Boushey et al. (1995), concluded that every 5μmol.dm^{-3} increase in plasma total homocysteine, conferred the same increase in risk for coronary artery disease as 0.5mmol.dm^{-3} (19 mg.dL^{-1}) increase in total cholesterol.

Elevated levels of cholesterol, low density lipoprotein and homocysteine with decreased levels of high density lipoprotein, folate and vitamin B_{12} are associated with increased risk of coronary atherosclerosis, cerebrovascular disease, peripheral vascular disease and thrombosis, (Ueland & Refsum, 1989; Boers et al., 1985). A study by Olszewski et al. (1989), found a correlation between plasma homocysteine and serum total cholesterol in 52 males aged 30 to 60 years old. They obtained a reduction in plasma homocysteine and serum total cholesterol levels when treating the patients with vitamins B_6, folate, B_{12}, B_2, choline and troxerutin.

Zulli et al. (1998), found a synergistic effect of homocysteine and cholesterol feeding upon the plasma total homocysteine, serum total cholesterol and triacylglycerol levels. When 4mmol.dm^{-3} (over 200 times the mild hyperhomocysteinemic level) was used to incubate human hepatoma cell line HepG2, there were significant increases in the production and secretion of cholesterol and the secretion of apolipoprotein B-100. Intracellular 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity was also increased, (Karmin et al., 1998). Thus implying that apart from a poor diet for

Authors	Thrombosis type	Age	Homocysteine measured	Homocysteine (μmol.dm^{-3})		Hyperhomocysteinemia cases		
				Patients (n)	Controls (n)	Patients %	Controls %	OR (95% CI)
Brattstrom et al., (1991)	Venous	<50	Fasting PML (Δ)* F+PML	13.4 (29)# 23.2# (42)##	10.6 (30) 20.4 (42)	14	5	3.1 (0.4, 27.7)
Amundsen et al., (1995)	Deep Vein	<56	Fasting PML (T)*	10.3±3.5 (35) 39.5±14.2	10.3±2.6 (39) 37.2±14.2	5.7 5.7	2.6 2.6	2.3 (0.1, 68.5) 2.3 (0.1, 68.5)
Bienvenu et al., (1993)	Venous + arterial	<58	Fasting	13.8±6.0 (50)	8.1±2.2 (49)	36	4	13.2 (2.82, 83.0)
Falcon et al., (1994)	Juvenile venous	<40	Fasting PML(Δ)*	7.4±5.5 (80) 10.3±5.1 (79)	7.9±2.1 (51) 8.3±3.4 (40)	8.8 18.8	0 2.5	4 (1.16, 4) 9.1 (1.38, 194)
Fermo et al., (1995)	Venous	<45	f+PML(T)*	N/A (107)	N/A (60)	13.1	5	2.9 (0.79, 12.1)
den Heijer et al., (1995)	Recurrent	67P 51C	Fasting	N/A (185)	N/A (220)	25	10	2.0 (1.5, 2.7)
den Heijer et al., (1996)	Deep vein	44	Fasting	12.9 (269)	12.3 (269)	10	5	2.5 (1.2, 5.2)
Cattaneo et al., (1996)	Deep vein	N/A	F+PML(T)	N/A (89)	N/A (89)	13.5	6.7	2.2 (0.8, 6.0)
Simioni et al., (1996)	Deep vein	62	Fasting	15 (60)	12 (148)	25	11	2.6 (1.1, 5.9)
Petri et al., §(1996)	Atherothrom-botic lupus	38	Fasting	10.26 (337)	7.41	15	10	3.49 (0.97, 12.54)

Table 1.4.7 Relationship between plasma homocysteine and thrombosis
(Selhub & D'Angelo, 1997)

Key:
males only;
males & females;
(Δ)* : net increase in homocysteine after methionine load, ie fasting levels substracted from total;
(T)* : total plasma homocysteine level after methionine load (fasting homocysteine level was not subtracted).
(T) : total plasma homocysteine level
§ prospective study;
P patients
C controls
N/A data not available
OR odds ratio
CI confidence interval
PML post methionine load
F fasting

the association between elevated plasma total homocysteine levels and serum total cholesterol, it could be that homocysteine increased the activity of hepatic intracellular HMG-CoA reductase and thus the synthesis of cholesterol.

1.5.2 Triacylglycerol

A study of peripheral arterial occlusive disease patients reported a significantly higher mean concentration of homocysteine than the control subjects. The patients also had significantly lower levels of high density lipoprotein cholesterol (HDL_C), and the subgroup $HDL_{3\,C}$ and the apolipoprotein A-1, whereas their triacylglycerol was elevated, (Rassoul et al., 2000).

1.5.3 Low Density Lipoprotein

Homocysteine binds to lipoproteins: low density lipoprotein, very low density lipoproteins and high density lipoprotein by peptide bonds (Olszewski & McCully, 1991). The 'free' amino groups of apolipoprotein B in low density lipoprotein are thiolated by homocysteine thiolactone (Vidal et al., 1986). This causes aggregation and increased uptake of low density lipoprotein by macrophages (Naruszewicz et al., 1994), and the formation of foam cells.

The highest concentration of thiolated proteins was present in the low density lipoprotein fraction and 4.1 times higher in hypercholesterolaemic to normolipaemic group. The atherogenic index for homocysteine, low density $lipoprotein_{homocysteine}$/high density $lipoprotein_{homocysteine}$ is 3.5 times higher in the hypercholesterolaemic than the normolipaemic group, (Olszewski & McCully, 1991).

Lipid peroxidation reduces membrane fluidity, permeability and excitability, (Vladimirov 1987, Poli et al., 1987). Low levels of homocysteine can oxidize low density lipoprotein in the presence of copper(II) ions, (6 and 25 $\mu mol.dm^{-3}$) and act as an

antioxidant in high concentrations as found in homocystinuria patients (100, 250 and 500 $\mu mol.dm^{-3}$), (Halvorsen et al., 1996). This implies that with the exception of homocystinuria patients, normal homocysteine levels are in the range that would produce oxidized low density lipoprotein in the presence of transition metals.

Raal et al. (1999), have shown using multivariate analysis, that for familial hypercholesterolemia subjects, homocysteine was not a predictive factor for carotid intima media thickness but low density lipoprotein cholesterol and cholesterol-years (appendix 1) score are.

1.5.4 Lipoprotein(a)

Olszewski and McCully (1993), state that thiolation of low density lipoprotein results in the formation of a peptide bond between the N-terminal end of apolipoprotein B-100. The sulphydryl group of the bonded homocysteine is free to form a disulphide bond with apolipoprotein (a). The apolipoprotein (a) molecule bonded this way, could explain how some low density lipoprotein appears to have two apolipoprotein (a) molecules per apolipoprotein B-100.

1.5.5 Radicals

Radicals are unstable, hydrogen peroxide formed in the mitochondria could oxidize homocysteine forming disulphides with membrane bound proteins, thereby altering their function. The sulphydryl group of homocysteine acts catalytically with Iron(III) and Copper(II) ions in a mixed function oxidation system to generate hydrogen peroxide, oxygen radicals and homocysteinyl radicals within cultured endothelial cells, (Starkebaum & Harlan, 1986). Two to five percent of the oxygen consumed might be reduced to the superoxide anion radical (Boveris and Chance 1973). The superoxide radical in the presence of the copper(II) ion modified low density lipoprotein which was taken up more readily by mouse peritoneal macrophages and degraded, than native low

density lipoproteins, (Heinecke et al., 1987).

1.5.6 Blood Pressure

Plasma total homocysteine is positively related to blood pressure, (Nygård et al., 1995).

1.5.7 Smoking

Plasma total homocysteine is also positively related to the number of cigarettes smoked daily, (Nygård et al., 1995). Targher et al. (2000), showed that plasma total homocysteine was significantly elevated in type 1 diabetic patients compared to nondiabetic subjects and that diabetic smokers had significantly elevated plasma total homocysteine verses nonsmokers. This could be due to their diet and lifestyle.

1.5.8 Psychosocial Factors

Stoney (1999), gave pre and postmenopausal women some psychological stressors (mental arithmetic and speech stressors) and measured plasma total homocysteine. She found that the plasma total homocysteine had significantly increased by the end of the ten minute acute psychological stress similarly in both groups, but only by 0.4 $\mu mol.dm^{-3}$, ($p = 0.002$). She found no effects of menopausal status or endogenous oestrogens on plasma total homocysteine. As there was an associated increase in heart rate and blood pressure, she suggests that the elevation of homocysteine could have been mediated sympathetically. So does the sympathetically generated influx of sodium ions, cause an egress of homocysteine as the sodium ions are returned extracellularly from smooth muscle cells? (ASC transport system - section 1.3.2), If this change in homocysteine is an actual change then it calls into question all the papers on homocysteine. If there was an elevation in homocysteine, was this due to the actual or perceived stress or was it the patients' actual baseline? This is not to deny the effects

of homocysteine upon endothelial cells, myocytes, lipids or nitrogen(II)oxide, what it implies is maybe that homocysteine is not continually high in the plasma, but fluctuates depending upon the persons' ability to deal with stress. This suggests that there are two groups of people:

- A - those with temporarily elevated level, because they were under a stressful situation, eg exercise.
- B - those with high plasma total homocysteine levels due to genetic mutation and/or vitamin deficiency.

1.6 The Effect of Diet

The British Heart Foundation (2000e) estimates that about 30% of deaths from coronary heart disease are due to unhealthy diets. The socioeconomic difference is quite marked in the consumption of fresh fruit and vegetables. The volume of leafy salads and fruit juice consumed is three times as great in the richest 10% of households compared to the poorest. Five percent of coronary heart disease is estimated to be due to obesity (Body Mass Index greater than 30 $kg.m^{-2}$).

The serum levels of several vitamins have been shown to effect the level of homocysteine (Woodside et al., 1998; Selhub et al., 1993). Both folate and B_{12} are required in the conversion of homocysteine to methionine and B_6 in the conversion of homocysteine to cysteine. Vitamin A in the conversion of homocysteine thiolactone to thioretinamide and ascorbic acid in the conversion of thioretinamide to sulphate(IV). Mildly elevated homocysteine can be caused by deficiencies in folic acid and/or vitamin B_{12} and/or vitamin B_6.

Malinow et al. (1997), found a negative correlation between total homocysteine and basal levels of folate, pyridoxal-5′-phosphate(V) (a form of vitamin B_6) and vitamin

B_{12}. Total homocysteine increases post puberty and is inversely related to parental educational level. It is also inversely related to intakes of folate, vitamin C, fruits and vegetables, a similar finding was found for serum folate and vitamin B_{12}.

The European Concerted Action Project study, found that red blood cell folate concentration below the lowest tenth percentile (<513nmol.dm^{-3}), and vitamin B_6 concentrations below the twentieth percentile (<23.3nmol.dm^{-3}) for control subjects were associated with increased risk of cardiovascular disease, which was independent of conventional risk factors. The risk factor for folate could be partially explained by increased homocysteine, whereas B_6 and the atherosclerosis relationship were independent of the homocysteine level, (Robinson et al., 1998). Vitamin B_6 deficiency would effect not just the trans-sulphuration of homocysteine, but it would also effect niacin production. Niacin is essential for almost all neurotransmitter synthesis or metabolism and in the synthesis of many hormones, such as insulin and growth hormone. A shortage of niacin can effect the circulation by increasing blood pressure and cholesterol.

Graham et al. (1997), found that patients using vitamin preparations (folate, vitamins B_{12} & B_6) experienced protection from vascular disease with a relative risk of 0.38 (95% CI, 0.2-0.72) compared with the non users of vitamin supplements. However, they stated that it was a small sample group and that the vitamin users may have been more health conscious than the non users. A reduction in homocysteine occurred when patients on haemodialysis took a standard multivitamin supplement containing B vitamins and 1mg of folate, (House & Donnelly, 1999).

A meta-analysis of the trials of vitamins (folate, B_{12} & B_6) upon plasma total homocysteine concentrations (Homocysteine Lowering Trialist's Collaboration, 1998) concluded that supplementation of folate reduced plasma total homocysteine concentrations, which was particularly noticeable in the highest quintile of homocysteine

(>18.5μmol.dm^{-3}) and the lowest quintile of serum folate (<6.9nmol.dm^{-3}). According to Clarke & Armitage (2000), there are a number of large-scale clinical trials that are ongoing or planned around the world. They are designed to answer the question of the importance of the vitamins separately or together upon the cardiovascular risk factors.

Verhoef et al. (1996) found a negative correlation between plasma total homocysteine and plasma folate and also vitamin B_{12}. Whereas, Quinlivan et al., (2002) found that the correlation between plasma total homocysteine and a high folate intake was poor, but the vitamin B_{12} showed a greater negative correlation.

1.6.1 Folate

One-carbon tetrahydropteroylglutamate (folates) are involved in many biochemical pathways that are necessary for growth, development and the homeostasis of humans. Unfortunately, humans are unable to synthesize folates *de novo*. A deficiency of this vitamin is associated with neural tube defects and cardiovascular disease whereas folate supplementation could affect zinc absorption. Iron deficiency can mask folate deficiency.

The folate stays in the red blood cell throughout the cells life of between 100 and 120 days, (Berlin et al., 1959). Red blood cell folate could be a reliable indicator of folate intake over time. Smoothing the troughs and peaks that could have occurred, as people tend not to eat the same foods every day, but on weekly and seasonal cycles.

Jacob et al. (1994), found that in ten healthy adult men, their plasma total homocysteine levels rose during folate depletion. In fact the response of homocysteine to changes in folate intake varied among individuals from very strong to absent. This illustrates people's genetic differences, which is their differences in folate-binding proteins in the enterocyte membranes, serum and their other tissues.

Folate deficiency can occur because of :

a) decreased dietary intake - alcoholics and the elderly who have inadequate or improper diets;

b) decreased intestinal absorption - as in coeliac disease or tropical sprue;

c) increased requirement - multiple pregnancies, lactation, haemolytic anaemia and in leukaemia;

Exercise causes an increase in plasma adrenaline (Kjaer & Dela 1996), which was synthesised from the methylation of noradrenaline. Running increases plasma total homocysteine (Weiß et al 1999, refer to section 1.7.3). Is there, therefore an increased need for folate?

d) effects of drugs - eg diphenylhydantoin (used in its sodium salt form, in the treatment of grand mal epilepsy);

e) alcoholism - chronic exposure to ethanol decreases folate transport into enterocytes, reduces hepatic uptake and increases the excretion of folic acid by the kidney, (as mentioned in a review by Halsted, 1990).

The United Kingdom set its reference nutrient intake at $200\mu g.dy^{-1}$ which is two standard deviations above estimated average requirement, ($150\mu g.dy^{-1}$).

Brattström et al. (1988b), found that enhanced folate intake reduced plasma total homocysteine concentration, since which, many other groups have also found that folate supplementation reduces plasma total homocysteine concentration (Riddell et al., 2000; Jacques et al., 1999; den Heijer et al., 1998; Malinow et al., 1997, 1998; Shimakawa et al., 1997; Ward et al., 1997; Landgren et al., 1995). Lewis et al. (1992), reported that subjects with a plasma folate $>15nmol.dm^{-3}$ had a low, plateau homocysteine level.

Malinow et al. (1998), fortified breakfast cereals with the recommended dietary allowances of vitamins B_6 (1.8 ± 0.1mg per 30g of cereal) and B_{12} (6.1 ± 0.3mg per 30g of cereal) and altered the level of folic acid. Seventy-five men and women with coronary artery disease ate breakfast cereals containing either zero (placebo), 127 ± 11, 499 ± 6, or

665±48μg of folic acid per 30g of cereal which approximately produced daily folic acid intakes of either 110-140μg.day^{-1}, three times the recommended dietary allowance and four times the recommended dietary allowance respectively. After taking the folate supplement their plasma folate increased by 30.8%, 64.8% and 105.7% respectively, and their plasma total homocysteine levels decreased by 3.7%, 11.0% and 14.0% respectively. Omenn et al. (1998), in their editorial stated that a supplement containing 400μg of folic acid would be expected to produce on average a total homocysteine reduction of 5μmol.dm^{-3} in non-supplement users and the serum folate should reach ≥ 15nmol.dm^{-3}, which was based on Boushey et al. (1995). Yet they recommend that folate should never be given on its own, but with vitamin B_{12}, because it could mask anaemia. So they recommend 200-1000μg of cobalamin (B_{12}) with the 400μg of folic acid supplement daily. The United States of America have now legalized the supplementation of certain foods with folate. Yet oral folate supplementation may reduce zinc absorption (Simmer et al., 1987).

The homocysteine lowering trialists' collaboration (1998), used meta-analysis on 12 trials (1114 people) and found that for Western populations, supplementation with folic acid of at least 0.5mg.day^{-1} and vitamin B_{12} about 0.5mg.day^{-1} would reduce blood homocysteine by about a quarter to a third, but they did not take into account variants in enzyme genotypes. Ward et al. (1997), tested 30 men aged between 34 and 65 (mean 45) years and divided them into three groups, based on their baseline total homocysteine level, (10.9±0.83; 9.11±0.49; 7.07±0.84). They were given 100, 200 and 400μg.day^{-1} of folic acid during the intervention period. Even though all tertiles showed reduced total homocysteine and increased serum folate levels, 400μg.day^{-1} did not produce a significant reduction in total homocysteine from that produced by 200μg.day^{-1} and only in those subjects that were in the top two tertiles. Thus, implying a threshold of plasma homocysteine in its ability to respond to folic acid. This variable response to folate intake as previously stated is most likely related to the folate binding proteins and therefore there is a genetical feature. Work by Mitchell et al. (1997), using twins found that 46% variance in red blood cell folate was attributed to additive genetic effects.

A study where hyperhomocysteinemic subjects were given folic acid for one year showed a decline in plasma total homocysteine concentrations and an improvement in flow-mediated dilation (Woo et al., 2002). Yet to just reduce the plasma total homocysteine levels and not the cholesterol levels as well appears not to reduce the cardiovascular risk. The effect of feeding an atherogenic diet for seventeen months with or without vitamin supplements (folic acid and vitamins B12 and 6) to 16 cynomolgus monkeys (*Macaca fascicularis*) with hypercholesterolaemia, showed that vitamin supplementation reduced the plasma homocysteine levels, but failed to prevent Tunica intimal thickening in the carotid or iliac arteries (Lentz et al., 2001). This could be interpreted that once the damage as occurred, initiated by hyperhomocysteine, to remove that risk, but leave hypercholesterolaemia, still poses a risk, but reduces the rate of progression. As the elevated cholesterol levels are associated with elevated low density lipoproteins which can become oxidised and form plaques.

Landgren et al. (1995), found that plasma Homocysteine levels post acute myocardial infarction increased over a period of six weeks from 13.1±4.6 to 14.8±4.8 $\mu mol.dm^{-3}$ ($p=0.001$). Folate was then administered for a further six weeks and this produced a decrease of $4.4 \mu mol.dm^{-3}$ in the homocysteine level.

1.6.1i Glutamine-Glutamate Cycle

Glutamate is important in the rate of reaction of folates and in preventing the cellular egress of N^5-methyltetrahydrofolate. Folate is only kept within the cell if bonded to at least three molecules of glutamate. Glutamate taken up by muscle is transported by System-X^-_{ac}, which in rats has a high affinity, low capacity and is sodium-independent, but H^+-dependent. It maintains a concentration gradient greater than 50-fold between muscle and blood, (Bergström et al., 1985).

Rohde et al. (1996), found that two hours after a long course triathlon (2.5km swim, 81km cycle and 19km run), the athletes had a significant decrease of nearly 50% in

plasma glutamine concentration. Lehmann et al. (1995), found that after the 1993 Colmar ultra-triathlon that lasted over 23 hours, serum glutamine concentration was unchanged, even though there was a significant 18% decrease in total amino acid levels. Neither paper appeared to consider any plasma volume shift that would have occurred due to sweating. So the variation in the effect of exercise duration, and intensity upon plasma glutamine concentration could be due at least partly by plasma volume shift. Yet if exercise causes a decrease in plasma glutamine concentration, this could cause a loss of glutamine from the cells and consequently might cause a loss of folate, which would result in an increase in cellular homocysteine and then its equilibration by egression to the plasma.

