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Niacin (pharmacologic dose levels)-induced hyperhomocysteinemia in rats

by

Neelam Makhani

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirement for the degree of Master of Science

in

Nutrition and Metabolism

Department of Agricultural, Food and Nutritional Science

Edmonton, Alberta

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Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled *Niacin (pharmacologic dose levels)-induced hyperhomocysteinemia in rats* submitted by *Neelam Makhani* in partial fulfillment of the requirements for the degree of *Master of Science* in *Nutrition and Metabolism.*

Dr. T. K. Basu, Supervisor

Dr. C. J. Field

Dr. J. Van Aerde

To my husband Karim whose love, understanding, encouragement and endless support have made this achievement possible.

ABSTRACT

Niacin (nicotinic acid) in large doses has been shown to be an effective lipid-lowering agent. The potential risks of the therapeutic utilization of the vitamin, however, have not been critically considered. Since niacin is excreted primarily as methylated pyridones requiring methionine as a methyl donor, the present study was undertaken to examine if the metabolism of the amino acid is altered in the presence of non-physiologic dose levels of niacin.

Male Sprague-Dawley rats were given nutritionally adequate, semi-synthetic diets containing large doses of niacin at a level of either 400 or 1000 mg/kg diet (compared to 30 mg/kg in the control diet) for up to three months.

Supplementation with large doses of niacin resulted in a significant increase in plasma and urinary total methionine, homocysteine and cysteine levels. The magnitude of increase in plasma homocysteine in relation to both plasma methionine and cysteine was significantly higher in the niacin treated groups. The niacin-associated increase in plasma homocysteine levels was further accentuated in the presence of a methionine load. This hyperhomocysteinemia was accompanied by a significant decrease in plasma concentrations of vitamins B_{12} and B_6, which are cofactors in the metabolism of homocysteine. The niacin-induced hyperhomocysteinemia was also associated with significantly raised triglyceride levels in the liver. A parallel increase in plasma bilirubin and alanine aminotransferase levels, suggestive of hepatic dysfunction, was observed in the presence of niacin supplementation.

Overall, these results suggest that niacin, in its non-physiologic dose levels interferes with the metabolism of methionine leading to hyperhomocysteinemia accompanied by hepatic hypertriglyceridemia. Hyperhomocysteinemia has an important toxicologic implication, as it is considered to be an independent risk factor for arterial occlusive diseases. It seems possible that the hyperhomocysteinemia in niacin-treated rats is caused by the decrease in vitamins B_6 and B_{12}; which may have been a result of a niacin-induced metabolic requirement of these vitamins. These untoward effects appear to be important limiting factors in the use of niacin as a hypolipidemic agent.

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ABBREVIATIONS

AGPA	L-α-amino-β-guandinopropionic acid
ACAT	Acyl coenzyme A: cholesterol acyl transferase
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
c-AMP	Cyclic 3',5'-adenosine monophosphate
CAD	Coronary artery disease
Cbl	Cobalamin
CBS	Cystathionine β-synthase
CHD	Coronary heart disease
CoA	Coenzyme A
CVD	Cardiovascular disease
HDL	High density lipoprotein
HMG CoA	3-hydroxy-3-methylglutaryl coenzyme A
HPLC	High Performance Liquid Chromatography
LDL	Low density lipoprotein
Lp(a)	Lipoprotein (a)
MTHFR	5,10-methylene tetrahydrofolate reductase
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (reduced form)
NADP	Nicotinamide adenine dinucleotide phosphate
NCEP	National Cholesterol Education Program

NTD	Neural tube defects
OPA	o-phthaldialdehyde
PHS	Public Health Service
PLP	Pyridoxal-5'-phosphate
Ppi	Pyrophosphate
PRPP	5-phosphoribosyl-1-pyrophosphate
RNI	Recommended Nutrient Intake
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SEM	Standard error of mean
SR	Sustained-Release
TG	Triglyceride
THF	Tetrahydrofolate
VLDL	Very low density lipoprotein
VLDL-TG	Very low density lipoprotein triglyceride

1. INTRODUCTION

Despite significant medical advances over the years, cardiovascular disease (CVD) remains the leading cause of death in Canada claiming 75,000 lives annually. Approximately 39% of total mortality in Canada is caused by heart and blood vessel disease and stroke (Heart and Stroke Foundation of Canada, 1997). The loss of quality of life and increasing health care expenditures as a result of this disease, demand cost efficient strategies which are aimed at prevention. Epidemiological, clinical and laboratory research has linked genetic, metabolic, nutritional and lifestyle factors to the development of CVD.

The landmark Framingham Heart Study (1984) originally identified three major risk factors that cause acceleration of the atherosclerotic process. These include high total plasma cholesterol (Table 1.1), hypertension and smoking. Other factors, such as family history of premature CVD, elevated plasma low density lipoprotein (LDL) levels, age, male gender, diabetes mellitus, obesity, alcoholism and lack of physical activity have also been associated with an increased risk of CVD (Consensus Conference, 1985; Shalowitz and Heaton, 1996; Stone, 1996). Thus, the relative strength of these risk factors in predicting CVD is complex due to its multifactorial nature.

According to a number of epidemiological, clinical, genetic and experimental studies, hypercholesterolemia associated with increased plasma LDL and very low density lipoprotein (VLDL), as well as, decreased high density lipoprotein (HDL) levels are consistently related to coronary atherosclerosis (Kane et al., 1981; Grundy, 1986; Prihoda and Illingworth, 1992; Holland et al., 1993). The Multiple Risk Factor Intervention Trial (1986) revealed that an increase in total serum cholesterol level is accompanied by an increase in mortality rate from coronary artery disease (CAD).