1.6.2 Vitamin B_{12}

Vitamin B_{12} is water soluble and found in liver, beef, pork, eggs milk, cheese and kidney, it is not recommended to be taken with alcohol because alcohol is believed to interact with the ileal B_{12}-intrinsic factor receptor (Halsted & McIntyre, 1972).

Changes in the oxidation state of the central cobalt ion affect the strength of the sigma bond between the cobalt and the R-group. The principle R groups are 5'-deoxyadenosyl (5'-deoxyadenosylcobalmin), $-CH_3$ (methylcobalamin) and -OH (hydroxocobalamin; Scott 2000). Methylcobalamin is the vitamin B_{12} coenzyme for methionine synthetase (homocysteine —> methionine), whereas 5'-deoxyadenosylcobalamin is the coenzyme for the mitochondrial methylmalonyl-CoA mutase, (methylmalonyl CoA —> succinyl-CoA).

Methylcob(III)alamin enzyme reacts with 5-methyltetrahydrofolate and homocysteine, the ternary complex forms cob(I)alamin enzyme. It then releases methionine and tetrahydrofolate reforming methylcob(III)alamin, (Banerjee et al., 1990). Occasionally the cob(I)alamin enzyme is oxidized to the inactive form cob(II)alamin

which is then reduced by flavodoxin and methylated by S-Adenosylmethionine (refer to Figures 1.3.1), thus S-Adenosylmethionine acts as cosubstrate.

Approximately 70% of the dietary vitamin B_{12} (physiological levels) is absorbed, but decreases to less than 10% at levels five times greater than the recommended dietary allowance (Herbert, 1987). Cooper & Rosenblatt (1987), demonstrated that at least 95% of vitamin B_{12} was bound to either cytoplasmic methionine synthase or to mitochondrial methylmalonyl-CoA mutase. The body pool of B_{12} is 2 to 3mg, of which 80% is in the liver and daily excretion was 1.2 to 1.3µg (Hall, 1964). The United Kingdom reference nutrient intake for vitamin B_{12} 1.5µg.day^{-1} for males and females 15 to 50^{+} years. Vegans should take 2-3µg.day^{-1} vitamin B_{12} and elderly people should take 10-25 µg.day^{-1} because of their impaired gut absorption preferably with calcium and iron which assist the absorption of this vitamin.

Heavy protein consumers may also require more vitamin B_{12}, because it works synergistically with almost all B vitamins, A, E and C vitamins. Smoking also affects vitamin B_{12} metabolism, (Flickinger et al., 1987).

Young (1996), found that sheep fed a cobalt deficient diet for twenty-eight weeks had elevated plasma homocysteine, increased hepatic triacylglycerol and free fatty acid concentrations.

Herzlich et al. (1996), reported a significant association between $B_{12} < 221$ pmol.dm^{-3} and Homocysteine > 16 µmol.dm^{-3} and low left ventricular ejection fraction.

1.6.3 Vitamin B_6

Vitamin B_6 is a group of substances that are water soluble and excreted within eight hours of ingestion. They are found in the following types of food; wheat bran and

germ, liver, kidney, heart, beef, pork, poultry, walnuts and banana. Yet only 60-80% of the dietary vitamin is absorbed in comparison to the pure form, (Tarr et al., 1981). Pregnant and lactating women, body builders and other people on high protein diets require higher levels of vitamin B_6. The recommended daily adult intake is 1.6 to 2.0mg. Doses greater than 50mg can affect sensory nerve function, (Schaumburg et al., 1983). Normal plasma pyridoxal-5′-phosphate(V), levels are 5 to 23ng.cm^{-3} (McCormick, 1988). Vitamin B_6, ten times or one hundred times the recommend dose fed to female rats, has been shown to affect concentrations of glutamate, taurine, methionine and the sum of the essential amino acids in the caudate nucleus, increase serum serine concentration and increase both the antagonist binding affinity and the maximal number of binding sites for cortical serotonin, (Schaeffer et al., 1998). As serotonin, has been implicated in central fatigue, an increase in number and affinity of receptors could initially increase the severity of the response and could be implicated in chronic fatigue syndrome.

The active form of vitamin B_6 is pyridoxal-5′-phosphate(V) and is a coenzyme for numerous reactions related to protein and glycogen metabolism, and sphingolipid synthesis in the nervous system (McCormick, 1988). Tryptophan can be converted to niacin (vitamin B_3) with the assistance of vitamin B_6. Vitamin B_6 is also required for haem, antibody synthesis and for proper absorption of vitamin B_{12}. Pyrodoxal-5'-phosphate(V)-dependent decarboxylation such as L-aromatic amino acid decarboxylase is involved in the synthesis of Dopamine from L-DOPA and adrenaline, and also 5-hydroxytryptamine from 5-hydroxytryptophan.

Pyridoxal-5'-phosphate(V) in the liver is bound to its apoenzymes, in red blood cells bound to haemoglobin, and circulates in the blood bound to serum albumin.

An association between vitamin B_6 deficiency and the accelerated development of atherosclerosis was first shown by McCully in 1969. Pyridoxine is the vitamin B_6 form

that is the cofactor for cystathionine β-synthase and cystathionine γ-lyase. One homocystinuric patient has been shown to have no active cystathionine β-synthase due to a mutant form missing about sixty amino acid residues (M_r 56,000 daltons), (Skovby et al., 1984a). The cystathionine β-synthase gene is located on chromosome 21, (Skovby et al., 1984b). In rats, the polypeptide translational (M_r 63,000 daltons) undergoes a slow post-translational proteolysis to produce the active form (M_r 48,000 daltons), (Skovby et al., 1984c).

Wilcken & Wilcken (1997), found that patients deficient in cystathionine β-synthase treated with pyridoxine, folic acid and hydroxocobalamin maintained on average plasma total homocysteine levels <20μmol.dm^{-3}. Non-pyridoxine-responsive patients were additionally given betaine and their plasma total homocysteine levels were 33±17μmol.dm^{-3}. During the treatment period there were fewer mortalities.

Homocysteine levels were higher in cardiovascular disease patients who smoked than in those who did not, and in non-cardiovascular disease adults who smoked or did not smoke. It was also discovered that smokers had lower levels of vitamin B_6, (Bergmark et al., 1993). 50 - 80% of chronic alcoholics have low serum levels of pyridoxal-5'-phosphate(V) (Halsted & Heise, 1987) caused by poor diet, decreased release of the vitamin from binding proteins in food, and increased degradation and excretion of the vitamin.

Pyridoxine supplementation does not affect fasting plasma total homocysteine because under fasting conditions methionine and transmethylation are still required so homocysteine-methionine pathway is favoured. Yet it lowers the post-methionine load total homocysteine peak because methionine loading favours the trans-sulphuration pathway and thus the vitamin B_6 dependent enzymes (cystathionine β-synthase & cystathionine γ-Lyase), (Ubbink et al., 1996). Bostom et al. (1995), found that in the Caucasian population, a 'normal' fasting plasma total homocysteine is not synonymous

with a 'normal' methionine load test whereas in the 'black' community the total homocysteine peak post methionine load test is significantly lower despite the lower vitamin B_6 status, (Ubbink et al., 1995). Ubbink (1997), suggested that a possible explanation for the racial difference could be due to genetic difference in the cystathionine β-synthase, that is the black people tend to have cystathionine β-synthase with higher affinity for pyridoxal-5'-phosphate(V).

1.6.4 Vitamin B_2

Vitamin B_2 (riboflavin) is phosphorylated to form flavin mononucleotide, or via condensation with adenosine diphosphate(V) to form flavin adenine dinucleotide. Both flavin mononucleotide and flavin dinucleotide are involved in the production of adenosine triphosphate(V) from glucose. Flavin dinucleotide also acts as co-factors for other enzyme complexes.

There are a number of places in the biochemistry of the methylation of Homocysteine that require the reduced form of flavin dinucleotide. Sometimes the methionine synthase is deactivated, that is the cobalt ion in the cobalamin enzyme complex is only oxidized to di- rather than trivalency. It then uses the reduced form of flavin dinucleotide and S-Adenosylmethionine to produce a methylcob(III)alamin enzyme complex, (refer to Figure 1.3.1).

1.6.5 Vitamin C

Herbert (1993) in his review of mega vitamin doses (>500mg.day-1) stated that oral vitamin C releases iron from body stores, causing a sharp increase in serum iron level which has been associated with increased risk of heart attack. It also increased absorption of iron. Iron oxidizes vitamin C, preventing it acting as an antioxidant. Olson and Hodges (1987), recommended against large daily intakes of vitamin C ingested routinely over several months or years because it had been found to lower plasma vitamin B_{12}. There is

a fine intracellular balance between the reactive oxygen species and the antioxidants, which is influenced by dietary intake of vitamins like vitamin C.

Scorbutic cartilage had decreased synthesis of chondroitin sulphate(VI), (Reddi & Norstrom, 1954). McCully (1971), found similar derivatives for ^{14}C-Homocysteine thiolactone as for ^{35}S-Homocysteine thiolactone in normal guinea-pig liver. They were converted to homocysteic acid and homocysteine sulphinic acid, whereas in scorbutic livers homocysteine accumulated and the derivatives were diminished. This implied that some homocysteine was converted to thiolactone and was used in the production of chondroitin sulphate(VI) as long as vitamin C was present, (refer to Figures 1.3.3 and 1.3.1). Whereas the studies of Bonham et al. (1998), and Chambers et al. (1999), showed that the homocysteine concentration was not affected by the addition of vitamin C.

1.7 Physical Exercise

According to Shepherd (1999), exercise is the voluntary performance of physical activity to improve fitness, physical performance or health. This can take the form of an acute or chronic response. An acute response is taken as a single bout of physical activity whereas a chronic response, consists of repeated bouts of physical activity (Shepherd, 1999).

Exercise stimulates the chromaffin system to release adrenaline, (Kjaer & Dela, 1996). Adrenaline reacts with β_2-adrenoceptors on smooth muscle and bronchial cells to cause dilation. This is achieved via cyclic adenosine monophosphate(V) which opens calcium ion channels and allows calcium influx. It is hypothetically possible that this localised alteration in ionic homeostasis is rebalanced using many membrane transport systems, one of which could be ASC system and the co-efflux of homocysteine and sodium ions. The intensity, duration and history of physical activity, appear to affect

the hormonal response. Trained athletes tend to have a lower hormonal response to a given absolute work load as compared to untrained subjects (Kjaer et al., 1988), but a higher response at similar work loads, (Kjaer, 1989).

Endurance trained individuals (80 - 115 km.wk^{-1}) have enhanced muscle capacity to metabolise fat, (Costill et al., 1979), which could be explained by increased sympathetic activity. Depending upon the type of training, they may have improved lactate tolerance and caused hypertrophy in specific subclasses of muscle fibres. In addition reduced resting heart rate, a strengthening of the respiratory muscles, and increased minute ventilation rate have been reported (Booth & Thomason, 1991).

1.7.1 Exercise and Cardiovascular Disease

According to Drygas et al. (2000), the greatest benefit from exercise upon cardiovascular risk factors occurs in those subjects that exercised most (>8.3MJ per week using the approximate metabolic costs of leisure time sports activities). A group of men aged 28 to 65 years were divided into four groups according to the amount of exercise they undertook per week over a two to nine year period. When the high exercise group were compared to the sedentary group, it was discovered that they had a lower risk of cardiovascular disease (refer to Table 1.7.1).

1.7.2 Lipid profile and exercise

Exercise increases the rate of lipolysis in adipose tissue, (Wolfe et al., 1990). Thirty minutes cycling was shown to produce a 35 -50% increase in glycerol in catecholamine-stimulated isolated (pre & post exercise) gluteal cells, (Wahrenberg et al., 1987 & 1991). It has been shown that trained individuals preferentially utilize fat during endurance exercise compared to sedentary individuals, (Costill et al., 1979). Lipoprotein lipase increases in response to exercise and most of the elevation occurs within the first week of training.

	High exercise group (mean±SD)	Sedentary group (mean±SD)
High density lipoprotein cholesterol mmol.dm^{-3} (mg.dl^{-1})	1.38±0.19 (53.4)	1.19±0.17 (46.0)
Cholesterol mmol.dm^{-3} (mg.dl^{-1})	5.29±0.77 (205)	5.41±0.89 (209)
smokers	11.3%	42.5%

Table 1.7.1 Comparison between the highest exercise group (>8.3MJ per week) and the sedentary group. (Dygas et al., 2000)

Hurley et al. (1984), found that steroid-free strength-trained athletes (bodybuilders) matched for age and body fat against runners and untrained controls had lipoprotein-lipid profiles significantly higher than runners. Lipson et al. (1980), found that when body mass was kept constant, exercise produced no significant change in plasma triacylglycerol, very low density lipoprotein cholesterol, low density lipoprotein cholesterol or high density lipoprotein cholesterol levels. High density lipoprotein two increased and there was no significant change in high density lipoprotein three or in their composition (proteins, phospholipids, cholesterol and triacylglycerol).

1.7.2i Total Cholesterol

Rimmer & Looney (1997), found that an aerobic exercise programme practised four times a week for fifteen weeks produced a reduction in the total cholesterol levels and no change in high density lipoprotein levels in adolescents (14-17 years old). Yet, in a review by Durstine and Haskell (1994), stated that when gender, dietary intake, plasma volume changes and body mass were allowed for, the physically active individuals did not have a significantly lower plasma cholesterol concentration than inactive individuals.

1.7.2ii Triacylglycerol

Haskell (1984), reported that exercise had little or no influence upon the plasma total cholesterol levels, whereas it did reduce triacylglycerol levels, when age, adiposity and pre-training cholesterol levels were allowed for. The reduction in triacylglycerol levels appeared to have both acute and chronic components and was not evident when pre-training levels were low. Lower levels of triacylglycerol were found in active middle-aged men, (Wood et al., 1976), elite marathon runners (Martin et al., 1977) and men and women runners (Wood et al., 1977).

1.7.2iii High Density Lipoprotein

Factors that affect high density lipoprotein concentrations are age, length and intensity of exercise, maximal oxygen uptake, body mass and the percentage of body fat. Active individuals had high density lipoprotein concentrations higher than sedentary individuals mean difference 13μmol.dm^{-3} (0.5mg.dl^{-1}), whereas endurance trained individuals had even higher differences of high density lipoprotein, mean differences 31 - 52μmol.dm^{-3} (1.2 - 2.0mg.dl^{-1}) (Haskell et al., 1980). Wood et al. (1983), found that runners who ran less than 12.9km per week produced no effect upon their high density lipoprotein concentration, whereas Williams et al. (1982), found no effect upon high density lipoprotein concentration for those who ran less than 16km per week. It appears that as long as the exercise is above a threshold level and maintained for at least nine months then the subject changes high density lipoprotein cholesterol and low density lipoprotein cholesterol. When a subject exercises above the weekly threshold there is a decrease in total cholesterol and triacylglycerol, and increased levels of high density lipoprotein which cause increases in lipoprotein lipase. Yet, when the subject ceases to exercise above the threshold level the benefits are lost completely. Kokkinos et al. (1995), showed a dose-response relationship between the miles run per week and high density lipoprotein cholesterol levels with a significant difference to the non-exercise group at seven miles or more per week (≥11.2km.wk^{-1}).

A study of middle age males who took up running for two years showed an increase in high density lipoprotein cholesterol and a decrease in low density lipoprotein cholesterol. In addition the percentage of body fat decreased and the caloric intake (mainly as carbohydrate) increased, (Wood et al., 1985).

1.7.2iv Low Density Lipoprotein

DuFaux et al. (1982), identified twenty-two studies that showed that endurance-trained people had lower levels of low density lipoprotein in comparison to sedentary

people. A review of the short term effects of exercise upon low density lipoproteins found no significant difference in twelve, significant decreases in seven and significant increases in three studies, over a range of exercises and intensities, (Pronk 1993).

1.7.2v Very Low Density Lipoprotein

Gyntelberg et al. (1977), found that people with type IV hyperlipoproteinemia, who had spent 30 minutes of treadmill walking daily for four days showed decreased very low density lipoprotein concentration.

1.7.2vi Apolipoprotein A

Apolipoprotein A-I levels was 32% higher in athletes than in age-matched controls, but there was no difference for apolipoprotein A-II levels, (Lehtonen et al., 1979; Thompson et al., 1983; Wood & Haskell, 1979).

1.7.2vii Free Fatty Acid (non esterified fatty acid)

Mild to moderate exercise increases fatty acid oxidation, which is obtained from the plasma free fatty acid pool. The initial uptake of free fatty acid from the plasma can be greater than that released from the adipocytes, so there is an initial drop in the plasma concentration. As the duration of the exercise continues the rate of free fatty acid released from adipose tissue outstrips muscle requirements so the concentration of plasma free fatty acid increases, (Saltin & Astrand, 1993).

1.7.3 Homocysteine and exercise

Nygård et al. (1995), found an inverse relationship between Homocysteine levels and physical activity levels. They used a very brief description for leisure time activity, namely: sedentary/no activity, level one; walking, cycling or other type of moderate

physical activity for at least four hours a week, level two; exercise, gardening, or similar activity for at least four hours a week, level three and regular heavy training or competitive sport several times a week level four. They argued that their physical activity questionnaire was validated because they also found an inverse relationship between activity level and triacylglycerol levels. Durstine and Haskell (1994), in their review stated that the reduction in plasma triacylglycerol concentration was dependent upon the pre-training concentration and the volume of exercise completed during the training program.

If the exercise session was anaerobic, it could reduce the spontaneous conversion of homocysteine thiolactone back to homocysteine, if the intensity was sufficient to cause a reduction in the pH below 7.4 . At pH 7.0, 37°C and low ionic strength homocysteine thiolactone has a half-life of more than thirty hours. Prolonged existence of homocysteine thiolactone in an environment of relative high concentrations of homocysteine would most likely increase the concentration of homocysteine diketopiperazine polymer, (refer to Figure 1.3.3). Homocysteine diketopiperazine polymer can damage the intimal surface of artery walls (Dudman & Wilcken, 1982).

Weiss et al. (1998, 1999), found homocysteine levels corrected for plasma volume shift, had increased post acute aerobic exercise (2.5h. run). They reckoned that the increase, lasting over 24h was due to a loss of serum serine which would affect the trans-sulphuration of homocysteine and the methylation of tetrahydrofolate (refer to Figure 1.3.1). This may not be the whole answer as the level of stress (cortisol) during the exercise would have increased and this impairs vitamin B_6 absorption that would affect the trans-sulphuration of homocysteine long term, or increased egress due to stress (refer to section 1.4.8) or muscle fibre damage due to the stress of the run. De Crée et al. (2000), found a non significant increase in plasma total homocysteine concentration in seven male subjects (21.6±1.3years) after performing one hour of submaximal exercise on a cycle ergometer (10.89±2.05 and 11.21±1.81μmol.dm^{-3}, pre and post respectively, 60% of their $\dot{V}O_2$ max). They stated that the intra-assay coefficient of variation (cv_i)

and inter-assay coefficient of variation (cv_b) for the homocysteine assay were 2.0 and 7.0% respectively which gives a power of the test of 0.33 for a significant level of 0.05. So with increased numbers (36 subjects) there may have been a significant change. Wright et al. (1998), had twenty subjects in their acute exercise study (24-39 years old). They found a significant increase in plasma total homocysteine (11.5%) which was still elevated thirty minutes later. When the plasma total homocysteine levels were corrected for plasma albumin changes, the increase was non significant.

A comparison study between fourteen elite athletes and fourteen leisure time athletes, found that strength training for eight weeks (2 - 6 sessions per week of 45 - 60 minutes each) produced no significant change in plasma total homocysteine. The elite athletes had significantly lower plasma total homocysteine level than the leisure time athletes. They believed it was due to addition of folate and vitamins B_6 and B_{12} to their diet, (Große-Scharmann et al., 1995).

The Heritage Family study found that females had lower levels of plasma total homocysteine levels than males (7.5 vs 8.8μmol.dm^{-3}, $p<0.001$). Parents had higher levels than their children (8.7 vs 7.8μmol.dm^{-3} $p<0.05$). Subjects responded to the twenty-week endurance training programme with a significant 5.5% mean increase in plasma total homocysteine, ($p = 0.0003$; Lussier-Cacan et al., 1998).

These studies imply that exercise as a stressor causes a change in plasma total homocysteine. There appears to be a threshold over which the exercise stressor produces a significant increase in plasma total homocysteine. The threshold appears to be affected by the subject's diet.

1.8 Hypoxia

Hypoxia is a deficiency of oxygen at tissue level (Vander, Sherman, Luciano

1994), that can occur naturally in the environment. At sea level assuming dry air, the inspired partial pressure of oxygen (P_IO_2) is 19.9kPa, at 1000m P_IO_2 17.6kPa and at 5500m P_IO_2 9.7kPa that is equivalent to the following percentages of oxygen at sea level barometric pressure: 20.9%, 18.5% and 10.2% respectively, (Roach, 2000). Any physiological changes that occur at altitude, may not be due to the decline in partial pressure of oxygen, but to the decrease in temperature, atmospheric pressure or the increase in certain hormonal secretions due to increased physical stress. At 5500m, atmospheric pressure is 50% that of sea level and the induced hypoxia is called hypobaric hypoxia because of the reduction in atmospheric pressure.

Due to the constant partial pressure of the water vapour in the alveoli (P_{wv} 6 kPa), and the partial pressure of carbon dioxide (PCO_2 5kPa), the alveoli oxygen partial pressure is about 12kPa, which can be slightly raised by hyperventilation (greater loss of CO_2, PO_2 15kPa). It can only really be increased by two ways, either by increasing the ambient pressure (hyperbaric) or by increasing the inspired oxygen fraction ($F_i > 0.21$). If the lungs are normal then there should be no decline in arterial oxygen tension during exercise (Harries, 1998).

Fick's law state that "the rate of transfer of a gas through a sheet of tissue is proportional to the tissue area and the difference in gas partial pressure between the two sides and inversely proportional to the tissue thickness", (Fick, 1855).

$$V_{gas} \propto \frac{A}{T}.D.(P_1 - P_2)$$

$$D \propto \frac{Sol}{\sqrt{M_r}}$$

Equation 1.8 Fick's Law

A - area of tissue through which diffusion occurs; D - diffusion constant, which is proportional to the solubility of the gas and inversely proportional to the square root of its relative molecular mass; P_1-P_2 - partial pressure difference either side of the tissue barrier; T - tissue thickness

1.8.1 Hypoxia and Physiological Changes

Moderate hypoxia has been shown to have no effect upon endothelial cells or smooth muscle cells viability, proliferation and migration, (Wall et al., 1981). Yet hypoxia causes the release of hypoxia-inducible factor 1α and the accumulation of p53 protein, (Graeber et al., 1994), via its stabilization (Won et al., 1998). Hypoxia-inducible factor-1 regulates elements of the endothelial layer, (Minchenko et al., 1994(a)). *In vivo*, hypoxia was a potent inducer of vascular endothelial growth factor expression in heart, brain, liver, kidney and muscle, (Minchenko et al., 1994(b), Levy et al., 1995). Hypoxia-inducible factor induces a number of genes that produce changes in enzymes, the vascular system and breathing, (refer to Figure 1.8.1). Hypoxia induces glycolytic enzymes and GLUT 1 genes, therefore hypoxic exercise would have a reduced effect upon lipid metabolism in comparison to normoxic exercise.

1.8.2 Hypoxia and Cardiovascular Disease

To the author's knowledge there is only one report of people suffering from cardiovascular disease at altitude, (Fisher, 1990). It looked exclusively at highlanders in Papua New Guinea (~4000m), and found cor pulmonale and valvular heart disease as the commonest heart diseases. They also had an increased ischaemic heart disease in relation to the other two studies in Papua New Guinea, but the numbers were very small (n=5). All the patients were male and under 45 years old, with sedentary lifestyles and a long history of smoking (n = 4) .

1.8.3 Homocysteine and hypoxia

Deussen et al. (1989), found that for guinea-pigs 34% of the cardiac adenosine relaxation under normoxic conditions was supplied by the transmethylation pathway (SAM ->SAH + A), whereas under hypoxic conditions the adenosine was predominantly derived from enhanced 5'AMP hydrolysis. Yet the rate of transmethylation in the *in*

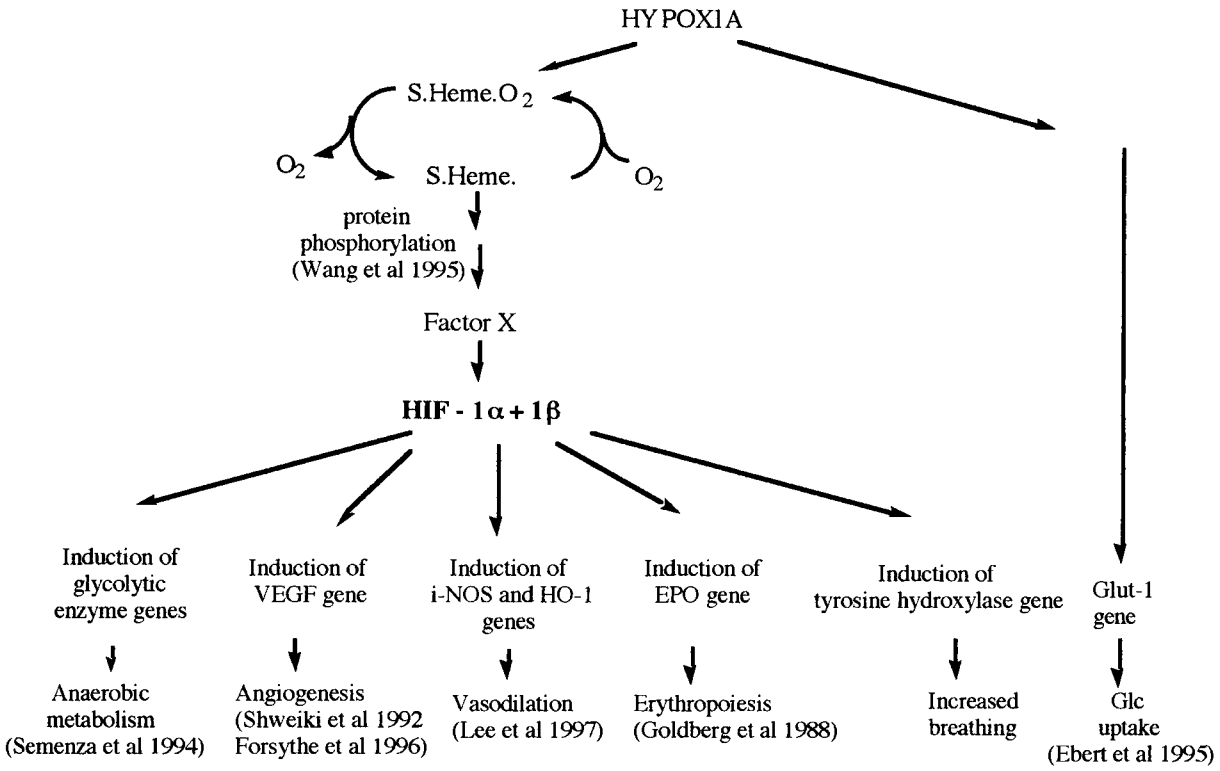


Figure 1.8.1 Model of the HIF-1 Hypoxia Response Pathway
(Guillemin & Krasnow 1997).

At reduced oxygen tension, oxygen leaves the haemoprotein oxygen sensor (S•Heme). The freed Haemoprotein triggers a protein phosphorylation signal cascade. This activates a hypothetical regulator (Factor X) which up regulates the expression of HIF-1α and -1ß subunits. The heterodimer then activates a number of genes.

Key:
HIF - hypoxia-inducible factor;
VEGF - vascular endothelial growth factor;
NOS - nitrogen(II)oxide synthase;
HO-1 - heme oxygenase-1;
EPO - erythropoietin;
Glut-1 - glucose transporter-1; Glc - glucose.

vitro heart was not enhanced under hypoxic conditions (Lloyd et al., 1988) although there was a twenty-fold increase in the free cytosolic adenosine level, (Deussen et al., 1988). In dogs under hypoxic conditions most of the vasodilation that occurs is due to adrenergic activation, but adenosine also assists in increasing coronary blood flow, (Herrmann & Feigl, 1992). The increase in adenosine appears related to adrenergic α-receptor activation (Hori et al., 1989, Kitakaze et al., 1987), and not from SAH (Herrmann & Feigl, 1992). Thus, at least in the heart under hypoxic conditions there appears to be no increase in intracellular homocysteine.

Enzymes involved in thiol biochemistry have been shown to be affected by hypoxia. This is usually the effect of very low oxygen levels of less than 10%, which is equivalent to oxygen levels experienced on top of Everest converted to sea level (7 % oxygen). Methionine adenosyltransferase mRNA was down-regulated in rat hepatocytes (*in vitro*) via reduced transcription and destabilization of the mRNA and induced the expression of nitrogen(II)oxide synthesis (NOS), (Avila et al., 1998).

Long duration exercise has been shown to cause an increase in plasma cortisol, (Kjaer, 1989). Sutton (1977), found that hypobaric hypoxic exercise (P_IO_2 11kPa), produced greater increased concentrations of plasma cortisol, whereas Kjaer et al. (1988), found no difference in plasma concentrations cortisol (P_IO_2 13kPa). Cortisone {can be converted to cortisol by 11 β-hydroxysteroid dehydrogenase type 1} impairs vitamin B_6 absorption. Vitamin B_6 is the coenzyme for cystathionine β-synthase and cystathionine γ-lyase. Vitamin B_6 is also required in the synthesis of niacin (Vitamin B_3) from tryptophan. Vitamin B_3 is essential for sex hormone synthesis as well as cortisone, thyroxin, and insulin, thereby providing a control upon vitamin B_6 absorption. Yet if the reduction is prolonged, insulin could be affected and therefore also carbohydrate, fat and cholesterol metabolism. Changes in the level of vitamin B_6 could alter the catabolism of homocysteine producing increased plasma total homocysteine.

1.8.4 Hypoxia and exercise

Hypobaric hypoxic exercise produced greater increases in the concentrations of plasma glucose, free fatty acids, lactate concentrations than normobaric normoxic conditions, implying increased fat mobilization and gluconeogenesis, (Sutton, 1977; Kjaer et al., 1988). Whereas there was no difference in plasma adrenaline in sedentary controls at rest and exercising in hypobaric hypoxia or normobaric normoxia, (Kjaer et al., 1988). Rostrup (1998), reviewed the effect of acute and prolonged hypoxia on catecholamines and found that 14 out of 15 laboratory studies showed no changes in plasma or urinary noradrenaline and only four showed significant increases in plasma or urinary adrenaline. Thirteen out of fourteen field studies with subjects staying for more than one week at high altitude showed increased plasma or urinary excretion of noradrenaline whereas only three studies showed increased plasma or urinary excretion of adrenaline. There appears to be a temporary reduction in plasma catecholamines in unacclimatized subjects that experience a short-term hypoxic effort, (Rostrup, 1998). Havel and Goldfien (1959), showed in dogs a relationship between adrenaline and free fatty acid mobilization. An acute hypoxic exercise challenge would not change sympathetic activity and therefore neither affect free fatty acid mobilization. Chronic exercise challenge would increase noradrenaline, but not adrenaline and therefore have no effect upon free fatty acid metabolism. The effect of acute and chronic hypoxic exercise upon adrenaline and therefore possibly homocysteine and free fatty acid metabolism is equivocal.

1.9 Summary

Methionine is an essential amino acid involved in protein synthesis, (the initiation of all peptide chains from mRNA require methionine) and methyl group transference via the formation of S-Adenosylmethionine. S-Adenosylmethionine has two very important roles namely as an allosteric inhibitor of N^5,N^{10}-methylenetetrahydrofolate reductase and as a cosubstrate for methionine synthase

Homocysteine is the lynchpin, as it is at the junction of two very important pathways. It can be methylated in one of two ways to form methionine or desulphurated to form cysteine that can be converted to glutathione and taurine important cellular antioxidants. Under certain cellular conditions homocysteine can be oxidized to homocysteine thiolactone and then to homocysteric acid.

The duality of homcysteine, is demonstrated in the finally balanced coagulation and fibrinolysis pathways, in that it activates the Hageman factor (Figures 1.4.5 & 1.4.6). It's negative side is demonstrated in that it is involved with oxidation of low density lipoprotein, the production of lipoprotein (a), hyperplasia of smooth muscle, inhibition of endothelial cell proliferation and the formation of atheromas. Whereas its positive side, is demonstrated in that it increases nitrogen(II)oxide synthesis and reacts with it to form a potent vasodilator, S-nitrosohomocysteine.

Poor diet that lacks fresh vegetables or meat can cause an increase in Homocysteine because of the reduction of the vitamin B complex (B_2, B_3, B_6, B_{12} and folate). Folate is required in the transmethylation reactions, (SAM + NA -> SAH + A), and vitamin B_6 for L-dopa to dopamine and homocysteine to cysteine. Excess homocysteine and methionine (high protein diet) are associated with changes in growth and organ development in young rats. Moderate to high alcohol intake raises serum homocysteine concentration.

The literature could be interpreted, that if the psychological and physiological stresses are summated and are above a certain intensity level (threshold), it causes an increase in plasma total homocysteine. This could be transitional, due to the transient increased sympathetic activity and egression and ingress into the liver and kidneys. The duration of the elevated plasma total homocysteine would depend upon folate and vitamins B_{12} and B_6 tissue levels, (Große-Scharmann et al., 1995). Chronic intermittent hypoxia would down regulate S-Adenosylmethionine synthase and therefore cause a change in the methionine biochemical pathways. The evidence that hypoxia alters

adrenaline secretion or affects free fatty acid metabolism is equivocal.

Adrenaline inhibits S-adenosylhomocysteinase, hypoxic and normoxic exercise would produce a temporary block in the cycle so that homocysteine could not be produced from S-adenosylhomocysteine. However, under hypoxic conditions adenosine is obtained from adenosine monophosphate and not from S-adenosylhomocysteine in cardiac muscle, and therefore would reduce the production of homocysteine.

Hypoxia increases vasodilation, by increasing constitutive nitrogen(II)oxide synthase and decreasing endothelin-1. It also increases Glut-1, so more glucose can be taken up by the cells. There is an increase in lactate dehydrogenase activity causing a decrease in lactate levels. Hypoxia also increases the net uptake of low density lipoprotein. The effect of hypoxia upon homocysteine is inconclusive.

It would appear that plasma homocysteine is a double-edge sword. The effect of acute variations in plasma total homocysteine upon cardiovascular disease would depend upon the total risk factors and would more than likely be a dependent as opposed to an independent factor. Yet chronic elevated homocysteine could be an independent factor for cardiovascular disease. The problem is the basal level of plasma total homocysteine. Is the measured basal plasma total homocysteine level, the actual level or an increased level due to the perceived stress of taking blood, recent history and what the person is about to do?

Exercise has been linked to a decrease in cardiovascular disease by altering the subject's lipid profile. This change appears to be significant when the exercise was over a threshold value, (Drygas et al., 2000; Kokkinos et al., 1995; Wood et al., 1983; Williams et al., 1982). The research investigating the relationship between homocysteine and exercise is at present, equivocal. The relationship appears to be dependent upon intensity, duration and repetition of the exercise stressor, (Nygård et al., 1995). It also

appears to be affected by other types of stress and emotional states, (Stoney, 1999; Stoney & Engebretson, 2000) and the person's vitamin B_{12}, B_6 and folate intake, (Große-Scharmann et al., 1995).

Plasma total homocysteine level is the subject of experimental measurement, not the intracellular. Therefore, any change in the plasma total homocysteine level would be due to the difference between the summation of production, subsequent "excretion" and uptake by cells.

The presented literature clearly suggests that plasma total homocysteine level is a risk factor for cardiovascular disease, (Kang et al., 1992). Therefore it is imperative that ways are found to decrease plasma total homocysteine.

The present study was designed to ascertain the influence of exercise on plasma total homocysteine levels. In addition, because highlanders (people who live at altitude) are believed to have a low incidence of cardiovascular disease, it was decided to examine the influence of hypoxia and exercise on the plasma total homocysteine levels.

Part II

"There is no scientific method.

There are only scientific methods"

Otta Neurath

Cartwright, N. et al., (1996)
Otta Neurath: Philosophy between Science and Politics
(Cambridge: Cambridge University Press).

Chapter 2

2.1 Aims of Study

1. To compare the responses of plasma total homocysteine levels and selected metabolic coronary heart disease risk factors following a single bout of exercise, under normoxic and hypoxic conditions.

2. To compare the metabolic responses of acute normoxic exercise following intermittent normoxic and hypoxic chronic exercise.

2.2 Null Hypotheses (H_0)

Null Hypothesis (1) There is no difference in the effect of acute and chronic normoxic and hypoxic exercise upon plasma total homocysteine concentration, as adrenaline released by the stress of exercise would inhibit S-adenosylhomocysteinase. Further more, the adenosine required by the cardiac muscle comes from two different sources, dependent upon hypoxic (adenosine monophosphate) and normoxic (S-adenosylhomocysteine) exercise; there is no difference in the affect upon homocysteine egression.

Null Hypothesis (2) There is no difference in the effect of chronic normoxic and hypoxic exercise upon the lipid profile, as both groups (normoxic and hypoxic) are exercising at the same relative intensity and duration.

Chapter 3

Methodology

3.1 Derivation of sample size

The study recruited young, non-smoking men (age range 18 to 28 years), with no known cardiovascular problem.

In order to calculate the minimum number of subjects required for statistical significance with the recognised variation, a number of factors were needed to be considered. These factors include, analytical variance (the variance that is inherent in the analysis of plasma total homocysteine concentration) and biological variance (the variance in the population within the normal range).

The minimal number (sample size - n) for each of the two independent groups for a given power against a specified difference (minimal detectable difference - δ_1)

$$n > 2\left[\frac{(z_{2\alpha} + z_{2\beta})\sigma}{\delta_1}\right]^2$$

(Armitage et al. 2002a)

An exercise study (Weiss et al., 1998) with 13 sports students (age 24.6 years) had a pre exercise plasma total homocysteine concentration of 9.3 $\mu mol.dm^{-3}$ with a standard deviation of 1.7$\mu mol.dm^{-3}$ (σ). The minimal detectable difference between the groups (δ_1) was obtained from the coefficients of variances based on previous work, (appendix VII, biological critical variance 0.081 and analytical critical variance 0.028). The sample size (n), for a significance of 5% (α = 0.05, $z_{2\alpha}$ = 1.960, Armitage et al., 2002b) for a given power (90%, $z_{2\beta}$=1.342, Armitage et al., 2002b), against the specified

difference (2.20μmol.dm^{-3}, appendix VII) was :

$$n > 2\left[\frac{(1.960 + 1.342)1.7}{2.20}\right]^2$$

$$n > 13$$

$$2n = 26$$

There should be at least thirteen subjects in each group and therefore a total of twenty-six subjects in the two groups.

3.2 Pre-Investigation to establish work loads

Eight male subjects in their late second or third decade of life undertook normobaric normoxic incremental submaximal cycle tests, (Monark 824E). The height of the saddle had been adjusted so that the leg was 150° when the pedal of the cycle, was in the fully down position. The subjects cycled for five, three minute stages, at a pedal frequency of seventy revolutions per minute, (refer to Table 3.2 for Mass, power output and times). A minute from the end of each stage their blood pressure was recorded, (using a mercury sphygmomanometer and stethoscope). Thirty seconds from the end of each stage, an 80mm^3 capillary blood sample was taken from the ear lobe. The sample was tested for lactate concentration (Analox P-LM5), packed cell volume and haemoglobin concentration (Hemocue-Hb). Heart rates were recorded at the end of each stage using a telemetry system (Polar), and the rate of perceived exertion was ascertained using a 1-20 scale, (Borg, 1982). The subjects repeated the cycle test on alternate days four times. A personal communication from Dr. D.M. Bailey stated that there was no change in the subjects performance in relation to their heart rate, blood pressure and oxygen capacity.