Table 1.1 Classification of plasma lipoprotein levels

Lipoprotein	Concentration (mmol/L)		
	Desirable	Borderline	High
Total cholesterol	<5.2	5.2-6.2	>6.2
LDL cholesterol	<3.4	3.4-4.1	>4.1
HDL cholesterol	>0.9		

National Cholesterol Education Program (NCEP, 1993)

Both primary prevention (The Lipid Research Clinics Coronary Primary Prevention trial, 1984; The Helsinki Heart Study, 1987) and secondary prevention (The Cholesterol Lowering Atherosclerosis Study, 1990; The Familial Atherosclerosis Treatment Study, 1990) trials have indicated that atherosclerotic morbidity and mortality rates can be reduced by altering plasma lipid levels. An approximate reduction in CAD risk of 2% for each 1% reduction in blood total cholesterol has been suggested (The Lipid Research Clinics Coronary Primary Prevention Trial, 1984). Furthermore, secondary prevention trials have shown that lowering cholesterol to desirable levels slows the progression or induces the regression of atherosclerosis (Rossouw and Rifkind, 1990). Management of lipid abnormalities is thus aimed at treating the underlying cause and normalizing plasma lipoprotein levels. Intervention may be either non-pharmacologic or pharmacologic in nature or a combination of both.

1.1 NONPHARMACOLOGIC MEASURES

Obesity, smoking and a sedentary lifestyle can potentiate the effects of lipid abnormalities. Accordingly, the nonpharmacologic approach emphasizes diet modification, exercise for loss of body fat and cessation of smoking.

Long term control of dyslipidemia begins by adjusting caloric intake and/or activity level to achieve desirable body weight. Diet modification occurs in two steps designed to progressively reduce intake of saturated fat and cholesterol and to eliminate excess total calories. Step 1 diet calls for a total fat intake of less than 30% of total calories, saturated fat intake of less than 10% and cholesterol intake

of less than 300 mg/day. Blood cholesterol levels should be measured at four to six weeks and at three months after diet initiation. If the minimal goals of therapy are not achieved by three months, the patient should proceed to a Step 2 diet. This diet calls for a further reduction in saturated fat intake to less than 7% of total calories and cholesterol intake of less than 200 mg/day (Naito, 1987). In clinical practice, the majority of dyslipidemic patients respond to the proposed diet regimens because, the major portion of their metabolic disturbance is caused by faulty dietary habits and overnutriton.

1.2 PHARMACOLOGIC MEASURES

The Adult Treatment Panel II (1993) recommends drug therapy if high risk for CAD persists after 6 months of intensive dietary therapy. Total risk status should decide the type and intensity of drug treatment and aggressive dietary therapy should be maintained. Although, the primary therapeutic goal is to lower LDL cholesterol, the presence of hypertriglyceridemia or low HDL cholesterol may influence the choice of drug regimen.

A variety of pharmacologic agents have been introduced for treatment of dyslipidemia of which, five categories of drugs have emerged as the mainstays of therapy. These include bile acid sequestrants, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, fibric acid derivatives, probucol and niacin.

1.2.1 Bile acid sequestrants

The major drugs in this category are cholestyramine and colestipol. Bile acid sequestrants are primarily effective in lowering LDL cholesterol. These agents are active entirely within the intestine where they bind bile acids and prevent their reuptake into the enterohepatic circulation. The resultant depletion of hepatic cholesterol stimulates cholesterol-7α-hydroxylase, the rate limiting enzyme of bile

acid synthesis. This further reduces intrahepatic cholesterol concentrations as more cholesterol is degraded into bile acids. Decreased hepatic cholesterol content stimulates the synthesis of LDL receptors on the surface of hepatocytes. This increases the uptake of LDL resulting in catabolism and removal of LDL from the blood which in turn, leads to a reduction in serum LDL cholesterol levels (Shepherd et al., 1980; Grundy, 1986).

Common side effects observed with bile acid sequestrants include changes in bowel function including constipation and bloating. Bile acid sequestrants also have the potential to bind with certain drugs and may decrease the absorption of folic acid and fat-soluble vitamins (Schachter, 1996). These drugs also inhibit lipoprotein lipase and tend to increase triglyceride (TG) levels in patients with mixed hyperlipidemia. Some biochemical side effects include mild increases in alkaline phosphatase and transaminase levels, suggesting hepatic dysfunction (Illingworth, 1987).

1.2.2 HMG-CoA reductase inhibitors

Current available agents in this class include fluvastatin, lovastatin, simvastatin and pravastatin (Grundy, 1988). HMG-CoA reductase is a rate limiting enzyme in cholesterol biosynthesis. These drugs function mainly by competitively inhibiting endogenous cholesterol production. Decreased cholesterol synthesis in hepatocytes stimulates the synthesis of LDL receptors which lower serum levels of both LDL and VLDL. These drugs significantly reduce total and LDL cholesterol levels and may also decrease TG and increase HDL cholesterol levels (Reihner et al., 1990; Bradford et al., 1991). The beneficial effects on TG and HDL levels may be marginal in patients with severe hypertriglyceridemia (Gates, 1994; Schachter, 1996).

Headache, myalgia, insomnia and gastrointestinal complaints are the most frequently reported side effects of these hypocholesterolemic agents (Bradford et

myopathy have been reported in up to 2% of patients. Skin rashes including hypersensitivity reactions have been seen in some patients (Dujovne et al., 1991).

1.2.3 Fibric acid derivatives

Drugs in this category include clofibrate, gemfibrozil, fenofibrate, bezafibrate and ciprofibrate (Grundy, 1987). In hypertriglyceridemic patients, these drugs lower very low density lipoprotein triglyceride (VLDL-TG) levels, both by decreasing the secretion rates of VLDL-TG and by promoting subsequent lipolysis. They appear to produce this effect by enhancing the activity of lipoprotein lipase and promoting the catabolism of TG from VLDL particles (Grundy and Vega, 1987). In hypercholesterolemic patients, these drugs also lower LDL cholesterol levels by 10-15% by mechanisms that remain unknown and are yet to be determined. In many patients of both types, fibric acid derivatives raise plasma concentrations of HDL cholesterol (Hunninghake and Peters, 1987).

Several side effects can occur with usage of fibric acid derivatives. They increase the risk of cholesterol gall stones by enhancing the secretion of cholesterol into bile. They can also cause gastrointestinal complaints, produce mild myopathy and have been reported to occasionally have other side effects such as water retention, impotence and skin rash (Grundy, 1990; Yeshurun et al., 1993).

1.2.4 Probucol

It lowers LDL cholesterol by promoting its clearance from circulation. A 10-15% reduction of LDL cholesterol can be expected. This agent, however, does not affect triglyceride (TG) levels, and more importantly, it decreases HDL cholesterol by 20-30% (Kesaniemi and Grundy, 1984; Gotto and Pownall, 1992). This HDL lowering effect has been considered by many health professionals to limit the usefulness of probucol as a lipid-lowering drug. However, probucol is

incorporated into LDL particles and prevents their oxidation. Since oxidation of LDL within the arterial wall has been postulated to be a key step in atherogenesis, probucol could be antiatherogenic beyond its action to lower LDL cholesterol level (Schwartz, 1988).

Gastrointestinal disturbances from probucol use are not uncommon but are usually mild, characterized mainly by diarrhea, flatulence and hyperperistalsis (Yeshurun and Gotto, 1995). Occasionally heartburn, nausea, headaches and palpitations have been reported. However, its effects on cardiac function are more alarming and include prolongation of the QT interval and rhythm disturbances (Naito, 1987).

In summary, the pharmacologic therapy for dyslipidemia with cholesterol lowering drugs is advantageous, but not without adverse effects. Lovastatin, colestipol and cholestyramine produce a maximum reduction in LDL cholesterol, whereas gemfibrozil produces a maximum increase in HDL cholesterol. Although, probucol causes a reduction in total cholesterol, much of its effect is due to a reduction in HDL cholesterol levels. Gastrointestinal symptoms are the most common side effects for these drugs. Other side effects include drug interaction and malabsorption of vitamins with bile acid sequestrants, hepatic injury with HMG-CoA reductase inhibitors, myopathy with fibric acid derivatives and cardiac dysfunction with probucol.

Progress has been made in the development and testing of pharmacologic agents towards the control of hypercholesterolemia and for the treatment and prevention of atherosclerotic complications. There is intense research activity aimed at improving the existing drugs. Side effects of these drugs, as well as, their beneficial effects on lipids and CAD are an important topic of current investigations. As a result, increased attention is being focused on attenuating the harmful side effects of these drugs for their long term usage.

1.3 NIACIN (NICOTINIC ACID)

Niacin has been found to be an effective agent for the control of hyperlipidemia. The lipid-lowering efficacy of niacin was first reported in the mid 1950's with the demonstration that niacin could lower cholesterol levels in hypercholesterolemic subject (Parsons et al., 1956; O'Reilly et al., 1957). We now know that niacin is also very effective in lowering TG and lipoprotein(a) [Lp(a)] and in increasing HDL levels (Illingworth, 1988; Carlson et al., 1989; Keenan et al., 1991; Seed et al., 1993).

Although usually considered an essential nutrient, niacin exerts its hypolipidemic action only when consumed in large amounts. Its pharmacologic dose level is usually in the range of 2 to 6 g/day, while the daily Recommended Nutrient Intake (RNI) is 7.2 mg/1000 kcal.

Efficient treatment strategies include two main factors: pharmacologic and cost effectiveness. Niacin possesses both these properties. It is effective in increasing HDL cholesterol and at the same time it is inexpensive relative to any other conventionally available hypolipidemic agents (Table 1.2). In fact, NCEP (1993) recommended niacin as a first-line therapy in the management of hyperlipidemia not adequately controlled by diet and exercise.

Table 1.2 Cost of drugs used to treat hyperlipidemia

Medication	Approximate cost/month ($US)
Lovastatin	78-154
Cholestyramine	70
Colestipol	47
Probucol	46
Gemfibrozil	43
Niacin	5-15

Data from Witztum, 1989

1.3.1 Proposed mechanisms of hypocholesterolemic action of niacin

The beneficial effects of niacin in treatment of hyperlipidemia are consequences of interrelated effects on lipid and lipoprotein metabolism. These include (i) inhibition of lipolysis in adipose tissue, (ii) inhibition of synthesis and secretion of VLDL by the liver, (iii) increased biliary lipid output, (iv) increase in serum levels of HDL accompanied by a shift in HDL subtype distribution, and (v) lowering of serum levels of Lp(a).

Inhibition of lipolysis in adipose tissue: It is thought that pharmacologic doses of niacin decrease mobilization of fatty acids from adipose tissue by inhibiting the breakdown of TG through lipolysis. Adipose tissue lipolysis is regulated by hormones and paraendocrine agents through the cyclic-adenosine monophosphate (c-AMP) system. Elevated levels of c-AMP activate a protein kinase that phosphorylates a hormone-sensitive lipase, thus activating the lipase to catalyze TG hydrolysis. Hormonal signals are transduced through the guanine nucleotide linked G-protein system, which interacts with adenyl cyclase to modulate cellular c-AMP levels. Both stimulatory (Gs) and inhibitory (Gi) pathways have been identified. In fat cells, G-proteins function as transducing elements for receptors that are either stimulatory (Rs) or inhibitory (Ri). Lipolytic agents such as catecholamines, adrenocorticotropic hormone and glucagon bind to distinct Rs receptors. The ligand-receptor complexes and then interact with a common Gs protein, leading to stimulation of adenyl cyclase activity, which results ultimately in fat breakdown. Antilipolytic agents such as adenosine, prostaglandin E_2 and niacin bind to Ri receptors, which in turn interact with the Gi protein. The resulting G-protein decreases c-AMP by inhibiting adenyl cyclase. Decreased c-AMP activity leads to a decrease in lipase activity and therefore, a decrease in the breakdown of fat is seen. This results in reduced circulation of both non-esterified fatty acids and TG (Casey et al., 1988; Birnbaumer, 1990; DiPalma and Thayer, 1991).

Inhibition of VLDL synthesis by the liver: It has been shown that niacin treatment decreases serum levels of both TG and cholesterol. These lipid changes are found mainly in the VLDL and LDL fractions. Turnover of lipoproteins in hyperlipidemic patients have shown that the rate of synthesis of VLDL is decreased by niacin. The predominant effect is a reduction in hepatic TG synthesis, thereby limiting overall assembly and secretion of VLDL from the liver. As niacin diminishes the flow of free fatty acids, the hepatic synthesis of VLDL, an important precursor of TG, is reduced. Since the majority of LDL is produced by the catabolism of VLDL, a decreased synthesis and secretion of VLDL by the liver leads to the lack of availability of VLDL for catabolism, thus reduced production of LDL indirectly (DiPalma and Thayer, 1991). Direct inhibition of LDL secretion has also been suggested (Carlson et al., 1989). Subsequently, concentrations of both VLDL and LDL are decreased in response to niacin therapy.