Exercise stage	Pedal time (min)	mass kg	Power W	Power increase W
1	0 - 3	0	-	0
2	3 - 6	1	70	70
3	6 - 9	1 + 0.7	119	49
4	9 - 12	1 + 1.4	168	49
5	12 - 15	1 + 2.1	217	49
6	15 - 16	1 + 2.5	245	28
7	16 - 17	1 + 2.9	273	28
8	17 - 18	1 + 3.2	294	21
9	18 - 19	1 +3.6	322	28
10	19 - 20	1 + 3.9	343	21
11	20 - 21	1 + 4.2	364	21
12	21 - 22	1 + 4.6	392	28
13	22 - 23	1 + 4.9	413	21
14	23 - 24	1 + 5.3	441	28

Table 3.2: Mass, Power output and times associated with each stage of the incremental cycle test to volitional exhaustion.

3.3 Subject selection

Ethical approval was obtained from Bro Taf Local Research Ethics Committee. The subjects were completely informed about the protocol and study. Forty Caucasian males between the age of eighteen and thirty volunteered to take part in the experiment and signed the consent form, (appendix 2). They were randomly assigned to either the normoxic or hypoxic exercising groups to produce a randomized double blind placebo controlled experiment. All subjects were non smokers and kept their own training and diet constant throughout the experimental period .

3.4 Outline of method

The subject's food and fluid intake were assessed using a seven day self-report questionnaire (NutriCheck, Health Options Ltd., UK) which they completed seven days before the experiment started. They also completed a health questionnaire. On the morning of the venepuncture the subjects had undertaken no physical activity for forty-eight hours and abstained from alcohol and caffeine for twenty-four hours. They arrived at the laboratory having fasted overnight for twelve hours. Post venepuncture they sat on the cycle ergometer (Monark 824E) and the saddle height was adjusted until the leg was 150° using a goniometer.

On one of the following two days the subjects ensured they were fully hydrated and consumed a high carbohydrate meal three hours before attending the laboratory. Upon arrival in the laboratory they were seated for fifteen minutes at the end of which their resting heart rate (Polar Vantage NV^{TM}, Polar Electro Oy, Finland) and systemic blood pressure using a mercury sphygmomanometer (Accoson Freestyle, Cardiokinetics, UK) and anaethescope (Littman^{TM}, 3M, USA) were determined. Following which the subject's anthropometric measurements were taken using a calibrated Harpenden skinfold caliper (British Indicators Ltd. UK) and balanced weighing scales and stadiometer (Seca, Cardiokinetics, UK).

Two or three days post venepuncture, the subjects ensured they were fully hydrated and consumed a high carbohydrate meal three hours before the maximal cycle test. They undertook the normobaric maximal cycle test to volitional exhaustion, either normoxically or hypoxically. Forty-eight hours later, those that were going to exercise hypoxically returned to repeat the cycling test, either normoxically or hypoxically, (refer to Table 3.4). Immediately post maximal cycling tests, the subjects were seated and the blood samples were drawn.

The intermittent exercise training was four weeks in duration and throughout the last week, a food and fluid diary (NutriCheck, Health Options Ltd., UK) was kept. Four days after the last exercise session, at the same time as the pre-intermittent exercise venepuncture (forty-eight hours of no physical activity, twelve hours fast, no alcohol or caffeine) the venepuncture was repeated, and a health questionnaire completed. Over the next two days anthropometric measurements, heart rate and blood pressure were taken. Two or three days post venepuncture, the subjects ensured they were fully hydrated and consumed a high carbohydrate meal three hours before the maximal cycle test. They undertook a normobaric normoxic maximal cycle test to volitional exhaustion at the same time of day as their pre-intermittent exercise test. Immediately post maximal cycle test, blood samples were drawn.

This was a randomised placebo controlled double-blind study. All measurements were conducted at the same time of day and by the same investigators to control for biological variation and minimise inter-subject analytical variation .

3.5 Dietary Analysis

The subjects completed a seven-day self-report dietary questionnaire seven days before the first resting blood samples and the last seven days of intermittent exercise.

Days	Control	Experimental	
1	Blood drawing in the morning - all subjects [A]		
2 3	Anthropometric data, resting blood pressure, orthostatic test and heart rate.		
4		Hypoxic maximal cycle test to volitional exhaustion. Blood drawn post test [C]	Normoxic maximal cycle test to volitional exhaustion. Blood drawn post test [B]
5	Normoxic maximal cycle test to volitional exhaustion. Blood drawn post acute maximal cycle test [B]		
6		Normoxic maximal cycle test to volitional exhaustion. Blood drawn post test [B]	Hypoxic maximal cycle test to volitional exhaustion. Blood drawn post test [C]
7 to 13 (wk1)	70% heart rate peak for 20 mins. 3 sessions per week		
14 to 20 (wk2)	75% heart rate peak for 20 mins. 3 sessions per week		
21 to 27 (wk3)	80% heart rate peak for 30 mins. 3 sessions per week		
28 to 34 (wk4)	85% heart rate peak for 30 mins. 3 sessions per week		
36	Blood drawing in the morning - all subjects [D]		
37 38	Anthropometric data, resting blood pressure, orthostatic test and heart rate.		
39 40	Normoxic maximal cycle test to volitional exhaustion Blood drawn post acute maximal cycle test [E]		

Table 3.4 Chronology of the experiment

Their average daily protein intake was calculated for each of the recorded weeks using a commercialized software package (NutricCheck, Health Options Ltd., UK). The subject's average daily protein intake was divided by his body mass at the commencement or termination of the experiment, ($g.day^{-1}$.$kg(BM)^{-1}$).

3.6 Blood sampling

Blood samples were taken early in the morning between 08.00 and 10.00h., upon arrival in the environmentally controlled laboratory (21±2 °C, relative humidity 40%±5%). The subjects sat and rested for thirty minutes before the blood sample was taken to control for plasma volume shifts. The blood was drawn from the cubital vein using a tourniquet and a vacutainer needle. The vacutainers were filled in the following sequence: Lithium Heparin (pale green traditional stopper), K_3EDTA (tripotassium 1,2-bis[bis(carboxymethyl)amino] ethane, hemogard purple closure) and lastly SST (serum separation tube, yellow/black traditional stopper). Each vacutainer was labelled with the subject's name, code, date and exercise code (pre-rest, pre-ex(N), pre-ex(H), post-rest and post-ex(N)), whereas each eppendorf was labelled with the subject's code, test date, exercise code, test name and number.

The filled vacutainers were immediately inverted eight times. One EDTA vacutainer was analysed for complete blood count and red blood cell folate. The other EDTA vacutainer was kept in the refrigerator and centrifuged within fifteen minutes of blood draw at 3000rpm for fifteen minutes at 4°C. The plasma was divided as required for each test, (Table 3.6). The lithium heparin and SST tubes were kept in the refrigerator until centrifuged. They were centrifuged within thirty minutes of blood draw at 4°C for ten minutes at 3500rpm. The plasma/serum was divided as required for each test (Table 3.6). The eppendorfs were stored in the -70°C freezer until analysed. The serum B_{12} eppendorf was wrapped in silver foil as it is photosensitive, stored in the refrigerator and analysed the next day.

Tube/test name		volume (cm^3)	rest	ex.
Li-Hep	Q	1.0	√	-
EDTA	CBC & rbc folate	4.5	√	-
EDTA	tHcy	0.5	√	√
	NEFA	1.0	√	-
SST	Lipids	1.0	√	-
	B_{12}	1.0	√	-
	Apo	1.0	√	-

Table 3.6 Test names and codes.

Key:

Li-Hep	Lithium Heparin
Q	glutamine
EDTA	1,2-bis[bis(carboxymethyl)amino]ethane
CBC	complete blood count;
rbc folate	red blood cell folate
tHcy	plasma total homocyst(e)ine
NEFA	non essential fatty acid;
SST	serum separation tube
B_{12}	vitamin B_{12}
Apo	apolipoproteins A_1 & B_{100}.

3.6.1 Complete Blood Count

The complete blood count required a whole blood sample collected in an EDTA tube. The tube was not spun, but placed in the refrigerator until required (within 24h). The cell types and numbers were counted using electrical impedance on a Coulter counter. Approximately 11mm^3 of whole blood was diluted by the isotonic solvent (1:50,000). Approximately 5mm^3 was analysed for erythrocytes and 5mm^3 analysed for white blood cells. If the sample was abnormal, then a blood smear was produced and the cells stained before examination using bright field light microscopy, but if due to coagulation a fresh sample was obtained.

The complete blood count was a multiple assay reported on a haemogram, including the following tests: erythrocyte, reticulocyte, leucocyte, monocyte, neutrophil, eosinophil, basophil, lymphocyte, platelet, differential white, cell counts and haemoglobin, packed cell volume, mean corpuscular haemoglobin, mean corpuscular volume, and mean corpuscular haemoglobin concentration.

3.6.2 Plasma volume shifts

3.6.2i Haemoglobin

The hemocue was checked for accuracy using a standard haemocuvette. A haemocuvette was filled with capillary blood drawn from the right ear lobe and placed in the hemocue. It gave a reading of the amount of haemoglobin in the sample in grams per millilitre.

3.6.2ii Packed Cell Volume

The packed cell volume was obtained from a Hawksley heparinized capillary tube, filled with capillary blood obtained from the right ear lobe. One end of the capillary

tube was gentle twisted in the CRISTA seal (Hawksley) to seal it. The sealed ends of the capillary tubes were placed against the rubber gasket, the lid of the rotor screwed on. It was then centrifuged (Hawksley microhaematocrit centrifuge) at 11,800rpm for five minutes. The tube was removed from the rotor and placed on the Hawksley reader and the cell percentage read.

The following equations were used to calculate the plasma volume post acute exercise, (Dill & Costill, 1974).

$$\text{Calculated blood volume}_{(after)} = Hb_{(before)} / Hb_{(after)} \times 100$$

{Hb = Haemoglobin quantity}

$$\text{Calculated cell volume}_{(after)} = \text{Calculated blood volume}_{(after)} \times PCV_{(after)}$$

{PCV = packed cell volume}

$$\text{Calculated plasma volume}_{(after)} = \text{Calculated blood volume}_{(after)} - \text{Calculated cell volume}_{(after)}$$

3.6.2iii Post-chronic exercise hypervolemia

Plasma volume shift was not measured, as Evans Blue dye is now considered carcinogenic, but an average blood volume expansion of 7% was used, (Convertino, 1991). In his review on blood volume, he stated that the increase in blood volume in the first two to four weeks was accounted for by exercise-induced hypervolemia, which was related to sympathetic activity and total body dehydration during the exercise training sessions. He also stated that the degree of hypervolemia induced by endurance exercise training was affected by the subject's initial level of fitness, history of exercise, and genetic factors.

3.6.3 red blood cell folate

The Abbott IMx Folate assay is based on ion capture, (Pennington et al., 1991; Wilson et al., 1993). It was used to measure the folate concentration in red blood cells, the assay was performed according to the manufacturer's method, which has been evaluated against cloned enzyme donor immunoassay (CEDIA, correlation r=0.97, Steijns et al., 1996). Whole blood was stored in the refrigerator at 4°C for less than twenty-four hours and protected from light. The whole blood sample was vortexed in the tripotassium 1,2-bis[bis(carboxymethyl)amino]ethane tube to ensure a homogeneous sample. Packed cell volume (haematocrit) was determined and then the red blood cells were lysed.

Into a clean test tube was pipetted $1cm^3$ of the lysis reagent (ascorbic acid and stabilizers) and then vortexed with $50mm^3$ blood sample. It was either frozen (-20°C) or the assay run. Into the sample well of the ion capture reaction cell was placed 150 mm^3 of the thawed haemolysate.

The predilution well of the ion capture reaction cell was filled with denaturant (dithiothreitol in ethanoate buffer), which was then transferred with the sample to the incubation well. Potassium hydroxide ($0.8mol.dm^{-3}$) was then added to the incubation well.

Mouse monoclonal anti-folate binding protein coupled to polyanion and folate binding protein in a borate buffer were added to the reaction well and incubated. An aliquot of the reaction was transferred to the positively charged glass fibre matrix that was pre-coated with a high molecular mass quaternary ammonium compound.

The positively charged matrix captures the polyanion folate binding protein analyte complexes. The matrix was washed to remove unbound materials and then the 'folate' reagent (folate $\{0.3ng.cm^{-3}\}$: alkaline phosphatase conjugate in TRIS buffer with

protein stabilizers and sodium azide) was added to the matrix and which bounds to the unoccupied folate binding protein sites.

The matrix was washed to remove the unbound conjugate. 4-Methylumbelliferyl Phosphate(V) was added to the matrix and the fluorescent product measured by the microparticle enzyme immunoassay optical assembly. The printed folate result was then used in the following equations to calculated the red blood cell folate concentration.

$$\text{rbc [folate]}(\text{ng.cm}^{-3}) = \frac{\text{IMx folate printed result}(\text{ng.cm}^{-3}) \times 21 \times 100}{\%\ \text{Packed cell volume}}$$

concentration in nmol.dm^{-3} = concentration in ng.cm^{-3} x 2.265

The National Health reference range of red blood cell folate is 408 - 1133 nmol.dm^{-3} (180 to 500$\mu\text{g.dm}^{-3}$), and the indeterminate (deficient-normal values) is 317 - 408 nmol.dm^{-3} (140-180$\mu\text{g.dm}^{-3}$).

The assay was calibrated using at least four known folate concentrations in duplicate, at the same time as the subjects' sample if the reagents and reaction cells have changed their set number, if not a control was run. All levels were run at least once during the eight-hour shift when the assay was run. If the control value was outside the specified range, the subject's samples were rerun with the calibration samples.

Blood from subjects that had received mouse monoclonal antibodies for diagnosis or therapy may contain human anti-mouse antibodies. Human anti-mouse antibodies could interfere with the immunoassay that uses mouse monoclonal antibodies.

3.6.4 serum vitamin B_{12}

The Abbott IMx B_{12} assay is based on the microparticle enzyme immunoassay technology, (Fiore et al., 1988). It was used to measure serum vitamin B_{12}

concentration, the assay was performed according to the manufacturer's method, which has been evaluated against cloned enzyme donor immunoassay (CEDIA, correlation r=0.97, Steijns et al., 1996).

Whole blood collected in serum separation vacutainer was centrifuged and $1cm^3$ of serum was placed in an eppendrof, wrapped in silver foil and stored in the refrigerator at 4°C for less than a day, (Mastropaola & Wilson, 1993).

The predilution well in the first reaction cell was filled (approximately twelve drops) with extractant 1 [cobinamide dicyanide in borate buffer with ovalbumin and sodium azide], and that in the second reaction cell, with extractant 2 {α-monothioglycerol in 1,2-bis[bis(carboxymethyl)amino]ethane}. Into the third reaction cell's predilution well was delivered via the probe/electrode assembly aliquots of extractant 1 and 2 which became the working extractant.

The sample ($150mm^3$ of subjects' serum) and denaturant reagent (sodium hydroxide $\{0.8mol.dm^{-3}\}$ with 0.005% potassium cyanide) was delivered to the third reaction cell's incubation well. Then the microparticles coated with intrinsic factor (in borate buffer with protein stabilizers and sodium azide) were added to the incubation well.

An aliquot of the B12: intrinsic factor coated microparticles reaction mixture was transferred to the glass fibre matrix, which bound irreversibly the microparticles. The matrix was washed and then B12: alkaline phosphatase conjugate in TRIS buffer with protein stabilizers and sodium azide was added to complex with the "free" intrinsic factor microparticle complex. The matrix was washed again to remove any unbound conjugate.

4-Methylumbelliferyl Phosphate(V) was added to the matrix and the fluorescent product measured by the microparticle enzyme immunoassay optical assembly.

B_{12} concentration in $pmol.dm^{-3}$ = B_{12} concentration in $pg.cm^{-3}$ x 0.7378

The National Health reference range of serum vitamin B_{12} is 163-590 $pmol.dm^{-3}$ (221 to 800$ng.dm^{-3}$), and the indeterminate (deficient-normal values) is 125-162 $pmol.dm^{-3}$ (170 to 220$ng.dm^{-3}$).

The assay was calibrated using at least four known B_{12} concentrations in duplicate, at the same time as the subjects' sample if the reagents and reaction cells have changed their set number, if not a control was ran. All levels were ran at least once during the eight-hour shift when the assay was ran. If the control value was outside the specified range, the subject's samples were rerun with the calibration samples.

3.6.5 plasma total homocysteine

Plasma homocysteine exists as free, dimerized and protein bound, the method releases homocysteine from proteins. S-adenosyl homocysteine hydrolase is not present in blood, there was no need to add an inhibitor to prevent the hydrolysis of S-adenosyl homocysteine.

To 150mm^3 of plasma or standard was added 15mm^3 of 10% tri-n-butyl-phosphine in dimethylformamide. The mixture was incubated at 4°C for thirty minutes to reduce the disulphide bonds between homocysteine and other thiols or albumin. 150mm^3 of 10% tricloroethanoic acid (containing 1mmol / 1 1,2-bis[bis (carboxymethyl) amino]ethane) was added. This mixture was centrifuged and 50mm^3 of the clear supernatant was added to 10mm^3 of 1.55$mol.dm^{-3}$ sodium hydroxide, 125 mm^3 of 0.125$mol.dm^{-3}$ borate buffer (pH 9.5) containing 4$mmol.dm^{-3}$ 1,2- bis[bis (carboxymethyl)amino]ethane and 50mm^3 of ammonium 7-fluorobenzo- 2-oxa-1,3-diazole-4-sulphonate (SBD-F) solution (1$mg.cm^{-3}$ dissolved in borate buffer). This mixture was incubated for 60 minutes at 60°C to completely derivatize homocysteine and the other plasma thiols. A 20mm^3 aliquot was used for reversed-phase High-

performance liquid chromatography, (Arki & Sako, 1987; Ubbink et al., 1991b; Kuo et al., 1997).

A 20mm^3 aliquot was autosampled (SA 360) onto the Spherisorb ODS2 [4.6mm internal diameter, 150mm long, 5μm particles] analytical column (Jones Chromatography). The SBD-derivative was eluted with the mobile phase, 0.1mol.dm^{-3} potassium dihydrogen phosphate(V), pH 2.0 containing 40cm^3.dm^{-3} ethanenitrile at a flow rate of 0.8cm^3.min^{-1} using High-Performance liquid chromatography pump (325-Kontron Instruments). The SBD-derivative's fluorescence was detected with SFM 25 spectrofluorometer (Kontron Instruments), excited at 385nm and emission at 516nm, (Ubbink et al., 1991b; Kuo et al., 1997). A mobile phase with a pH 2.1 ensures that SBD-Homocysteine is completely resolved from SBD-cysteinylglycine, (Ubbink et al., 1991b).

Kuo et al. (1997), used cysteamine as an internal standard. It is a naturally occurring substance but in very low concentrations, (<0.1μmol.dm^{-3} Smolin & Schneider, 1988). It improves the within-batch imprecision (critical variance) was 2.8% (for low plasma homocysteine concentration 7.8μmol.dm^{-3}, n=10) and 2.2% (for high plasma homocysteine concentration 23.6μmol.dm^{-3}, n=10) from 8.3% and 5.3% respectively. The between-batch critical variances was 6.9% (n=75) and 7.9% (n=75) for the different plasma homocysteine concentrations with respect to 11.0% (n=142) and 11.3% (n= 125) without the internal standard. The internal standard compensates for variations in injection volume or detector sensitivity. The internal standard was added to the plasma or standard before reducing the disulphide bonds, (30mm^3 of 50μmol.dm^{-3} of cysteamine hydrochloride was added to 120mm^3 of plasma or standard to produce a concentration of the internal standard of 10μmol.dm^{-3}).

There is little fluctuation in plasma total homocysteine during the day (mean total homocysteine±10%, Guttormsen et al., 1994). Fasting plasma total homocysteine was used because of the variability of postpranially plasma total homocysteine (Ueland et al.,

1993). As it is related to food intake, food intake and eating times must be kept constant.