Increased biliary lipid output: Niacin increases biliary cholesterol output. Biliary cholesterol is derived from both *de novo* synthesis in the liver and pre-formed hepatic cholesterol pool. The contribution from *de novo* synthesis is relatively small (approximately 20%) and varies with the activity of HMG-CoA reductase, the rate limiting enzyme in cholesterol synthesis. It is possible that niacin increases biliary cholesterol output either by increasing HMG-CoA reductase activity or by inhibiting acyl coenzyme A:cholesterol acyl transferase (ACAT) activity. ACAT is an enzyme that esterifies cholesterol in the body, a process believed to be essential for cholesterol absorption. However, this is unlikely since this effect would increase serum cholesterol concentrations. It has been observed that c-AMP analogues produce a sustained reduction in biliary lipid output without a sustained effect of biliary bile acid output (Hamlin et al., 1990). Therefore, by analogy to the known ability of niacin to reduce c-AMP concentrations in adipose tissue (DiPalma and Thayer, 1991), it is speculated that niacin may similarly decrease c-AMP concentration in the liver. Such an effect

would be expected to increase the biliary output of cholesterol from pre-formed sources (Holland et al., 1993).

Increase in HDL_2 concentration: The HDL subclasses have been shown to be strong determinants for the severity, as well as, the progression of myocardial infarction. The HDL_2 subclass is regarded as a beneficial form of the lipoprotein that is possibly active in removing cholesterol from atherosclerotic lesions. It has been strongly and inversely related to the degree, severity and further progression of coronary atherosclerosis. In contrast, HDL_3 has been positively correlated to the progression of atherosclerosis (DiPalma and Thayer, 1991). Johansson and Carlson (1990) found a pronounced increase in HDL_2 fraction by niacin, while HDL_3 fraction remained unchanged. The exact mechanism related to the increase in HDL_2 is not known, but it has been speculated by these investigators that niacin inhibits catabolism of HDL_2 by decreasing hepatic lipase activity. Reduction in hepatic lipase has been suggested to be instrumental in converting HDL_2 to HDL_3.

Lowering of Lp(a): A high plasma concentration of Lp(a) is becoming widely accepted as a risk factor for atherosclerotic and thrombotic CVD (Armstrong et al., 1986; Scanu et al., 1991). Lp(a) is a variant form of LDL that contains apolipoprotein B (apo B), a normal constituent of VLDL and apo(a). Apo(a) shows structural homology to plasminogen and is bonded to apo(B) by disulfide linkages in Lp(a). A clinical study conducted by Carlson et al., (1989) revealed a significant reduction in serum Lp(a) in hyperlipidemic subjects taking niacin. There was a linear relationship between the percent decrease of Lp(a) and the reduction in LDL cholesterol levels. The authors suggested that niacin may act by inhibiting the synthesis of apo(B), since it is the common component of both Lp(a) and LDL cholesterol. Lp(a) may also play a key role in thrombosis due to its structural similarity to plasminogen. Lp(a) competes with plasminogen for binding to surface receptors on endothelial cells, thereby impeding activation of plasminogen to plasmin. The consequence of such an action would be inhibition

of thrombolysis and thereby favoring thrombosis (Crouse, 1997). The ability of niacin therapy to decrease serum levels of Lp(a) is distinctive among antihyperlipidemic drugs.

1.3.2 Biosynthesis of niacin

Niacin is a generic term that includes both nicotinic acid (pyridine-3-carboxylic acid) and nicotinamide (pyridine-3-carboxamide). Coenzymes that are the metabolically active forms of niacin include nicotinamide adenine dinucleotide (NAD) and NAD phosphate (NADP). In addition to dietary intake, niacin is also available through synthesis in the liver from the essential amino acid, tryptophan. It is estimated that 1 mg of niacin activity (niacin equivalent) can be synthesized from ingestion of 60 mg of dietary tryptophan (Van der Berg, 1997).

The first step in the metabolic pathway is the conversion of tryptophan to N'-formylkynurenine. This conversion is catalyzed by the enzyme tryptophan pyrrolase, a rate-limiting enzyme in this pathway. 3-hydroxyanthranilic is the most immediate precursor which undergoes an oxidation reaction to form α-amino-β-carboxymuconic-γ-semialdehyde. This primary product of the oxidation reaction forms quinolinic acid (Nishizuka and Hayaishi, 1963). In mammalian liver and kidney, the enzyme, quinolinate phosphoribosyltransferase, converts quinolinic acid to niacin ribonucleotide from which the coenzymes NAD and NADP are synthesized (Dietrich, 1971).

The efficiency of formation of these coenzymes is affected by both nutritional and hormonal factors. Deficiencies of vitamin B_6 and/or riboflavin slow the conversion, as these vitamins are cofactors for enzymes acting in the tryptophan metabolic pathway. Activities of enzymes such as tryptophan pyrrolase and quinolinate phosphoribosyltransferase are modulated by protein, energy and niacin intakes (Henderson, 1983).

At least 200 enzymes are known to be dependent on NAD and NADP with the nicotinamide moiety acting as an electron acceptor or hydrogen donor. Most of the NAD-dependent enzymes are involved in catabolic reactions, such as the oxidation of fuel molecules. Whereas, NADP more commonly functions in reductive biosynthesis of compounds such as fatty acids and steroids. Due to their involvement in hydrogen transfer reactions, pyridine nucleotides function in the synthesis and degradation of macronutrients (DiPalma and Thayer, 1991).