3.6.6 Lipid profile

3.6.6i Serum total Cholesterol

Cholesterol is present in tissues and in plasma lipoprotein either as cholesterol or as cholesterol esters bound to proteins. The frozen serum was allowed to warmup gradually in the laboratory. $10mm^3$ of the serum was placed on a Kodak Ektachem Clinical Chemistry Slide (CHOL). The slide was a dry, multilayered analytical element coated on a clear polyester support. Above the support layer was a gelatin buffer, the base layer, above that, was the spreading layer containing barium sulphate(VI), surfactant, cholesterol ester hydrolase, cholesterol oxidase, peroxidase, leuco dye (2-(3,5-dimethoxy-4 hydroxyphenol)-4,5-bis(4-dimethylaminophenyl) imidazol) and buffer.

$$\text{Lipoprotein} \xrightarrow[\text{TX-100}]{\text{Surfactant}} \text{Cholesterol} + \text{Cholesterol Esters} + \text{Proteins}$$

Cholesterol ester hydrolase hydrolyses the cholesterol esters to cholesterol.

$$\text{Cholesterol Esters} + H_2O \xrightarrow[\text{ester hydrolase}]{\text{Cholesterol}} \text{Cholesterol} + \text{Fatty acids}$$

The cholesterol was oxidized by cholesterol oxidase to cholestenone and hydrogen peroxide.

$$\text{Cholesterol} + O_2 \xrightarrow[\text{oxidase}]{\text{Cholesterol}} \text{Cholest-4-en-3-one} + H_2O_2$$

The generated hydrogen peroxide oxidized the leuco dye in the presence of the peroxidase to generate a coloured dye.

$$H_2O_2 + \text{Leuco dye} \xrightarrow{\text{peroxidase}} \text{Dye} + H_2O$$

The density of the coloured dye was proportional to total cholesterol concentration and was measured by reflectance spectrophotometry at 540nm.

Mean value of the internal quality control was 4.24mmol.dm^{-3}, with a critical variance of 2.7%. Kodak Ektachem Calibrators 1,2 and 6 were reconstituted and used to calibrate Kodak Ektachem 700 Analyser.

$$1\text{mmol.dm}^{-3} = 38.67\ \text{mg.dL}^{-1} \times 0.02586$$

3.6.6ii Serum Triacylglycerol

Triacylglycerol is present in tissues and in the plasma lipoprotein which dependant upon the rate of entry and removal. The frozen serum was allowed to warmup gradually in the laboratory. 10mm^3 of the serum was placed on a Kodak Ektachem Clinical Chemistry Slide (TRIG). The slide was a dry, multilayered analytical element coated on a clear polyester support. Above the support layer was the reagent layer, containing leuco dye (2-(3,5-dimethoxy-4-hydroxyphenol)-4,5-bis(4-dimethylamino-phenyl)imidazole), peroxidase, glycerol kinase, L-α-glycero-phosphate(V) oxidase, adenosine triphosphate(V), magnesium chloride and buffer. The next layer, was the Scavenger layer containing ascorbate oxidase and buffer. The top layer, the spreading layer contains Titanium(IV)oxide, surfactant and lipase.

The surfactant helps to spread the sample and in dissociating the triacylglycerol from the lipoprotein. The lipase hydrolyses the triacylglycerol to yield glycerol and fatty acids.

The glycerol diffuses through the scavenger layer to the reagent layer. It was phosphorylated by glycerol kinase in the presence of magnesium adenosine triphosphate(V). L-α-glycerophosphate(V) was oxidized by L-α-glycero-phosphate(V) oxidase to yield dihydroxypropanone phosphate(V) and hydrogen peroxide.

The generated hydrogen peroxide oxidized the leuco dye in the presence of the peroxidase to generate a coloured dye.

The density of the coloured dye was therefore proportional to total cholesterol concentration and was measured by reflectance spectrophotometry at 540nm.

The analyser's dynamic range is 0.11-5.93mmol.dm^{-3} while the expected values for males are between 0.45 and 1.81mmol.dm^{-3}, (Tietz 1986). Mean value of the internal quality control was 1.29mmol.dm^{-3}, with a critical variance of 3.0%. Kodak Ektachem Calibrators 1,2 and 4 were reconstituted and used to calibrate Kodak Ektachem 700 Analyser.

Limitation of the procedure was that free (non-esterified) serum glycerol was also measured with the glycerol obtained from the hydrolysis of triacylglycerol and diacylglycerol.

$$1\text{mmol.dm}^{-3} = 88.57\text{mg.dL}^{-1} \times 0.01129$$

3.6.6iii Serum High Density Lipoprotein Cholesterol

The frozen serum was allowed to warmup gradually in the laboratory. 200 mm^3 of the serum was placed in a plastic centrifuge tube with 200mm^3 of poly(ethen-1,2-diol), (M_r 6000) with a final concentration of 100g.dm^{-3} (used to precipitate low and very-low density lipoprotein). The mixture was vortexed for five minutes and then centrifuged at 2000g for twenty minutes. The supernatant was aspirated at room temperature. The CentrifiChem pipetter takes 15mm^3 of the supernatant, 45mm^3 of de-ionized water and 350mm^3 of combined "CHOD-PAP" reagent. [The kit provide by Boehringer Mannheim (Roache Diagonistic) contains cholesterol ester hydrolase, cholesterol oxidase which produces hydrogen peroxide. The hydrogen peroxide in the presence of horseradish peroxidase (EC.1.11.1.7) reacts with 4-aminophenazone and

phenol to yield 4-(p-benzoquinone-monoimino)phenazone]. The reaction takes place at a temperature of 30°C. Using a wavelength of 500nm, the absorbance was read at five seconds and then every two minutes for twelve minutes.

High density lipoprotein cholesterol concentration was calculated from 1.30 $mmol.dm^{-3}$ ($50mg.100cm^{-3}$) and 2.59$mmol.dm^{-3}$ ($100mg.100cm^{-3}$) cholesterol standards.

Allen et al. (1979), found that for 539 men, the high density lipoprotein cholesterol was 1.20±0.31$mmol.dm^{-3}$, the within-run reproducibility (critical variance) was 0.8% and the between-run reproducibility (critical variance) was 3.6%.

3.6.6iv Calculated Low Density Lipoprotein Cholesterol

Low density lipoprotein cholesterol was derived using the Friedwald et al. (1972), formula:

$$LDL_C = tC - HDL_C - \frac{TG}{2.2} \quad mmol.dm^{-3}$$

Key
LDL_C = Low density lipoprotein cholesterol
tC = total Cholesterol
HDL_C = High density lipoprotein cholesterol
TG = Triacylglycerol.

3.6.6v Apolipoprotein A_1

Apolipoprotein A_1 was measured directly from serum micrcosamples, by rate immunonephelometry using a Bechman ARRAY 360 system (Beckman Instruments, Inc.) using antibodies and reagents supplied by Beckman Coulter(UK) limited.

The samples were defrosted, vortexed, barcoded and loaded onto the sample wheel. The preprogrammed test was selected and it automatically runs the required test using the apo diluent, buffer and antigens. It automatically retested out-of-range results, and tested for excess antigen. Single-point calibration for proteins were stable for two weeks. Lyophilized apolipoprotein A_1 was used as the serum reference material. Low-volume samples were diluted before being placed in the sample wheel.

Maciejko et al. (1987), have found that for the assay of apolipoprotein A-1 or B using the Beckman reagents, the within-run and between-run coefficients of variation was less than 5% over the physiological range, with an accuracy of 101 to 109% compared to purified protein assayed by the method of Lowry.

3.6.6vi Apolipoprotein B_{100}

Apolipoprotein B_{100} was measured by rate immunonephelometry using a Bechman ARRAY analyser using antibodies and reagents supplied by BeckmanCoulter(UK) limited.

Similar protocol as for apolipoprotein A_1, but a different programmed test was used and the liquid stabilized apo B_{100} control serum for calibration.

3.6.7 Glutamine

To determine the plasma glutamine concentration, the plasma was first deproteinized (Bernt & Bergmeyer, 1974) and then under went the asparaginase assay (Windmueller & Spaeth, 1974).

To remove any fatty deposits, the frozen plasma obtained from the lithium-heparin vacutainers was allowed to thaw out to laboratory temperature and then

centrifuged for thirty seconds at 1400 revolutions per minute in a micro-centrifuge (Heraeus Biofuge A).

300mm^3 of the centrifuged plasma was placed in an eppendrof tube (1.5cm^3) with 300mm^3 of chloric(VII) acid. This was repeated in another eppendrof, they were both centrifuged immediately for ten minutes at 3000g.

15mm^3 of Universal indicator was placed in an eppendrof tube (1.5cm^3), with 50mm^3 of triethanolamine buffer (0.5mmol.dm^{-3}) and 100mm^3 of potassium hydroxide. This was repeated in another eppendrof.

Precisely 460mm^3 of the supernatant was added to each eppendrof containing the universal indicator mixture and vortexed. The pH was adjusted to 7.0 to 7.5 by the dropwise addition of 20% potassium hydroxide and/or supernatant and the quantity recorded.

The deproteinized samples were aspirated with a plastic pipe and stored at -20°C in new eppendrof tubes.

The asparaginase was dialysed for a day against two changes of potassium dihydrogen phosphate(V) buffer (80mmol.dm^{-3}, pH 6.5) before use.

1.06cm^3 of the reaction mixture (20 units of asparaginase, 0.5mg glutamate dehydrogenase, bovine serum albumin 0.05%, glycerol 8%, 2-oxoglutarate 3.6 mmol.dm^{-3}, oxidized nicotinamide adenine dinucleotide 172μmol.dm^{-3} and potassium dihydrogen phosphate(V) 45mmol.dm^{-3}) was added to the thawed deproteinized sample. The asparaginase hydrolyses glutamine to glutamate and the ammonium ion, where upon the glutamate dehydrogenase oxidises glutamate to 2-oxoglutarate and reduces nicotinamide adenine dinucleotide. The reduction of the nicotinamide adenine dinucleotide was detected spectrophotometrically at 340nm (Gilford, Stasar III, UK).

Plasma glutamine concentration is 614±27μmol.dm^{-3} using this enzyme method (Rowbottom et al., 1996).

3.6.8 Lactate concentration

The Lactate analyser (Analox P-LM5) was calibrated using Lactate standard 5 and 8mmol.dm^{-3}, after Lactate II reagent and standards had attained to the laboratory temperature. The assay was performed according to the manufacturer's method, which has been utilized in a number of studies (Gullstrand et al., 1994, 1996; Khanna et al., 1996; Murphy & Hardman 1998; Fernandez-Garcia et al., 2000).

The right ear lobe was held firmly in the fingers and the lowest part cleaned with an alcohol swab and then stabbed with an Analox sterile lancet. The first few drops of blood were wiped away, before the heparinized capillary tube (Analox) was filled. The capillary tube was gently rotated for 4 minutes, and using a Gilson microman 7mm^3 placed in the lactate analyser. Thirty seconds later the lactate concentration was given.

The assay is a one-step procedure. The Lactate II reagent is a buffered solution and can be stored in the refrigerator indefinitely, but the vial containing the enzyme - L-lactate: oxygen oxidoreductase (LOD) has a shelve life of about six months to a year. Once the vial has been added to the reagent the working life of the reagent: enzyme mixture is at least two months if stored at between 0 and 5°C. The buffered reagent: enzyme mixture was entrained into the analyser and the reaction is activated by the injection of the blood sample or standard.

$$\text{L-lactate} + O_2 \xrightarrow[\text{pH } 6.5]{\text{LOD}} \text{Pyruvate} + H_2O_2$$

The maximum rate of oxygen consumption was directly related to the lactate concentration.

The calibration was checked with the standard after every subject, and at the beginning of each day with the lactate/pyruvate quality control serum.

The within-run precision standard deviation (SD), shows negligible variation over the entire assay concentration range, SD 0.05 - 0.07mmol.dm^{-3}. After calibration using a 7mm^3 standard and a sample of 7mm^3, then the linearity limit was 10 mmol.dm^{-3}.

The subject's lactate concentration was plotted against the work load, to find the critical load at which lactate level increased markedly (the lactate inflection point). This is called the lactate threshold (onset of blood lactate accumulation).

3.7 Heart Rate

The heart rate was monitored using a short-range telemetry system (Polar Vantage NV™, Polar Electro Oy, Finland). The heart rate was sampled and averaged every 5 seconds by the chest belt and then via transmitter to the monitor (watch).

3.8 Blood pressure

Blood pressure was measured by the same investigator to standardize the investigator potential measurement difference. A pressure cuff (correct size for subject's arm girth) was placed on the subject's left arm around the biceps and triceps. Auscultatory blood pressure measurement was recorded by occlusion of the brachial artery using a mercury sphygmomanometer (Accoson Freestyle, Cardiokinetics, UK) and anaethoscope (Littmann™, 3M, USA). The pressure cuff was quickly inflated to a pressure of about 100mmHg and the anaethoscope placed on the brachial artery in the cubital fossa on the straight left arm. When the heart sound was heard, the pressure in the cuff was increased until just above the pressure when the sound could not be heard. The cuff was slowly deflated at about 2mmHg per second until the sound was heard again (Korotkoff phase 1 - Systolic) and the pressure recorded, it continued to be

deflated until the sound disappeared - Korotkoff phase 5 (Diastolic) (George et al., 1994).

The subject was then asked to stand and thirty seconds later the heart rate was recorded with blood pressure.

The cuff contains a bladder in a cloth sheath. The length of the bladder should be sufficient to encircle nearly or completely the subject's upper arm.

standard adult size - 120 x 260mm
"obese" size bladder - 120 x 400mm
"small" size bladder - 120 x 180mm.

If the cuff was too wide for the arm, it was folded over itself. Yet, if it was too short and there were no large ones available then the bladder was placed over the artery. The bladder width should be at least 80% of the arm's circumference, (O'Brien, 1996).

3.9 Anthropometric measurements

Anthropometric measurements were undertaken in an environmentally controlled laboratory (21±2 °C, relative humidity 40%± 5%).

3.9.1 Body Mass

The body mass was measured with a calibrated Seca beam pillar balance and recorded to the nearest tenth of a kilogram (Ross & Marfell-Jones, 1991). The subjects were weighed in shorts.

3.9.2 Height

Height was measured using a stadiometer attached to beam-pillar balance. The subjects stood erect, barefoot with heels together and arms hanging naturally by their sides. They were then told to look straight ahead and take a deep breath. Their height was measured from the balance platform to their vertex, at the end of a maximal inspiration

The vertex is the highest point on the skull when the head is held in the Frankfort plane, (the orbitale to the tragion is horizontal and at right angles to the long axis of the body. The orbitale is located on the most inferior position on the margin of the eye socket. The tragion is the notch superior to the tragus of the ear, at the superior aspect of the zygomatic bone), (Ross & Marfell-Jones, 1991).

3.9.3 Body fat

Four skinfolds were measured using a calibrated Harpenden caliper (British Indicators Ltd.) and recorded as the sum of four skinfolds, (Durnin & Womersley, 1974). The procedure was explained to the subject and the sites were marked with a water soluble, non toxic marker pen. All measurements were taken on the right side of the body with the subject standing in a relaxed condition. Each site was measured by drawing up a layer of skin (pulling the skin together and slightly away from the body), with the thumb and forefinger of the left hand. The calipers were placed perpendicular to the fold, approximately 10mm distally from the examiner's left hand. The pressure on the calipers was released and likewise the examiner's left hand. Two seconds were allowed before the measurement in millimetres (interpolated to the nearest 0.1mm) being recorded The calipers were completely removed from the site and the procedure was repeated. Three readings were obtained and the closest two, if different by $\leq$1mm were averaged, if not more measurements were recorded. For reliability and validity, the repeated measurements were attempted to be taken at the same time of day, preferably in

the morning, (Ross & Marfell-Jones, 1991).

The anatomical position of the skinfolds were identified as follows:
Biceps fold: - the anterior side of the right arm (right angles) and one centimetre above the level of the triceps mark, over the belly of the biceps muscle, (midway between the acromion process and the head of the radiale), (Robergs & Roberts, 1997).

Triceps fold: - the posterior side of the straightened, relaxed arm, a vertical fold mid region between the acromion and olecranon processes, (Robergs & Roberts 1997).

Subscapular fold: - the arm was drawn up behind the back and then relaxed, an angular fold taken at 45° one to two centimetres below the inferior angle of the scapula, (Robergs & Roberts, 1997).

Supraspinale :- Relax abdominal muscles and raise a fold at the intersection of the border of the ilium (about 70mm above the spinale) on a line from the spinale to the anterior axillary border (armpit). The caliper was applied 10mm anteriorly to the left thumb and index finger, (Ross & Marfell-Jones, 1991).

3.10 Exercise

3.10.1 Acute maximal exercise

Before commencing the cycle test the cycles were calibrated. Four kilograms were placed on the basket and the cranks rotated by hand. The tension on the cord raised the basket so that it was between 30 and 80mm above the flywheel, if not the tension in the cord was altered.

The first stage the three minute stage was without any resistance on the cycle ergometer, the second three minute stage was with no load on the kilogram basket and the

next three sub-maximal three minute stages the mass increased by 0.7kg. Then the stages were reduced to one minute and the mass increased by either 0.4 or 0.3kg, (refer to Table 3.2).

For the first five stages, the following submaximal data was collected: - Heart rate was recorded using a bipolar electrocardiograph (3 lead electrocardiograph - Lifepulse LP10, HME Ltd. Cardiokinetics, UK), rate of perceived physical exertion (Borg, 1982), blood pressure, arterial oxygen saturation, capillary lactate concentration, haemoglobin and pack cell volume. During the last minute of each exercise stage the expired air was collected and analysed for oxygen and carbon dioxide percentages and the gas volume was measured. The stages decreased to one minute duration, and only the blood pressure, arterial oxygen saturation, heart rate and the relative rate of perceived physical exertion (Borg, 1982) were collected. The subjects indicated one minute before exhaustion and the expired air was collected in a Douglas bag for analysis at the end of the test. The expired air was analysed using fast responding paramagnetic oxygen and infrared carbon dioxide analysers (Servomex 1400 Series Analyser, UK). These had been calibrated with precision-analysed quality gas mixtures containing pure nitrogen, 17% oxygen and 5% carbon dioxide. The expired air volume was measured using a dry gas meter (Harvard LTD, UK) which had been calibrated against a 600L Tissot spirometer (Collins Limited, USA). The duration of the test, maximum lactate concentration, packed cell volume, haemoglobin, maximum heart rate, blood pressure and rate of perceived exertion were all recorded.

The test was terminated if the subject experienced chest pains, dyspnoea, exhaustion or recorded a systolic blood pressure $\geq$250mmHg or the subject had reached peak oxygen consumption ($\dot{V}O_2$ peak). Peak oxygen capacity was considered to be attained if as a result of an increase in workload, three of the following were obtained: no change in the volume of oxygen consumed ($2cm^3.kg^{-1}.min^{-1}$), maximum heart rate attained during the stage, respiratory exchange ratio (VCO_2/VO_2) above 1.15, rate of perceived

exertion >20 (Borg, 1982) and a lactic acid concentration above 8mmol.dm^{-3}.

3.10.2 Chronic intermittent exercise

The subjects exercised for three sessions a week for four weeks using heart rate monitors (Polar). The intensity of the exercise was based upon the maximal heart rate they had obtained in their first acute maximal exercise session, breathing the same gas composition as they were to breathe during the intermittent exercise sessions. For the first and second weeks, they exercised at 70% and 75% of their maximal heart rate respectively, for twenty minutes, whereas for weeks three and four it was increased to 80% and 85% respectively, for thirty minutes. The hypoxic group of students breathed a dry gas mixture of 84% nitrogen and 16% oxygen on average over the exercise period. (16% oxygen is approximately equivalent to 1.7km altitude in relation to the partial pressure of inspired oxygen of about 16kPa).

Blood pressure and an ear capillary sample for lactate analysis were taken in the last minute of the last exercise session in weeks three and four.

3.11 Statistics

3.11.1. Missing Data

Due to vein collapse, or insufficient plasma/serum obtained, some data was missing. Missing values were calculated using either the least squares regression line of x on y or y on x (appendix VI). If both the pre and post data were missing or if there was only one set of data, then the arithmetical mean was used. If a missing value was derived from another missing value, then the derived value was not calculated directly, but calculated from the calculated first missing value.

3.11.2 Normality

The data's symmetry (skewness) and kurtosis were obtained using StatView 512+ for MAC (appendix III and IV). The Shapiro-Wilks test for normality was calculated for all the data (appendix VI), which showed that some of the data was non normal. Normal and log-normal data was tested parametrically and non-normal data was tested non-parametrically using STATISTICA 4.1 for MAC (1994).

3.11.3 Parametric tests

The following parametric tests were used: The unpaired Student's 't' test for independent samples; The paired Student's 't' test for dependent samples and the Pearson correlation coefficient between two variables. Significance was taken as $p < 0.05$.