1.3.3 Metabolism

As niacin is a water-soluble vitamin, it is not stored in the body beyond its tissue saturation level. Niacin is metabolized by two pathways: it may be converted either to NAD or to nicotinuric acid (Stern et al., 1992). The principle aspects of niacin metabolism in human are summarized in Figure 1.1. The first step involves the cytosolic pyrophosphorylase (phosphoribosyltransferase) catalyzed reaction of niacin with 5-phosphoribosyl-1-pyrophosphate (PRPP) to form pyrophosphate (PPi) and niacin ribonucleoside (White et al., 1978). The niacin ribonucleoside produced is converted to deamidoNAD in the presence of adenosine triphosphate (ATP) by a pyrophosphorylase. The deamido compound subsequently reacts with glutamine (GLN) in a cytosolic ATP-dependent synthase step to yield NAD, glutamate (GLU), adenosine monophosphate (AMP) and PPi (Nishizuka and Hayaishi, 1963). The NAD produced here and that produced in the liver by tryptophan, can be degraded by glycohydrolases to produce nicotinamide. The released nicotinamide undergoes methylation to produce N'-methylnicotinamide. Methionine, in the form of S-adenosylmethionine (SAM) donates the methyl groups for this methylation reaction (Shibata and Matsuo, 1989). N'-methylnicotinamide undergoes oxidation in the presence of aldehyde oxidase to form 4-pyridone [N-methyl-4-pyridone-5-carboxamide] and 2-pyridone [N-methyl-2-pyridone-5-carboxamide].

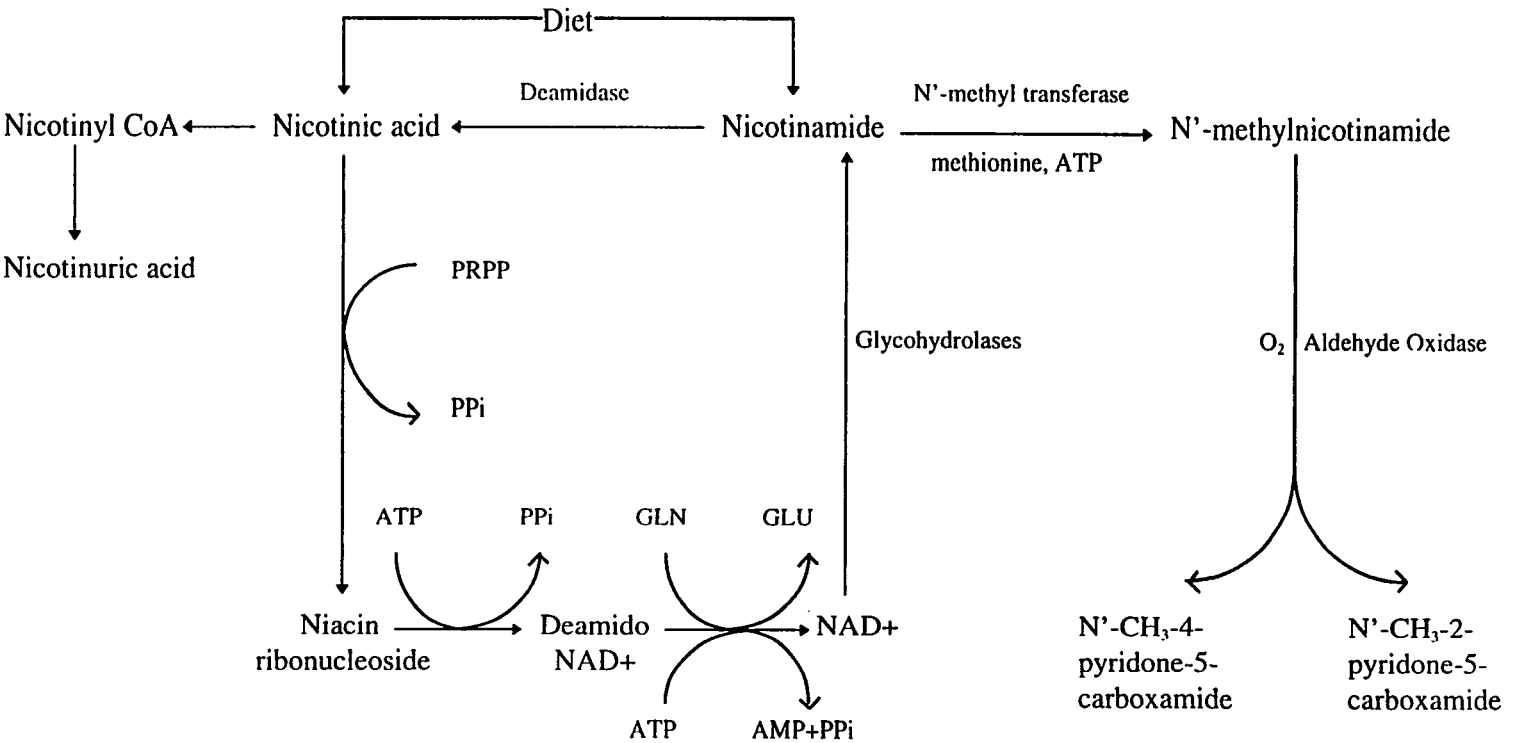

Figure 1.1 Niacin metabolic pathway
(modified from Shibata and Matsuo, 1989)

The alternative niacin metabolism pathway consists of the formation of nicotinyl CoA followed by conjugation with glycine to form nicotinuric acid (Mrochek et al., 1976). N'-methylnicotinamide is the major niacin metabolite excreted in urine. Other metabolites found in urine include 2-pyridone, 4-pyridone and nicotinuric acid (Van den Berg, 1997).

1.3.4 Adverse effects of niacin

Niacin is highly effective as a lipid-lowering drug, but unfortunately it causes a variety of side effects. The most common adverse effects associated with its therapeutic use are cutaneous flushing, pruritus, skin rash and abdominal pain. Other side effects include nausea, gastrointestinal irritation, hyperuricemia, glucose intolerance and modest elevations of hepatic enzyme levels (Reimund and Ramos, 1994; Brown, 1995). These undesirable effects are generally reversible with reduction of the dose or discontinuation of the vitamin. The cutaneous reactions reported are prostaglandin mediated. The NCEP (1988) recommended that treatment with niacin be preceded by a dose of aspirin, as it inhibits prostaglandin synthesis. The flushing symptoms become less bothersome with time and are ameliorated by taking niacin with meals, avoiding concurrent use of hot liquids and alcohol and taking an aspirin tablet 30 to 60 minutes before taking niacin (Whelan et al., 1992; Etchason et al., 1991).

Hepatotoxicity related to niacin has gained more attention with its increased use. Liver dysfunction can range from asymptomatic serum aminotransferase elevation to symptomatic hepatitis with widely varied liver enzyme patterns and even liver failure in rare cases (Mullin et al., 1989; Lawrence, 1993). The hepatotoxic effects are usually transient with evidence of both cholestasis and parenchymous necrosis (Patterson et al., 1983). Although the mechanism of hepatic injury remains unknown, it has been suggested to be a dose-related, direct toxic effect rather than an idiosyncratic drug reaction (Clementz and Holmes, 1987; Henkin et al., 1990).

A recent study has shown that feeding male Sprague-Dawley rats a semi-synthetic diet supplemented with either 400 or 4000 mg niacin/kg diet for 3 weeks caused an increase in plasma and urinary methionine and homocysteine levels (Basu and Mann, 1997). This niacin-induced hyperhomocysteinemia is an important observation, and may have serious health implications due to the potential toxic effects of homocysteine (see Section 1.5).

1.4 METABOLISM OF HOMOCYSTEINE

Dietary methionine is essential for normal growth and development of mammals. However, the minimum daily requirement for this amino acid varies depending on the availability of other nutrients. Homocysteine is a metabolic intermediary, formed by the demethylation of dietary methionine (Figure 1.2). Methionine is activated in an ATP-dependent reaction to form SAM which is converted to S-adenosylhomocysteine (SAH). SAH is subsequently hydrolyzed to adenosine and homocysteine.

Homocysteine metabolism consists primarily of two pathways: 1) the transsulfuration of homocysteine via cystathionine to cysteine, a catabolic pathway, and 2) the remethylation of homocysteine to form methionine, a recycling pathway (Miller et al., 1994).

The transsulfuration reaction is catalyzed by cystathionine β-synthase (CBS), which condenses homocysteine and serine in the presence of pyridoxal-5'-phosphate (PLP) to form cystathionine, which is subsequently cleaved by γ-cystathionase to form cysteine and α-ketobutyrate. The critical step in this pathway is the formation of cystathionine from homocysteine, as this step is irreversible in humans (Masser et al., 1994).

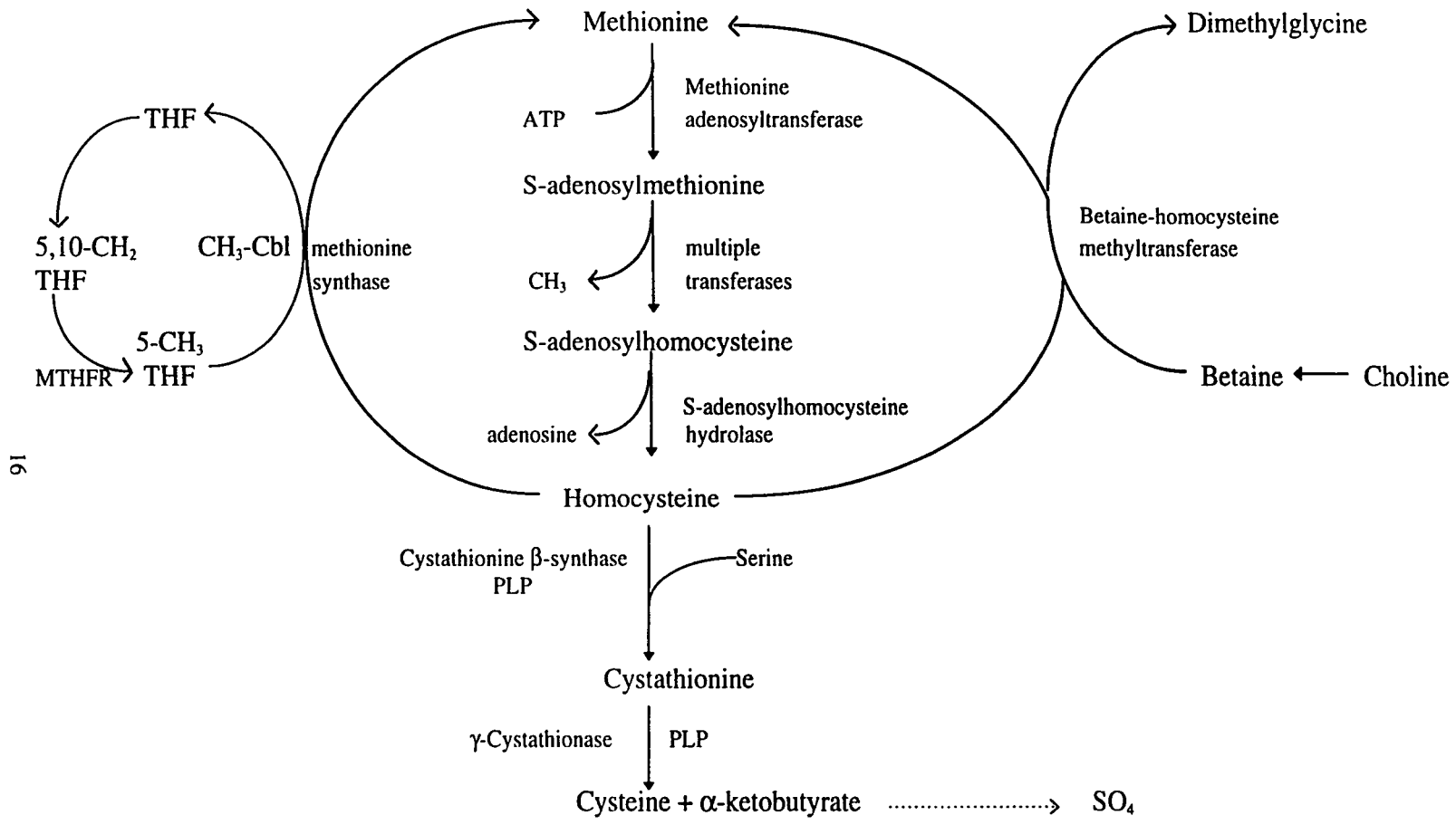

Figure 1.2 Methionine metabolic pathway
(modified from Stabler et al., 1996)

Homocysteine can be remethylated to reform methionine by means of reactions that require the enzymes methionine synthase, 5,10-methylene tetrahydrofolate reductase (MTHFR) and betaine-homocysteine methyltransferase and vitamins folate, cobalamin (Cbl), betaine and choline. The first reaction is catalyzed by a methylcobalamin-dependent enzyme, methionine synthase, which converts homocysteine and 5-methyl tetrahydrofolate to methionine and tetrahydrofolate (THF), respectively (Allen et al., 1993).