3.11.4 Non-parametric tests

The following non-parametric tests were used: Mann-Whitney U, Wald-Wolfowitz run test and Kolmogorov-Smirnov for independent samples (so not just means or rank sums can be compared, but also the overall shape of the distribution - skewness); Wilcoxon signed-rank for dependent samples and the Spearman Rank correlation coefficient between two variables. Significance was taken as $p < 0.05$.

3.11.5 Analysis of Variance

The nonparametric analysis of variance (ANOVA) tests used are Kruskal-Wallis for between groups and Friedman for within-subjects (repeated measures) which will be compared to a 2x2 repeated measures ANOVA.

Chapter 4

Results

Of the forty subjects that commenced the study, ten did not complete the study or were withdrawn for not meeting the criteria. Data was obtained on 30 young adult males of whom fourteen exercised normoxically and sixteen exercised hypoxically for four weeks and will be expressed as mean ± standard deviation(SD), unless otherwise stated.

4.1 Statistical Analysis

Not all the data were normally distributed.

Within the control group the following factors did not show normality: -

- age.
- plasma total homocysteine concentration post-acute cycle test, pre-chronic exercise.
- post-chronic exercise body mass index (BMI).
- pre- chronic exercise plasma glutamine concentrations.
- post-chronic exercise high density lipoprotein cholesterol (HDL_C) concentrations.
- post-chronic exercise non-esterified fatty acid (NEFA) concentrations.

Similarly within the hypoxic group the following were not normally distributed: -

- maximum heart rate at the end of the normoxic acute cycle tests pre-chronic exercise.
- maximum heart rate at the end of the hypoxic acute cycle tests pre-chronic exercise.
- pre-chronic exercise triacylglycerol (TG) concentrations.

The logarithmic transformation ($X' = \log(X + 1)$ Zar 1996b), transformed some

of the data into normality, such as: -

- age.
- post-chronic exercise HDL_C concentrations.
- pre-chronic exercise TG concentrations.

Where the logarithmic transformation produced a reduction in the variation from a normal distribution, then logarithmic transformed data was investigated parametrically and non-parametrically (plasma total homocysteine concentrations and serum NEFA concentrations). If the transformation did not reduce the variation from a normal distribution, the data was investigated parametrically and non-parametrically using the original data (heart rate, plasma glutamine and BMI).

Independent samples were compared non-parametrically using Mann-Whitney U test and parametrically using the Student's 't' test. Matched samples were compared non parametrically using Wilcoxon matched pairs and parametrically using the paired Student's 't' test. Interactions were investigated using a two way ANOVA and relationships checked using *post hoc* Tukey honest significant difference tests and non-parametrically using the Kruskal-Wallis ANOVA by ranks (independent samples), and the Friedman ANOVA with the Kendall's coefficient of concordance (paired samples).

All blood metabolites obtained post-chronic exercise, were assumed to have experienced a 7% blood volume expansion and so were multiplied by 1.07, (refer to section 3.6.2iii).

4.2 Baseline data

4.2.1 Anthropometric Data

The non-paired Student's 't' test showed no significant difference between the normoxic and hypoxic groups for their log-normal age (means±SD 22±2 and 22±3 years

old respectively, p =0.849); body mass (means±SD 74.5±6.3 and 74.6±8.6 kg respectively, p =0.980) or sum of skinfolds, (means±SD 30.1±7.3 and 30.4±7.5 mm respectively, p =0.932).

Their body mass index (BMI, means±SD 23.90±1.26 and 23.23±1.88 $kg.m^{-2}$ respectively) showed no significant difference parametrically or non-parametrically (p=0.267 and p=0.261 respectively).

4.2.2 Homocysteine and related compounds

There was no difference between the normoxic and hypoxic groups' logarithmic plasma total homocysteine concentrations and related compounds, (refer to Table 4.2.2)

However, there tended to be a stronger correlation (Pearson r) in the hypoxic group than the normoxic group with respect to: red blood cell folate concentration and logarithmic plasma total homocysteine concentration (r = -0.716 and -0.081 respectively); rbcfolate concentration and red blood cell number (r = -0.043 and -0.256 respectively); rbcfolate concentration and plasma glutamine concentration (r = +0.363 and +0.177 respectively); serum vitamin B_{12} concentration and logarithmic plasma total homocysteine concentration (r = -0.570 and -0.411 respectively); Vitamin B_{12} concentration and daily protein intake per kilogram body mass (r = +0.294 and -0.071 respectively).

4.2.3 Plasma Lipid Data

There was no difference between the normoxic and hypoxic groups' pre-chronic exercise log-normal lipid profiles, (refer to Table 4.2.3).

There tended to be strong correlations (Pearson r) in the normoxic and hypoxic groups between their logarithmic serum HDL cholesterol concentration and

compound	mean±SD		Logarithmic transformation	Statistic	
	Normoxic	Hypoxic		parametric	non-parametric
[tHCy]	6.9±1.2	7.5±2.2	yes	p=0.673	p=0.244
[rbcfolate]	869±252	804±231	n/a	p=0.468	
[vitaminB_{12}]	383±129	373±129	n/a	p=0.832	
#rbc	5.10±0.38 x10^{12}	5.02±0.23 x10^{12}	n/a	p=0.486	
[Gln]	633±83	605±107	no	p=0.426	p=0.146
Protein intake	1.24±0.22	1.27±0.42	n/a	p=0.775	

Table 4.2.2 Statistical significance for logarithmic plasma total homocysteine concentration and related compounds

Key:

[tHCy] plasma total homocysteine concentration (μmol.dm^{-3})

[rbcfolate] red blood cell folate concentration (nmol.dm^{-3})

[vitaminB_{12}] serum vitamin B_{12} concentration (pmol.dm^{-3})

#rbc number of red blood cells per dm^3

[Gln] plasma glutamine concentration (μmol.dm^{-3})

Protein intake daily protein intake per kilogram body mass (g.day^{-1}.kg(BM)$^{-1}$)

n/a not applicable, data was normally distribute

If the logarithmic transformation produced normality, then it was used with parametric statistics.

If the logarithmic transformation of the data had not produced normal distribution, but had reduced the variation, parametric and non-parametric statistics were used on the logarithmic transformed data.

If the logarithmic transformation of the data had not produced normal distribution, nor had reduced the variation, parametric and non-parametric statistics were used on the original data.

compound	mean±SD		Logarithmic transformation	Statistic	
	Normoxic	Hypoxic		parametric	non-parametric
A_1	1.44±0.16	1.37±0.16		p=0.269	
B_{100}	0.86±0.25	0.83±0.22		p=0.775	
[tC]	4.36±0.94	4.56±0.68	yes	p=0.427	
$[HDL_C]$	1.6±0.4	1.6±0.4	yes	p=0.952	
$[LDL_C]$	2.59±0.95	2.77±0.82	yes	p=0.494	
[TG]	0.93±0.33	0.98±0.40	yes	p=0.720	
[NEFA]	0.26±0.15	0.33±0.13	yes	p=0.116	p=0.096

Table 4.2.3 Statistical significance for apolipoproteins and log-normal lipids

Key:

A_1	serum apolipoprotein A_1 ($g.dm^{-3}$)
B_{100}	serum apolipoprotein B_{100} ($g.dm^{-3}$)
[tC]	serum total cholesterol concentration ($mmol.dm^{-3}$)
$[HDL_C]$	serum high density lipoprotein cholesterol concentration ($mmol.dm^{-3}$)
$[LDL_C]$	calculated low density lipoprotein cholesterol concentration ($mmol.dm^{-3}$)
[NEFA]	non-esterified fatty acid concentration ($mmol.dm^{-3}$)
n/a	not applicable, data was normally distribute

If the logarithmic transformation produced normality, then it was used with parametric statistics.

If the logarithmic transformation of the data had not produced normal distribution, but had reduced the variation, parametric and non-parametric statistics were used on the logarithmic transformed data.

If the logarithmic transformation of the data had not produced normal distribution, nor had reduced the variation, parametric and non-parametric statistics were used on the original data.

apolipoprotein A_1 (r =+0.906 and +0.757 respectively) and between their logarithmic calculated LDL cholesterol concentration and apolipoprotein B_{100} (r =+0.886 and +0.954 respectively).

4.3 The effect of exercise and hypoxia upon plasma total homocysteine concentration

4.3.1 The effect of acute cycle test upon plasma total homocysteine concentration

4.3.1i Pre-chronic, acute normoxic cycle test

A two-way ANOVA, comparing the normoxic and hypoxic groups' logarithmic plasma total homocysteine concentrations, pre- and post-acute cycle test showed no interaction (p=0.286). Only the normoxic group's non-parametric Friedman ANOVA (pre-post acute cycle test), showed a significant increase in plasma total homocysteine ($p<0.033$, refer to Table 4.3).

4.3.1ii Post-chronic, acute normoxic cycle test

A two-way ANOVA, comparing the normoxic and hypoxic groups' logarithmic plasma total homocysteine concentrations, pre- and post-acute cycle test showed no interaction (p=0.121). Only the hypoxic group's non-parametric Friedman ANOVA (pre-post acute cycle test), showed a significant increase in plasma total homocysteine ($p<0.012$, refer to Table 4.3).

	mean±SD		Kruskal-Wallis ANOVA
	Normoxic n=14	Hypoxic n=16	
[tHCy]A	6.9±1.2	7.5±2.2	p=0.244
[tHCy]B	7.6±1.8	7.3±2.0	p=0.787
Friedman ANOVA - AB Kendall's coefficient	***p<0.033** W = 0.327	p<0.439 W = 0.038	
[tHCy]C		7.7±2.1	
Wilcoxon -AC		p=0.408	
[tHCy]D	8.2±1.9	7.1±1.9	p=0.081
Friedman ANOVA - AD Kendall's coefficient	****p<0.001** W=0.735	p<0.617 W=0.016	
[tHCy]E	8.1±1.5	8.2±2.5	p=0.723
Friedman ANOVA - DE Kendall's coefficient	p<0.593 W = 0.020	***p<0.012** W = 0.391	
[tHCy]AB	-0.6±1.3	0.2±1.5	***p=0.044**
[tHCy]DE	0.1±1.8	-1.2±2.1	p=0.064
Friedman ANOVA Kendall's coefficient	p<0.109 W=0.184	p<0.134 W=0.141	

Table 4.3: Non-parametric statistics for plasma total homocysteine concentration

Key:

[tHCy]	plasma total homocysteine concentration (μmol.dm^{-3})
A	at rest, pre-chronic exercise, pre-acute cycle test.
B	pre-chronic exercise, post-acute normoxic cycle test.
C	pre-chronic exercise, post-acute hypoxic cycle test.
D	at rest, post-chronic exercise, pre-acute cycle test.
E	pre-chronic exercise, post-acute normoxic cycle test.
AB	difference between [tHCy]A-[tHCy]B
DE	difference between [tHCy]D-[tHCy]E
*	p<0.05 - significant
**	p<0.01 - very significant

4.3.1iii Pre-chronic, acute hypoxic cycle test

The hypoxic acute cycle test produced no change in logarithmic plasma total homocysteine concentration using parametric (Student's 't' test p=0.376) and non-parametric (refer to Table 4.3) statistics.

4.3.2 The effect of chronic exercise upon plasma total homocysteine concentration

4.3.2i Pre-/Post-chronic exercise

A 2-way ANOVA, with the group as the independent variable and the logarithmic plasma total homocysteine concentration pre- and post-chronic exercise as the dependent variables showed a significant interaction, (p=0.015). The *post hoc* Tukey honest significant difference test for the normoxic group pre-/post-chronic exercise plasma total homocysteine concentration significantly increased (p=0.043), whereas the hypoxic group did not change (p=0.857). The *post hoc* Tukey honest significant difference for unequal sample size test, comparing the normoxic and hypoxic groups' post-chronic exercise plasma total homocysteine concentrations were significantly different (p=0.045), whereas the pre-chronic exercise were not (p=0.868). Only the non-parametric Friedman ANOVA for the normoxic group showed a significant increase in plasma total homocysteine concentration (p<0.001, refer to Table 4.3).

4.3.2ii Comparison of acute normoxic cycle test, pre/-post-chronic exercise

A 2-way ANOVA, with the group as the independent variable and the plasma total homocysteine concentration differences (pre-/post-acute normoxic cycle test), pre- and post-chronic exercise as the dependent variables showed no significant interaction, (p=0.050). The *post hoc* Tukey honest significant difference tests showed no significant

differences. The Kruskal-Wallis ANOVA non-parametric test showed a significant difference between the groups' pre-chronic exercise acute cycle test plasma total homocysteine concentration difference ($p=0.044$, refer to Table 4.3).

4.4 The effect of chronic exercise upon the Lipid profile

4.4.1 Serum total Cholesterol

A 2-way ANOVA fixed effects (independent variable -normoxic/hypoxic; dependent variables - logarithmic total cholesterol) had no significant interaction ($p=0.301$, normoxic group pre-chronic exercise 4.36±0.94 $mmol.dm^{-3}$; post- 3.86±0.61 $mmol.dm^{-3}$; hypoxic group pre- 4.56±0.68 $mmol.dm^{-3}$; post- 4.24±0.53 $mmol.dm^{-3}$).

4.4.2 Serum Apolipoprotein A_1

A 2-way ANOVA fixed effects (independent variable -normoxic/hypoxic; dependent variables - apolipoprotein A_1) had a significant interaction ($p<0.001$, normoxic group pre-chronic exercise 1.44±0.16 $g.dm^{-3}$; post- 1.24±0.12 $g.dm^{-3}$; hypoxic group pre- 1.37±0.16 $g.dm^{-3}$; post- 1.37±0.24 $g.dm^{-3}$). *Post hoc* Tukey tests showed that there were significant changes in the normoxic pre-/post-chronic exercise apolipoprotein A_1 concentration ($p<0.001$), and between the normoxic and hypoxic post-chronic exercise concentrations ($p=0.005$).

4.4.3 Serum High Density Lipoprotein

A 2-way ANOVA fixed effects (independent variable -normoxic/hypoxic; dependent variables - log-normal high density lipoprotein cholesterol) had showed no interaction ($p=0.577$, normoxic group pre-chronic exercise 1.6±0.4 $mmol.dm^{-3}$; post-

1.5±0.3 mmol.dm^{-3}; hypoxic group pre- 1.6±0.4 mmol.dm^{-3}; post- 1.6±0.5 mmol.dm^{-3}).

4.4.4 Serum Apolipoprotein B_{100}

A 2-way ANOVA fixed effects (independent variable -normoxic/hypoxic; dependent variables - apolipoprotein B_{100}) had a significant interaction (p=0.003, normoxic group pre-chronic exercise 0.86±0.25 g.dm^{-3}; post- 0.76±0.15 g.dm^{-3}; hypoxic group pre- 0.83±0.22 g.dm^{-3}; post- 0.88±0.24 g.dm^{-3}). *Post hoc* Tukey tests showed that there were significant changes in the normoxic pre-/post-chronic exercise apolipoprotein B_{100} concentration (p=0.0005), and between the post-chronic exercise concentrations, (p=0.005).

4.4.5 Calculated serum Low Density Lipoprotein

A 2-way ANOVA fixed effects (independent variable -normoxic/hypoxic; dependent variables - logarithmic calculated low density lipoprotein cholesterol) had no interaction (p=0.923, normoxic group pre-chronic exercise 2.59±0.95 mmol.dm^{-3}; post 2.47±0.53 mmol.dm^{-3}; hypoxic group pre- 2.77±0.82 mmol.dm^{-3}; post- 2.68±0.68 mmol.dm^{-3}).

4.4.6 Serum triacylglycerol

A 2-way ANOVA fixed effects (independent variable -normoxic/hypoxic; dependent variables log-normal serum triacylglycerol) had a significant interaction (p=0.034, normoxic group pre-chronic exercise 0.93±0.33 mmol.dm^{-3}; post- 0.99±0.40 mmol.dm^{-3}; hypoxic group pre- 0.98±0.40 mmol.dm^{-3}; post- 1.23±0.40 mmol.dm^{-3}). *Post hoc* Tukey tests showed that there were significant changes in the hypoxic pre-/post-chronic exercise serum triacylglycerol concentration (p=0.028), and between the post-chronic exercise concentrations, (p=0.004).

4.4.7 Serum non-esterified fatty acid

A 2-way ANOVA fixed effects (independent variable -normoxic/hypoxic; dependent variables - serum non-esterified fatty acid) had a significant interaction ($p=0.028$, normoxic group pre-chronic exercise 0.26 ± 0.15 mmol.dm^{-3}; post- 0.18 ± 0.09 mmol.dm^{-3}; hypoxic group pre- 0.33 ± 0.13 mmol.dm^{-3}; post- 0.16 ± 0.09 mmol.dm^{-3}). The *post hoc* comparison Tukey honest significant difference for equal samples showed a significant decrease in the normoxic group's non-esterified fatty acid concentration pre-/post-chronic ($p=0.022$) and hypoxic group's ($p=0.0002$). Whereas, non-parametric analysis showed no difference between the normoxic and hypoxic NEFA concentrations pre- and post-chronic exercise ($p=0.096$ and $p=0.835$, respectively). The effect of chronic exercise upon the hypoxic group's NEFA concentration was to significantly reduce it ($p<0.0001$, $W=0.938$).

4.5 The effect of chronic exercise upon aerobic power, lactate threshold and heart rate.

4.5.1. Comparison of the Normoxic acute cycle tests pre-/post-chronic exercise

4.5.1i Aerobic power

A 2-way ANOVA fixed effects (independent variable -normoxic/hypoxic; dependent variables - normoxic acute cycle tests) had no interaction ($p=0.568$, refer to Table 4.5.1).

4.5.1ii Lactate

A 2-way ANOVA fixed effects (independent variable -normoxic/hypoxic;

		mean±1SD		Tukey HSD (unequal sample sizes)	Kruskal-Wallis ANOVA
		N n=14	H n=16		
$\dot{V}O_{2\,peak}$ (BM) ($cm^3.kg^{-1}.min^{-1}$)	B	53.3±9.4	48.0±9.1	p=0.116	
	E	56.6±8.7	53.1±7.4	p=0.431	
Tukey HSD		p=0.502	p=0.109	interaction p=0.568	
[La] $mmol.dm^{-3}$	B	9.8±2.0	8.2±1.5	**p=0.019**	
	E	8.7±1.6	9.1±1.5	p=0.927	
Tukey HSD		p=0.194	p=0.277	interaction ***p=0.010**	
HR_{max} ($beats.min^{-1}$)	B	191±13	190±7	p=0.886	p=0.900
	E	193±10	192±7	p=0.924	p=0.851
Tukey HSD		p=0.507	p=0.389	interaction p=0.941	
Friedman ANOVA Kendall's coef. concordance		p<0.593 W=0.020	p<0.197 W=0.104		

Table 4.5.1 The effect of chronic exercise upon certain parameters of the acute cycle test

Key:

$\dot{V}O_{2\,peak}$ (BM) = aerobic power
[La] = maximum lactate concentration
HR_{max} = maximum heart rate at exhaustion.
N = normoxically exercised
H = Hypoxically exercised subjects.
B = acute normoxic cycle test, pre-chronic exercise
E = acute normoxic cycle test, post-chronic exercise.
HSD = honest significant difference
coef = coefficient
* p<0.05 = significant difference

[maximum heart rate data was not normally distributed, therefore it was investigated using parametric and non-parametric statistics]

dependent variables - normoxic acute cycle tests maximum lactate concentrations) had a significant interaction (p=0.010). The *post hoc* comparison Tukey test showed a significant difference (pre-chronic exercise) between the normoxic and hypoxic groups, (p=0.019, refer to Table 4.5.1).

4.5.1iii Heart rate

A 2-way ANOVA fixed effects (independent variable -normoxic/hypoxic; dependent variables - normoxic acute cycle tests' maximum heart rates) showed no significant interaction (p=0.941). Non-parametrically, the groups did not show any change in their maximum heart rates pre- to post-chronic exercise (refer to Table 4.5.1).

4.5.2. Comparison of the Normoxic and Hypoxic acute cycle tests pre-chronic exercise

4.5.2i Aerobic power

There was no change between the aerobic power obtained from the normoxic acute cycle test and that obtained from the hypoxic test (refer to Table 4.5.2).