The second synthetic reaction involving homocysteine as a substrate is catalyzed by betaine-homocysteine methyltransferase, which catalyzes the conversion of betaine and homocysteine to dimethylglycine and methionine, respectively. This reaction is not dependent on either cobalamin or folate.

The metabolic locus formed in mammalian liver by the enzymes which metabolize homocysteine possess the necessary regulatory properties. At this point, homocysteine may be converted irreversibly to cystathionine. Alternatively, remethylation by means of either methionine synthase or betaine-homocysteine methyltransferase retains the homocysteine moiety within the methionine cycle. The hepatic content of the competing enzymes is only one factor in determining the *in vivo* rates of these reactions. Concentrations of both substrates and other metabolites which affect enzyme reactivity are of considerable importance as well (Finkelstein and Martin, 1986).

1.5 HYPERHOMOCYSTEINEMIA

A deficiency of the above mentioned enzymes or vitamins results in an abnormal accumulation of homocysteine (Masser et al., 1994). The fact that vitamins B_{12}, B_6 and folate all play important roles as either coenzymes or substrates in the metabolism of homocysteine has greatly added to the interest in this amino acid. Deficiencies of anyone of these vitamins, or supplementation with them, have been found to have a substantial impact on circulating concentrations of homocysteine (Mason and Miller, 1992).

Of the enzymes involved in recycling or removing homocysteine, severe genetic defects resulting in loss of function are known to affect CBS and MTHFR. Individuals homozygous for severe mutations of either the CBS or MTHFR genes have homocystinuria with associated premature vascular disease and thromboembolism affecting both large and small arteries and veins (Mudd et al., 1989).

The classic inborn error of metabolism that leads to homocysteinemia is a homozygous deficiency of CBS. One in 300,000 newborns is homozygous for CBS (Mudd et al., 1989). The importance of identifying heterozygotes for homocystinuria, which may affect as many as 1 in 70 individuals, is reflected by studies demonstrating high prevalence of premature vascular disease associated with this condition. Boers et al., (1985) have shown that heterozygotes tend be at an increased risk for premature peripheral and cerebral occlusive arterial disease whereas, Clark et al., (1991) have reported that heterozygosity is an independent risk factor for coronary, peripheral and cerebrovascular diseases.

A phenotypic variant of MTHFR with characteristic thermolability after partial denaturation at 46° C for 15 minutes, has been identified in 5-8% of the healthy population (Kang et al., 1988). This variant has been shown to cause mild hyperhomocysteinemia without homocystinuria in healthy controls, as well as, in

CAD patients (Kang et al., 1993). This biochemically defined thermolabile variant is the most probable cause of mild hyperhomocysteinemia in 28% of hyperhomocysteinemic vascular disease patients (Engbersen et al., 1995).

Vitamins B_6, B_{12} and folate are involved as cofactors in the metabolism of homocysteine. The catabolism of homocysteine through the synthesis of cystathionine requires vitamin B_6 in the form of PLP, whereas the remethylation of homocysteine to methionine requires vitamin B_{12} in the form of methylcobalamin and folate in the form of 5-methyl THF (Ueland et al., 1992).

Park and Linkswiler (1970) reported that urinary homocysteine excretion increased considerably when diets depleted in vitamin B_6 were consumed by humans. Several studies on experimental animals have suggested that a vitamin B_6 deficiency results in hyperhomocysteinemia (Slavik et al., 1982; Smolin and Benevenga, 1982). In a newborn with homocystinuria and CBS deficiency, methionine restriction and mega doses of vitamin B_6 have been shown to ameliorate hyperhomocysteinemia (Mudd et al., 1985). In the case of vitamin B_6 deficiency or a heterozygous CBS defect, homocysteine transsulfuration is inhibited by the deficiency or defect, but remethylation continues unimpeded (Miller et al., 1994).

Homocysteine remethylation via the enzyme methionine synthase requires both vitamin B_{12} and folate. Thus, folate deficiency or a disturbed folate metabolism leads to a reduced availability of 5-methyl THF. This results in decreased homocysteine remethylation causing hyperhomocysteinemia (Mudd et al., 1989). Kang et al., (1987) provided the first evidence that marginal plasma folate status could result in moderate hyperhomocysteinemia. Soon afterward, Brattstrom et al., (1988) demonstrated that isolated vitamin B_{12} deficiency could cause moderate-to-intermediate fasting hyperhomocysteinemia, the fasting

homocysteine levels greatly exceeding those found in heterozygotes for CBS deficiency.

A close synergistic relationship exists between folate and vitamin B_{12}. Adequate vitamin B_{12} must be present for the activity of methionine synthase, the enzyme that removes the methyl group from 5-methyl THF and delivers it to homocysteine, thereby converting homocysteine to methionine and regenerating free THF from which the 5,10-methylene THF involved in thymidylate synthesis is made. As 5-methyl THF may only return to the body's folate pool via a vitamin B_{12}-dependent step, a deficiency of vitamin B_{12} traps body folate in the methyl form. This is known as the folate trap hypothesis. A deficiency of either folate or vitamin B_{12} impairs synthesis of purines and thymidine, as a result, symptoms of macrocytic anemia occur (Herbert and Coleman, 1988).

Vitamin B_{12} is involved in protein synthesis through its role in the synthesis of methionine. An inadequate amount of methionine caused by a deficiency of vitamin B_{12} decreases the availability of SAM required for choline formation. As methionine is involved in making available lipotropic factor such as choline, vitamin B_{12} plays a role in lipid metabolism indirectly (Davis, 1984). Feeding diets deficient in methyl donor compounds such as choline and methionine have been shown to produce fatty liver in rats (Griffith and Dyer, 1968). This increase in the level of hepatic TG in choline deficient rats is believed to be caused by an impaired transport of lipids, as lipoproteins, from the hepatic cells into the serum (Chalvardjian, 1970).