4.5.2ii Lactate

There was no change between the maximum lactate concentration obtained from the normoxic acute cycle test and that obtained from the hypoxic test (refer to Table 4.5.2).

4.5.2iii Heart rate

There was no change between the maximum heart rate obtained from the normoxic and hypoxic acute cycle tests, either parametrically or non-parametrically (refer to Table 4.5.2).

Hypoxic group (n=16)	mean±1SD		Student's 't' test	Wilcoxon
	B	C		
$\dot{V}O_{2\ peak}$ (BM) ($cm^3.kg^{-1}.min^{-1}$)	48.0±9.1	42.2±7.8	p=0.094	
[La] $mmol.dm^{-3}$	8.2±1.5	8.7±1.5	p=0.068	
HR_{max} ($beats.min^{-1}$)	190±7	187±15	p=0.321	p=0.514

Table 4.5.2 Comparison of the Normoxic and Hypoxic acute cycle tests pre-chronic exercise

Key:

$\dot{V}O_{2\ peak}$ (BM)	= aerobic power
[La]	= maximum lactate concentration
HR_{max}	= maximum heart rate at exhaustion.
B	= acute normoxic cycle test, pre-chronic exercise
C	= acute hypoxic cycle test, pre-chronic exercise.
HSD	= honest significant difference
*	$p<0.05$ = significant difference

[maximum heart rate data was not normally distributed, therefore it was investigated using parametric and non-parametric statistics]

Part III

Science needs to be lived alongside religion, philosophy,

history and aesthetic experience; alone it can lead

to great harm.'

Needham, J

1900 - 1998

(*Science and Civilization in China*

with the research assistance of Wang Ling

London, Cambridge University Press, 1954)

Chapter 5

Discussion

5.1 General Findings

The following inferences, can in my opinion be drawn from the results of this experiment as the normoxic and hypoxic groups' baseline data showed no significant differences.

- Acute normoxic cycle test produces a significant but not physiological increase in plasma total homocysteine concentration.
- Chronic normoxic intermittent exercise produces a significant but not physiological increase in plasma total homocysteine concentration
- Hypoxia inhibits the effects of exercise stress upon plasma total homocysteine concentration.
- Chronic intermittent exercise had no effect upon the serum cholesterol concentrations.
- Chronic intermittent hypoxic exercise caused a significant increase in triacylglycerol concentration, but not a physiological increase.
- Chronic normoxic and hypoxic intermittent exercise produced a significant decrease in serum non-esterified fatty acid.
- The acute cycle tests produced no significant difference between the normoxic and hypoxic groups for aerobic power and maximum heart rate.
- The normoxic acute cycle tests pre and post chronic intermittent exercise showed a significant interaction for maximum lactate concentration. The *post hoc* test showed a significant difference between the groups pre-chronic exercise. The normoxic group decreased their maximum lactate concentrations, whereas the hypoxic group increased theirs.
- There was no significant difference between the normoxic and hypoxic acute cycle

tests pre-chronic intermittent exercise for aerobic power, maximum lactate concentration and heart rate.

5.2 Homocysteine

5.2.1 Baseline Homocysteine

There was no significant difference in the means of the plasma total homocysteine concentrations between the normoxic and hypoxic groups at the beginning of this study, (mean±SD 6.9±1.2 $\mu mol.dm^{-3}$, 7.5±2.2 $\mu mol.dm^{-3}$ respectively), thereby implying that the two groups represented the same population. The National Centre for Health Statistics reported that the geometric mean for serum homocysteine concentration in the second decade of life was 7.7 $\mu mol.dm^{-3}$ and in the third decade of life was 9.3 $\mu mol.dm^{-3}$ (DHHS 1992). This study's groups spanned both decades (19-28 years old), but their geometric means were lower (6.8 $\mu mol.dm^{-3}$ normoxic; 7.1 $\mu mol.dm^{-3}$ hypoxic), which could be due to their healthier lifestyles of diet and exercise. Große-Scharmann et al. (1995) found that top athletes had significantly lower levels of homocysteine than leisure time athletes.

This study found that logarithmic plasma total homocysteine concentration was negatively correlated to red blood cell folate in the hypoxic group (Pearson r =-0.081 normoxic; r = -0.716 hypoxic) and also serum vitamin B_{12} (Pearson r = -0.411 normoxic; r = -0.570 hypoxic). A negative correlation between plasma total homocysteine concentration and plasma folate and also vitamin B_{12} had previously been reported (Verhoef et al., 1996). Quinlivan et al., (2002) found that the correlation between plasma total homocysteine and a high folate intake was poor, but the vitamin B_{12} showed a greater negative correlation. Dietary experiments pointed to a plateau, above which, further consumption of folate and serum vitamin B_{12} concentrations produced no further reduction in plasma total homocysteine concentration (Ward et al.,

1997). In this study, the groups' geometric means for red blood cell folate concentration (837 and 773 nmol.dm^{-3} normoxic and hypoxic groups' respectively) were in top end of the National Health reference range (folate 408 - 1133 nmol.dm^{-3}), whereas this study's vitamin B_{12} levels (362 and 355pmol.dm^{-3} normoxic and hypoxic groups' respectively) were at the lower end of the National Health reference range (B_{12} 163 - 590 pmol.dm^{-3}). The lack of correlation between the plasma total homocysteine and rbc folate concentrations in the normoxic group, but not the hypoxic group could be due to the relatively high levels of folate and low levels of serum vitamin B_{12}. Also, the poor correlation between logarithmic plasma total homocysteine and red blood cell folate could be related to factors such as methylenetetrahydrofolate reductase (MTHFR) genotype and the type and quantity of homocysteine transport channels in the plasma membrane. The poor correlation was unlikely to be related to exercise, as the subjects had not undertaken any physical activity for forty-eight hours and Weiß et al. (1999) had shown that twenty-four hours post acute exercise, the plasma total homocysteine concentration would have started to return to pre-acute exercise levels and would be no longer significantly different.

The serum total cholesterol concentration was not correlated to plasma total homocysteine concentration in the normoxic group (Pearson, $r = -0.028$ normoxic group; $r = 0.421$ hypoxic group). Yet the high density lipoprotein cholesterol concentration was significantly correlated to plasma total homocysteine in the normoxic group, but not in the hypoxic group ($r = 0.690$, normoxic group; $r = 0.333$ hypoxic group). The normoxic group subjects did have a higher normoxic aerobic fitness and a lower negative correlation to plasma total homocysteine (normoxic group 53.3±9.4 cm^3.kg^{-1}.min^{-1}, $r = -0.300$; hypoxic group 48.0±9.1 cm^3.kg^{-1}.min^{-1}, $r = -0.599$) and this could be interpreted as that the fitness level and type of training affects the clearance of plasma total homocysteine to the kidneys and liver and/or that fitter people tend to have a better diet, but that there could be a plateau effect. The correlation between rbc folate and normoxic aerobic fitness appears to be dependent upon their mean rbc folate concentration and a possible plateau effect ($r = -0.017$ normoxic group; $r = 0.425$ hypoxic group). Myocardial infarction

patients have been found to have a significant correlation between their serum total homocysteine concentration and their low-density lipoprotein cholesterol and high-density lipoprotein cholesterol concentrations, (Qujeq et al., 2001). This study's serum triacylglycerol concentrations were not strongly correlated, particularly in the hypoxic group, ($r = -0.211$ normoxic group; $r = 0.024$ hypoxic group).

The four year follow-up of the Hordaland study showed that the progressive increase in plasma homocysteine increased the risk of mortality, in cardiovascular and non cardiovascular causes of death (Vollset et al., 2001). A $5\mu mol.dm^{-3}$ was associated with a 49% increase in all-cause mortality, (50% increase in cardiovascular mortality, 26% increase in cancer, and a 104% increase in non-cancer, non-cardiovascular mortality). Fenech (2001) commented in his review that human intervention studies show that red cell folate concentration above $700nmol.dm^{-3}$, plasma B_{12} concentrations above 300 $pmol.dm^{-3}$ and plasma homocysteine less than $7.5\mu mol.dm^{-3}$ are associated with minimization of DNA hypomethylation and chromosomal breaks. DNA hypomethylation has been associated with cancer (Ehrlich 2002) and atherosclerosis (Hiltunen et al. 2002). The baseline plasma total homocysteine, rbc folate and vitamin B_{12} status implies that the students in this study would have minimal DNA hypomethylation and chromosomal breaks.

5.2.2 Homocysteine and normoxic exercise

Many of the studies looking at normoxic exercise with different durations and intensities have shown that exercise increases plasma total homocysteine concentration, (Große-Scharmann et al. 1995; Weiss et al. 1998; Lussier-Cacan et al. 1998; Weiß et al. 1999). The study by De Crée et al. (2000) was a one hour acute submaximal exercise and produced no change in plasma total homocysteine concentration in seven young males (mean age 21.6 years), but it could be because it was at a low intensity (60% $\dot{V}O_{2max}$). Yet when they (DeCrée et al. 1999) had looked at acute exercise to exhaustion in fifteen

eumenorrheic women (mean age 18.7 years), they had shown a significant increase in plasma homocysteine. Whereas the study by Wright et al. (1998), concluded that the increase in plasma homocysteine post exercise was due to plasma albumin changes. The implication that plasma total homocysteine concentration post exercise stress above a threshold causes a significant increase, albeit for a short time was born out partly by this study's data and that this increase could be related to rbc folate concentration and exercise induced changes in plasma albumin concentration.

5.2.2i Homocysteine and acute normoxic exercise

Pre-chronic, acute normobaric normoxic cycle test affected the groups differently. The normoxic group's logarithmic plasma total homocysteine concentration significantly increased non-parametrically post acute exercise, but not paramertrically (mean±SD; pre=6.9±1.2μmol.dm^{-3}, post=7.6±1.8μmol.dm^{-3}; Wilcoxon matched pairs p=0.035 and t-test p=0.095), whereas the hypoxic group showed no significant difference (mean±SD; pre=7.5±1.8μmol.dm^{-3}, post=7.3±2.0μmol.dm^{-3}, Wilcoxon matched pairs p=0.570 and t-test p=0.904). The two subjects in the normoxic group that showed the largest increase in plasma homocysteine also had the lowest rbc folate concentrations (564-566 nmol.dm^{-3}). The hypoxic group's difference was not just affected by their rbc folate levels, but probably also whether they undertook the normoxic or hypoxic acute cycle test first.

Post-chronic, acute normobaric normoxic cycle test also affected the groups differently. The normoxic group's logarithmic plasma total homocysteine concentration did not change significantly (mean±SD; pre=8.2±1.9 μmol.dm^{-3}, post=8.1±1.5 μmol.dm^{-3}; Wilcoxon matched pairs p = 0.638 and t-test p = 0.938), whereas the hypoxic group showed a significant increase (mean±SD; pre=7.1±1.9μmol.dm^{-3}, post=8.2±2.5μmol.dm^{-3}, Wilcoxon matched pairs p=0.044 and t-test p=0.057).

This study shows that an acute normoxic exercise can cause a significant increase in plasma total homocysteine concentration. When it did not occur there were two possible reasons: 1. In the hypoxic group pre-chronic exercise, some of the group had undertaken the hypoxic acute cycle test first; 2. In the normoxic group post-chronic exercise, the subjects were already at an elevated homocysteine concentration. This could indicate that exercise causes an increase in plasma total homocysteine concentration to a plateau and that further exercise stress does not increase the level further. This plateau concept requires further investigation as it could be related to the level of folate and vitamin B_{12}, that is, the higher the rbc folate concentration, the lower the increase in plasma total homocysteine concentration caused by the particular intensity and duration of the acute exercise session.

5.2.2ii Homocysteine and chronic normoxic exercise

Chronic normoxic intermittent exercise produced a significant, but not a physiological increase in logarithmic plasma total homocysteine concentration. (pre-chronic exercise 6.9±1.2 $\mu mol.dm^{-3}$, post-chronic exercise 8.2±1.9 $\mu mol.dm^{-3}$, Wilcoxon matched pairs p=0.002), but this was less than the calculated minimal detectable difference (2.20 $\mu mol.dm^{-3}$, appendix VII). Große-Scharmann et al., (1995) only found a slight increase in plasma total homocysteine concentration over an eight week training period. Whereas Nygård et al., (1995) - found an inverse relationship between plasma total homocysteine concentration and physical activity levels, but people who tend to exercise also tend to have a healthier lifestyle. Lussier-Cacan et al. (1998) found that a twenty-week endurance programme produced a 5.5% mean increase in plasma total homocysteine. A short term increase could be beneficial, in producing more glutathione (Figure 1.3.1), or glycosaminoglycan sulphate(VI) (Figure 1.3.3).

5.2.3 Homocysteine and hypoxic exercise

5.2.3i Homocysteine and acute hypoxic exercise

Acute normobaric hypoxic cycle test did not produce a significant change in logarithmic plasma total homocysteine concentration (mean±SD; pre - 7.5±2.2 μmol.dm^{-3}, post-acute cycle test 7.7±2.1 μmol.dm^{-3}, t-test p=0.376 and Wilcoxon matched pairs p=0.408). This was different to the effect of acute normoxic exercise and may be related to a hypoxic induced vasodilation by increasing nitrogen(II)oxide synthase and decreasing endothelin-1, which changes blood volume and flow rates (Lee et al. 1997).

5.2.3ii Homocysteine and chronic hypoxic exercise

Chronic hypoxic intermittent exercise produced no significant change (pre-chronic exercise 7.5±2.2 μmol.dm^{-3}, post-chronic exercise 7.1±1.9 μmol.dm^{-3}, p=0.361). Hypoxia was thought to increase i-NOS and HO-1 genes and thus vasodilation as well as VEGF gene causing angiogenesis, (refer to Figure 1.8.1), which could improve blood flow during exercise and thus removal of the egressed homocysteine more efficiently. Work on wild type (myo$^{+/+}$) and myoglobin knockout (myo$^{-/-}$) mice found no change in the hypoxia-inducible factor 1alpha under hypoxic conditions, (HIF-1α, Schlieper et al., 2002). This would indicate that reduced arterial oxygen partial pressure is not required to up regulate HIF-1α. Yet a similar duration and level of hypoxia in rats produced a 25-fold increase in HIF-1 mRNA and an increase in mass of the right ventricle, (Forkel et al., 2002). Thus the hypoxic induced bronchial constriction caused the enlargement of the right ventricle. It also implied that hypoxia might not have been a direct stimulus to up-regulate HIF-1. It would have been interesting to use ultrasound, to see if the chronic hypoxic exercise had caused a right ventricular enlargement and the effect of a possible increase in erythrocytes (Goldberg et al. 1988), and angiogenesis (Shweiki et al. 1992) due to hypoxia upon blood volume.

5.2.4 Summary of the effect of exercise upon plasma homocysteine

A 2-way ANOVA showed a significant interaction (p=0.026) between pre and post chronic exercise for normoxic and hypoxic groups. Post hoc Tukey tests only showed significance for the normoxic group pre/post chronic exercise (p=0.022) but not for the hypoxic group (p=998), nor between the groups pre- (p=0.948) and post-chronic exercise (p=0.051). Whereas, there were no significant interactions between pre- and post acute cycle test pre- (p=361) and post-chronic exercise (p=0.217)

The acute changes in plasma total homocysteine concentration might be related to the hydration, sodium and albumin plasma status of the subject, which would affect the sodium transporter channels. Channels which might be involved with the transport of homocysteine across the plasma membrane and albumin, the transport of homocysteine in the plasma. The hydration and sodium status are affected by exercise and the subjects were asked to be fully hydrated before the acute cycle tests. The chronic changes in plasma total homocysteine concentration is probably more related to rbc folate concentration and this could account for individual variation, but would not account for the apparent significant increase, but not physiological increase in plasma total homocysteine concentration post acute and chronic normoxic exercises and apparent no change post acute and chronic hypoxic exercises. Thus implying that the hypoxic stimulus affects the thiol biochemistry.

Diet (Verhoef et al. 1996), exercise (Große-Scharmann et al., 1995; Weiß et al 1999), mental stress (Stoney et al 1999, 2000), genetics (Goyatta et al., 1994; Frosst et al., 1995), transmethylation reactions (guanidinoacetate/creatine Stead et al., 2001) or adenosine (Harrington et al., 2000) and the ratio of S-adenosylmethionine / S-adenosylhomocysteine appear to affect cellular homocysteine. S-adenosylmethionine is an allosteric inhibitor of methylenetetrahydrofolate reductase (Sumner et al., 1986), an in vitro inhibitor of betaine-homocysteine methyltransferase (Finkelstein & Martin 1984) and an activator of cystathionine β-synthase (Finkelstein et al., 1975). Heart localised

circulation is affected by adenosine, which is provided by S-adenosylhomocysteine during normoxic exercise, but from 5' adenosine monophosphate during hypoxic exercise (Deussen et al., 1989). This difference could be part of the reason for the significant effect upon plasma total homocysteine concentration in normoxically exercising subjects in comparison to hypoxically exercising subjects. The effect of this might be able to be reduced if the subject's rbc folate concentration was relatively high. Further study is required to look at the effect of adenosine in myocardial contraction and folate status in relation to plasma total homocysteine concentration in the athlete as the vasodilation of coronary vessels is not fully understood (Tune et al., 2002).

5.3 The effect of acute and chronic exercise upon the lipid profile

5.3.1 Total Cholesterol

The subjects' serum total cholesterol concentrations were below the recommend maximum level ($\leq$4.7 mmol.dm^{-3}). There was no significant difference between the normoxic and hypoxic group's pre-chronic exercise logarithmic serum total cholesterol concentration ($p=0.427$, refer to Table 4.2.3). A 2-way ANOVA showed that chronic exercise did not produce a significant change in the logarithmic serum total cholesterol ($p=0.301$). Wood et al. (1976) found lower quantities of cholesterol, triacylglycerol and low density lipoprotein cholesterol in very active middle age male runners, (running more than twenty-four kilometres per week), whereas Williams et al. (1982) found that the threshold for reductions in cholesterol was sixteen kilometres per week. This is in agreement with the findings of Durstine and Haskell (1994) that physically active individuals do not significantly lower their plasma total cholesterol concentration in comparison to inactive individuals. The subjects in this study were already physically active before under taking the study and probably already had an increased lipolysis.

5.3.2 High Density lipoproteins and apolipoprotein A_1

The subjects' tended to have relatively high levels of high density lipoprotein cholesterol (1.6 mmol.dm^{-3}), and this study took place in the summer months when cholesterol values tend to be reduced. This implied that their lipid transport system was favoured in cholesterol removal from tissues and transport to the liver.

There was no significant difference between the normoxic and hypoxic group's pre-chronic exercise logarithmic serum high density lipoprotein cholesterol concentration ($p=0.952$, refer to Table 4.2.3) or apolipoprotein A_1 concentration ($p=0.269$). As expected, serum high density lipoprotein cholesterol and apolipoprotein A1 are significantly correlated (normoxic group: Pearson's $r=+0.906$; hypoxic group: $r=+0.757$).

A 2-way ANOVA for Apolipoprotein A_1 showed a significant interaction ($p<0.001$) and Tukey tests showed significant differences between pre-/post-chronic exercise in the normoxic group ($p<0.001$) and between the groups post-chronic exercise ($p=0.005$). The hypoxically exercised group did not change their level of Apolipoprotein A_1 whereas the normoxic group decreased it. Yet the ratio of apolipoprotein A_1:B_{100} which Naito (1985) stated was a better predictor of coronary artery disease showed no great change. The normoxic group (pre-chronic exercise 1:0.61, post-chronic exercise 1:0.58) and the hypoxic group (pre-chronic exercise 1:0.61, post-chronic exercise 1:0.62).

A 2-way ANOVA showed there was no interactive effect. Hypoxic and normoxic chronic exercise had no effect upon high density lipoprotein concentration. As the subjects were already active, this additional activity did not increase the removal of cholesterol from the tissue and its transport to the liver.

5.3.3 Low Density Lipoproteins and apolipoprotein B_{100}

There was no significant difference between the normoxic and hypoxic group's pre-chronic exercise serum calculated low-density lipoprotein cholesterol concentration ($p=0.494$, refer to Table 4.2.3) or apolipoprotein B_{100} concentration ($p=0.775$). As expected, serum calculated low-density lipoprotein cholesterol and apolipoprotein B_{100} are significantly correlated, (normoxic group: Pearson's $r=+0.886$; hypoxic group: $r=+0.954$).

A 2-way ANOVA for Apolipoprotein B_{100} showed a significant interaction ($p=0.003$) and *Post hoc* Tukey tests showed significant differences between pre-/post-chronic exercise in the normoxic group ($p=0.0005$) and between the groups post-chronic exercise ($p=0.005$). The hypoxically exercised group did not significantly increase their level of Apolipoprotein B_{100} whereas the normoxic group decreased it. As their low density lipoprotein concentrations are below the upper value for low cardiovascular disease risk (3.4 $mmol.dm^{-3}$), and their total and classes of cholesterol (HDL, and LDL) showed no significant change. The difference in apolipoprotein B_{100} could be due to recent dietary intake, as it is found on all the lipoprotein fractions except high density lipoprotein.

A 2-way ANOVA showed there was no interactive effect ($p=0.923$). Hypoxic and normoxic chronic exercise had no effect upon low density lipoprotein concentration. As the subjects were already physical active before this study, and tended to eat a balanced diet, this study had no effect upon their cholesterol levels.

5.3.4 Triacylglycerol

There was no significant difference between the normoxic and hypoxic groups' pre-chronic exercise serum triacylglycerol concentration, ($p=0.720$, refer to Table 4.2.3).

A 2-way ANOVA showed there was a significant interactive effect (p=0.034). Both groups had increased their triacylglycerol concentrations by the end of the chronic exercise period, but only the hypoxic group's *post hoc* Tukey test was significant increase (p=0.028). Also, there was significance between the groups post-chronic exercise triacylglycerol levels (p=0.004). Low levels have been reported in athletes (Martin et al., 1977; Wood et al., 1977) and as the subjects had low levels to begin with, it implies that they were already physical active. The difference could be due to changes in their meals prior to the blood sampling at the end of the chronic exercise period. Yet these variations are not important in relation to cardiovascular disease risk as they are well below the low risk factor's level (TG< 2.26 mmol.dm^{-3} NCEP 1993).

5.3.5 Non esterified fatty Acid

There was no significant difference between the normoxic and hypoxic groups' pre-chronic exercise serum non-esterified fatty acid (NEFA) concentration (p=0.096, refer to Table 4.2.3).

A 2-way ANOVA for Apolipoprotein A_1 showed a significant interactive effect (p=0.028) and the *post hoc* Tukey tests showed significant decreases in both groups pre-/post-chronic exercise (normoxic group p=0.022 and hypoxic group p=0.0002). Non-parametrically, only the hypoxic group showed a significant reduction in their NEFA concentration pre/post chronic intermittent hypoxic exercise, (p<0.0001).

Saltin and Astrand (1993) stated that a mild to moderate increase in exercise levels could cause an initial drop in the plasma NEFA concentration. As the exercise duration increases, the rate of NEFA released from the cells exceeds the muscle cell uptake, thus causing an increase in plasma levels. It could be that this study's chronic exercise stimulus was only mild and therefore caused a decrease in NEFA concentration. Previous studies' results are equivocal in regards to NEFA mobilization (refer to section 1.8.4), this study implies that an intermittent hypoxic chronic exercise challenge like

aerobic exercise could be mobilizing lipids, but that the challenge was not sufficient to show conclusively either way.

5.4 Exercise

5.4.1 Normoxic exercise

5.4.1i Aerobic Power

A 2 way ANOVA showed there was no interactive effect due to the chronic exercise between the two groups ($p=0.568$). Both groups increased their aerobic power, but not significantly, which can be explained by a small decrease in their body mass and a slight improvement in cardiac capacity. Their aerobic power was at the top end of the physiological norm range for males, 40 to 55 $cm^3.kg^{-1}.min^{-1}$ (Hollmann et al. 1988), it therefore appears that this exercise protocol was not strenuous enough on top of their own training programme to produce significant changes.

5.4.1ii Maximum Lactate

A 2 way ANOVA showed there was a significant interactive effect between the two groups ($p=0.010$). *Post hoc* Tukey tests showed that there was a significant difference between the two groups maximum lactate levels pre-chronic exercise. Implying that either the hypoxic subjects were not as lactate tolerant as the normoxic subjects or that they did not push themselves as hard as they could have. The acute cycle tests were to volitional exhaustion and therefore the subject's psychological state could affect the end point.

5.4.1iii Maximum Heart rate

A 2 way ANOVA showed there was no significant interactive effect

between the two groups (p=0.941).

5.4.2 Comparison between Hypoxic and Normoxic exercise

5.4.2i Aerobic Power

A student's 't' comparing the aerobic power within the hypoxic group between their normoxic and hypoxic tests, showed no significant difference (p=0.094). The hypoxic aerobic power was 88% of its normoxic value. Roach (2000) found that the additional stress of the hypoxic challenge would increase ventilation rate and decrease aerobic power by approximately 96% of that obtain using P_IO_2 19.8kPa.

5.4.2ii Maximum Lactate

A student's 't' comparing the maximum lactate concentration within the hypoxic group between their normoxic and hypoxic acute cycle tests, showed no significant difference (p=0.068).

5.4.2iii Maximum Heart rate

The parametric test (student's 't' test) and the non-parametric test (Wilcoxon matched pairs test) comparing the maximum heart rate for the normoxic and hypoxic acute cycle tests pre-chronic exercise, showed no significant difference (refer to Table 4.5.2). As the acute cycle test is under taken to volitional exhaustion, if they were close to their actual maximum heart rate, changes in the subject's psychological state and therefore effort, would not produce a significant difference.

5.5 Limitations

The main limitation in this study was due to costs and time. Both groups should have undertaken the acute cycle test hypoxic and normoxic challenge pre and post-chronic exercise. So that they both experienced the same stresses except for the chronic exercise. As one group undertook two acute challenges before the chronic period, it could have affected the data.

The blood sample for homocysteine analysis was taken immediately post acute exercise. A graph drawn of plasma homocysteine concentrations post acute exercise (Weiss et al. 1998), showed that the maximum homocysteine concentration was obtained five hours post exercise, and that even after 24 hours it had not returned to the pre exercise level. It can be interpreted in two ways either it takes longer than 24 hours to return to pre-exercise levels or that it does not return to pre exercise levels. Further work is required to see what happens to the plasma total homocysteine concentration after twenty-four hours post acute exercise. The normoxic group of subjects had increased plasma total homocysteine concentration post chronic exercise and showed no additional increase post acute exercise. This could mean that there is an individual upper limit which could possibly be minimised by increasing folate prior to the exercise session.

Further work is required to investigate the effect of hypoxic exercise upon plasma total homocysteine concentration in terms of exercise duration, intensity and lower partial oxygen pressure. This study showed no change in homocysteine concentration post acute and chronic hypoxic exercise. Further work is therefore required to see how long the effect lasts. The normoxic acute cycle test five days post last hypoxic training session produced a statistically significant increase in plasma total homocysteine (7.1 to 8.2 $\mu mol.dm^{-3}$, $p<0.012$), but not a physiological one (±2.2 mmol.dm-3).

Further work is required to see if it would not be more beneficial to recommend a healthier diet to patients with a high risk of cardiovascular disease, rather than normoxic

exercise.

5.6 Conclusion

This study found that acute and chronic normoxic exercise produced a statistically significant increase in plasma total homocysteine, but not a physiological one.

Hypoxic acute and chronic exercise did not produce a significant change in plasma total homocysteine.

The exercise (hypoxic or normoxic) had no affect upon the lipid profile as the subjects were already under taking exercise prior to the study and tended to have balanced diets.

This shows that all the cardiovascular risk factors are inter-related and are positively affected by a healthy lifestyle (exercise, diet and relaxation).

An advantage of training under hypoxic conditions is that it minimises the fluctuations in plasma total homocysteine concentration that would be obtained when training normoxically.

Appendices

Appendix I

Glossary

acute phase proteins:

serum proteins, mostly produced in the liver, which rapidly change in concentration, (some increases, others decrease) during the initiation of an inflammatory response.

Analysis of Variance (ANOVA):

ANOVA is a statistical method used to obtain inferences when more than two samples are used.

1-way ANOVA - tests the effect of one criterion (independent variable) upon another (dependent variable).

2-way ANOVA - tests the effect of two criteria (independent variables) upon another dependent variable).

C-reactive protein (CRP):

An acute phase protein which is able to bind to the surface of microorganisms where it functions as a stimulator of the classical pathway of complement activation, and as an opsonin for phagocytosis.

Cholesterol-years Score (CYS):

CYS is an estimate of the lifetime vascular exposure to hypercholesterolemia.

CYS ($mmol.dm^{-3}.yrs$) = (total cholesterol concentration at the time of diagnosis$\{mmol.dm^{-3}\}$ x age of patient at diagnosis {years}) + (average cholesterol concentration present after the introduction of lipid-lowering drug therapy$\{mmol.dm^{-3}\}$ x number of years of lipid-lowering drug therapy{years}) Hoeg et al. 1994, (Raal et al., 1999).

Friedman's test:

A nonparametric analysis that is used when the data is not normally distributed, nor the samples have the same variance and is based on ranking the data.

geometric means:

the nth root of the product of the n data.

$$\overline{X}_G = \sqrt[n]{X_1 X_2 X_3 \ldots\ldots X_n} = \sqrt[n]{\prod_{i=1}^{n} X_i}$$

When the data is positively skewed, it can be mathematical changed by taking logarithms causes the data to take on a more nearly symmetrical distribution.

Geometric mean can be calculated from the antilog of the mean of the log data.

intermittent claudication:

exercise induced pain caused by localised ischaemia because of atheroma.

Kruskal-Wallis test

It can be used instead of the parametric 1-way ANOVA. A nonparametric statistical test used when the samples are not normally distributed. It is also called analysis of variance by ranks.

Mann-Whitney-U

A non-parametric test that ranks the data and then numbers it.

Mann-Whitney statistic $$U = n_1 n_2 + \frac{n_1(n_1+1)}{2} - R_1$$

n_1 and n_2 are the number of observations in samples one and two respectively. R_1 is the sum of the ranks of the observations in samples one. The calculated U is compared with he two-tailed value of $U_{\alpha(2),n_1 n_2}$ as the critical value, if $n_1 > n_2$ and $U_{\alpha(2),n_2 n_1}$ if $n_2 > n_1$.

maximum volume of oxygen per minute ($\dot{V}O_2max$):

The maximal aerobic power for a subject is equal to the maximum voluntary oxygen exchanged per minute while exercising the large muscle groups by progressively increasing the intensity until exhausted. Usually reported as an absolute volume per minute ($dm^3.min^{-1}$), but in sports where body mass is supported during performance, then as volume per minute per kilogram body mass ($cm^3.kg^{-1}.min^{-1}$; nonSI units $ml.kg^{-1}.min^{-1}$).

Physiological difference

This is the minium difference that should occur, in order to state clearly that the difference occurred because the experiment. Smaller changes can be accounted for by biological and analytical differences

plasma total homocyst(e)ine (tHcy):

total homocysteine is the sum of plasma homocysteine, homocystine, cysteine-homocysteine and homocysteinyl moieties of oxidized disulphide, (protein bound).

Protein C - Protein S system:

Protein C is a plasma protein activated by the thrombomodulin / thrombin complex. Activated protein C binds with protein S and inactivates factors VIII and V in the coagulation pathway.

Statistic significant difference:

Statistics is a mathematic technique that calculates the probability that the difference between two or more samples happened by chance. Before looking at the data, a null hypothesis is formulated and the significance level is stated where the null hypothesis would be rejected. This was stated as $\alpha < 0.05$, that is there is less than a 5% chance that the difference between the two samples happened by chance.

Student's 't' test:

A mathematical technique that calculates the t distribution and allows inferences to be made about the sample. It assumes that the distribution of the cases is normal (gaussian distribution).

total cholesterol (tC):

total cholesterol is the sum of cholesterol in the serum: very low density lipoprotein cholesterol, low density lipoprotein cholesterol, high density lipoprotein cholesterol and intermediate density lipoprotein cholesterol.

Wilcoxon matched pairs test:

A nonparametric test for dependent samples, that is used instead of the paired 't' test when the data is not normally distributed.

Appendix II

Informed Consent Form

Health & Exercise Science
School of Applied Sciences
Glamorgan University
Trefforest, Pontypridd
CF37 1DL

Dear Sir,

Thank you for agreeing to take part in this study. Your participation is greatly appreciated. The study has been ethically approved by the ethics committee of the University of Glamorgan and we are therefore obliged to obtain a signed informed consent form from you. All information we obtain will be treated as strictly confidential.

I understand that I will be asked to give a small quantity of blood and will be requested to perform various physical activities requiring maximal levels of my fitness. I understand that these tests will be administered by the staff of Health and Exercise Science department of Glamorgan University.

I understand that I am free to ask any questions, about any of the tests performed. If my health status changes I will promptly report it to Drew Heusch.

My participation in this study is voluntary and I realize that I am free to withdraw from the study at any time.

If I have any further questions regarding this study I am free to contact Drew Heusch 01443 482873 or Professor Bruce Davies 01443 482 577

I have read this form and give written consent to participate in this study

_____ Date _____

Signature of Subject, Print name

_____

Signature of Witness Print name

Appendix III

Testing Symmetry

The sample's symmetry measure g_1

$$g_1 = \frac{k_3}{s^3} = \frac{k_3}{\sqrt{(s^2)^3}}$$

H_o : The sample is symmetrically distributed

H_A : The sample is not symmetrically distributed

g_1 was calculated by the computer statistical package StatView 512 for MAC.

The null hypothesis was rejected if $\alpha(2) \leq 0.05$ and g_1 value depends upon n, (Statistic Tables B.22, Zar 1996d).

Skewness

The skewness gives an indication of where the majority of the samples are in relation to the mean.

If g_1 was significantly less than 0, it indicated that the sample distribution was skewed to the left, that is, the mean was less than median. If g_1 was significantly greater than 0, then the distribution was skewed to the right, that is, the mean was greater than the median. If g_1 is not significantly different from 0, then the sample comes from a population distributed symmetrically around the mean.

The square of the normal deviate for symmetry (Z^2_{g1})

Method proposed by D'Agostino (1970, 1986; D'Agostino, Belanger, and D'Agostino, 1990) when $n \geq 9$.

$$\sqrt{b_1} = \frac{(n-2)g_1}{\sqrt{n(n-1)}}$$

$$A = \sqrt{\sqrt{b_1}\sqrt{\frac{(n+1)(n+3)}{6(n-2)}}}$$

$$B = \frac{3(n^2+27n-70)(n+1)(n+3)}{(n-2)(n+5)(n+7)(n+9)}$$

$$C = \sqrt{2(B-1)} - 1$$

$$D = \sqrt{C}$$

$$E = \frac{1}{\sqrt{\ln D}}$$

$$F = \frac{A}{\sqrt{\frac{2}{C-1}}}$$

$$Z_{g1} = E \ln (F + \sqrt{F^2 + 1})$$

$$Z^2_{g1} = (Z_{g1})^2$$

Appendix IV

Testing Kurtosis

The sample's Kurtosis measure, (g_2) , otherwise known as "peakedness" or "tailedness" that is, dispersion around μ - s and μ + s , (Moors, 1986).

$$g_2 = \frac{k_4}{s^4}$$

H_o : The sample is mesokurtic (normal) distribution

H_A : The sample is not mesokurtic distributed

g_2 was calculated by the computer statistical package StatView 512 for MAC.

The null hypothesis was rejected if $a(2) \leq 0.05$ and g_2 value depends upon n, (Statistic Tables B.23, Zar 1996c).

If g_2 was significantly less than 0, it indicated that the sample was platykurtic (Moors 1986). If g_2 was significantly greater than 0, then the sample was leptokurtic If g_2 is not significantly different from 0, then the sample comes from a population normally distributed, that is mesokurtic.

The square of the normal deviate for Kurtosis (Z^2_{g2})

Method proposed by Anscombe and Glynn (1983) for $n \geq 20$

$$G = \frac{24n(n-2)(n-3)}{(n+1)^2(n+3)(n+5)}$$

$$H = \frac{(n-2)(n-3)|g_2|}{(n+1)(n-1)\sqrt{G}}$$

$$J = \frac{6(n^2-5n+2)}{(n+7)(n+9)}\sqrt{\frac{6(n+3)(n+5)}{n(n-2)(n-3)}}$$

$$K = 6 + \frac{8}{J}\left[\frac{2}{J} + \sqrt{1+\frac{4}{J^2}}\right]$$

$$L = \frac{1-\frac{2}{K}}{1+H\sqrt{\frac{2}{K-4}}}$$

$$Z_{g2} = \frac{1-\frac{2}{9K}-\sqrt[3]{L}}{\sqrt{\frac{2}{9K}}}$$

$$Z^2_{g2} = (Z_{g2})^2$$

Appendix V

Assessing Normality using Symmetry and kurtosis measures

D'Agostino (1986) reviewed the available methods for the testing of normality and concluded that the D'Agostino and Pearson (1973) test was the best, and works well for $n \geq 20$.

The null hypothesis of population normality is tested using the statistic

$$K^2 = Z_{g1}^2 + Z_{g2}^2$$

H_0 : The sampled population is normally distributed

H_A : The sample population is not normally distributed

Type 1 error H_0 is true and it is rejected

Type 2 error H_0 is false and is not rejected

Zar (1996a) stated that as the level of significance is arbitrary, test results near the level (0.04 to 0.06, when significance is set at 0.05) then it should be repeated with additional data than declare that the null hypothesis is or is not true.

The significance of K^2 is determined using the chi-squared distribution (χ^2) with 2 degrees of freedom (υ). For $\alpha \leq 0.05$ then $K^2 \geq 5.991$ and the null hypothesis would be rejected, using statistical table B.1 (Zar 1996a).

Appendix VI

Calculating missing values

Least squares regression line of x on y

$$x = cy + d$$

$$\Sigma x = c\Sigma y + nd$$

n = number of pairs of values

$$\Sigma xy = c\Sigma y^2 + d\Sigma y$$

solve simultaneously to find c and d

for degrees of freedom for p remove one

Least squares regression line of y on x

$$y = ax + b$$

$$\Sigma y = a\Sigma x + nb$$

$$\Sigma xy = a\Sigma x^2 + b\Sigma x$$

solve simultaneously to find a and b

Crawshaw and Chambers Concise Course in A level statistics Stanley Thorne

Appendix VH

The critical difference

According to Costongs et al. (1985), the minimum detectable difference between two independent groups when the variable is continuous, can be calculated, assuming that the variable has a normal distribution for a significance of 0.05 (5%).

$$\delta = K\sqrt{CV_a^2 + CV_b^2}$$

δ = critical difference,
K = 2.77 for $p<0.05$,
CV_a = coefficient of analytical variation
CV_b= coefficient of biological variation.

$$CV_a = \frac{SD_a}{\overline{X}} \qquad CV_b = \frac{SD_b}{\overline{X}}$$

Coefficient of variance

The coefficient of variance was calculated from the following papers

paper	number	male	Age range	Biological variance within-person	Analytic variance - within
Garg et al., 1997	20	6	21 - 65	0.070	0.043
Clarke et al., 1998	96		65 - 74	0.089	0.0146
Rossi et al., 1999	20	9	31 - 55	0.083	0.031
Kuo et al., 1997	75 & 10	-	-	-	0.028
Pfeiffer et al., 1999	20	-	-	-	0.011
Average				0.081	2.0

The biological variance CV_b

$$CV_b = \sqrt{\frac{0.0703^2 + 0.089^2 + 0.083^2}{3}}$$

$$= 0.081$$

The analytic variance CV_a

$$CV_a = \sqrt{\frac{0.043^2 + 0.0146^2 + 0.031^2 + 0.028^2 + 0.011}{5}}$$

$$= 0.028$$

Therefore the critical difference:

$$\delta = k\sqrt{CV_a + CV_b}$$

$$= 2.77\sqrt{0.081^2 + 0.028^2}$$

$$= 0.237$$

Minimal detectable difference

minimal detectable difference = mean x critical difference

$\delta_1 = \overline{X} \times \delta$

$\delta_1 = 9.3 \times 0.237 = 2.20\ \mu mol.dm^{-3}$

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