A renewed interest in the sulfur containing amino acid, homocysteine, has emerged over the past few years. Mild hyperhomocysteinemia is now being recognized as an important risk factor for coronary artery disease, peripheral vascular disease, cerebrovascular disease and recurrent venous thrombosis (Malinow et al., 1989; Genest et al., 1990; Taylor et al., 1991; Den Heijer et al.,

1995). Elevated maternal levels of homocysteine have recently been associated with the occurrence of neural tube defects (Mills et al., 1995).

1.5.1 Vascular disease

McCully made the initial observation linking plasma homocysteine concentrations and arteriosclerotic vascular disease more than 25 years ago (McCully, 1969). He reported that an infant with homocystinuria who died as a result of a rare inborn error of cobalamin metabolism had widespread, severe arteriosclerosis analogous to the lesions seen in cases of homocystinuria caused by CBS deficiency. As elevated plasma homocysteine concentrations were the only metabolic abnormality common to these two hereditary enzyme disorders, McCully proposed that hyperhomocysteinemia resulted in arteriosclerotic disease.

Substantial research activity has resulted in the development of the 'Homocysteine Theory of Atherosclerosis', and the recognition that elevated plasma homocysteine can be considered as an independent risk factor for cardiovascular disease (Kang et al., 1992; Malinow and Stampfer, 1994; Selhub et al., 1995). Boushey et al., (1995) conducted a meta analysis of 27 studies and concluded that homocysteine is an independent risk factor for arteriosclerotic vascular disease unrelated to hyperlipidemia, hypertension, diabetes and smoking. The authors estimated that 10% of the populations' risk for CAD appears to be attributable to elevated homocysteine levels. With each 5 *u*mol/L rise in total homocysteine levels, the risk for CAD was increased by 60% for men and by 80% for women.

Both retrospective (Ueland et al., 1992; Malinow and Stampfer, 1994) and prospective (Stampfer et al., 1992; Arnesen et al., 1995) studies have demonstrated a relation between moderate hyperhomocysteinemia and premature vascular disease in coronary, cerebral and peripheral arteries. The association between plasma homocysteine concentration and atherosclerosis has more recently become the subject of a number of clinical studies, which have

vascular, cerebrovascular and coronary heart disease (Ueland et al., 1992; Kang et al., 1992).

Etiology of vascular disease: The pathogenesis of homocysteine-induced vascular damage is for the most part unknown. Plausible biological evidence propose a variety of mechanisms. Evidence suggests that accumulation of homocysteine in plasma may damage endothelial cells thereby, compromising endothelial cell function (Wall et al., 1980; De Groot et al., 1983). A currently favored theory of atherogenesis considers endothelial cell damage to be an early event in this process (Ross, 1986). Disruption of endothelial cell integrity is also believed to be a consequence of oxidative stress and oxygen radical mediated effects (Weiss and LoBuglio, 1982; Crapo, 1986). In this context it is well known that H_2O_2 is readily formed from thiol containing amino acids by mechanisms which also generate and perpetuate the formation of free radicals (Saez et al., 1982; Harman et al., 1984). Homocysteine could thus well exert its toxicity by this general mechanism.

In addition, recent data suggest that elevated homocysteine levels may induce a procoagulatory state and contribute to atherogenesis (Ross, 1986; Fuster et al., 1992). Homocysteine may have a deleterious effect on the normal prothrombolytic and anticoagulant activities of endothelial cells. Elevated levels of homocysteine can act as thrombogenic agents by affecting the expression of thrombomodulin and the activation of protein C (Rodgers and Cohn, 1990; Stampfer and Malinow, 1995). Homocysteine has also been shown to inhibit the cofactor activity of thrombomodulin thus, reducing the antithrombotic property of endothelial cells (Masser et al., 1994). Another potential mechanism described by Tsai et al., (1994) is that homocysteine stimulates the proliferation of smooth-muscle cells, a key component in atherogenesis. When put together, these data suggest that elevated homocysteine levels act atherogenic by inducing a procoagulatory state.

1.5.2 Neural tube defects

Neural tube defects (NTD) are among the most devastating of all birth defects, causing death or major disability in almost all affected children. In addition, they have tremendous costs to society both in terms of years of life lost and treatment costs. Worldwide, over 95% of such infants are born to families with no history of NTD (Little, 1992).

NTD result when the neural tube fails to close early in pregnancy; normally, the tube is closed by the end of the sixth week of gestation. Complete failure of closure at the rostral end results in anencephaly, localized defects in this location result in encephalocoele, and failure of closure anywhere along the spine results in spina bifida. All infants with anencephaly are either still-born or die soon after birth. A significant proportion of infants with encephalocoele and spina bifida survive, usually with lifelong physical and often with cognitive disabilities (Bower, 1995).

Etiology of NTD: It has now been well established that maternal folic acid supplementation during the periconceptional period significantly reduces the risk of giving birth to a baby with NTD (MRC Vitamin Study Research Group, 1991; Czeizel and Dudas, 1992). Pregnant women are prone to develop a folate deficiency due to increased folate needs for the growing fetal and maternal tissues (Bruinse et al., 1985). Supplementation of 0.4 mg/day to women of childbearing age reduces the chance of NTD (Swain and St. Clair, 1997).

The mechanism by which folic acid reduces the incidence of NTD at birth has not yet been fully elucidated. However, homocysteine metabolism is the focus of current investigation, in part because of evidence that folate, as well as, vitamin B_{12} may be important in the etiology of NTD.

There is growing evidence that vitamin B_{12} may also play a role in the etiology of NTD as well (Magnus et al., 1991; Kirke et al., 1993). The vitamin B_{12}-dependent enzyme methionine synthase is a key enzyme in methylation reactions. The function of methionine synthase is to provide sufficient methionine through homocysteine remethylation (Figure 1.3). Methionine is activated in a reaction with ATP to provide SAM, which is subsequently used as a methyl group donor in all biological methylation reactions (Zubay, 1983). This includes those producing myelin basic protein (Benjamins et al., 1984) which is required for proper neural tube formation. Furthermore, methionine synthase is also essential in the production of THF, required for DNA biosynthesis.

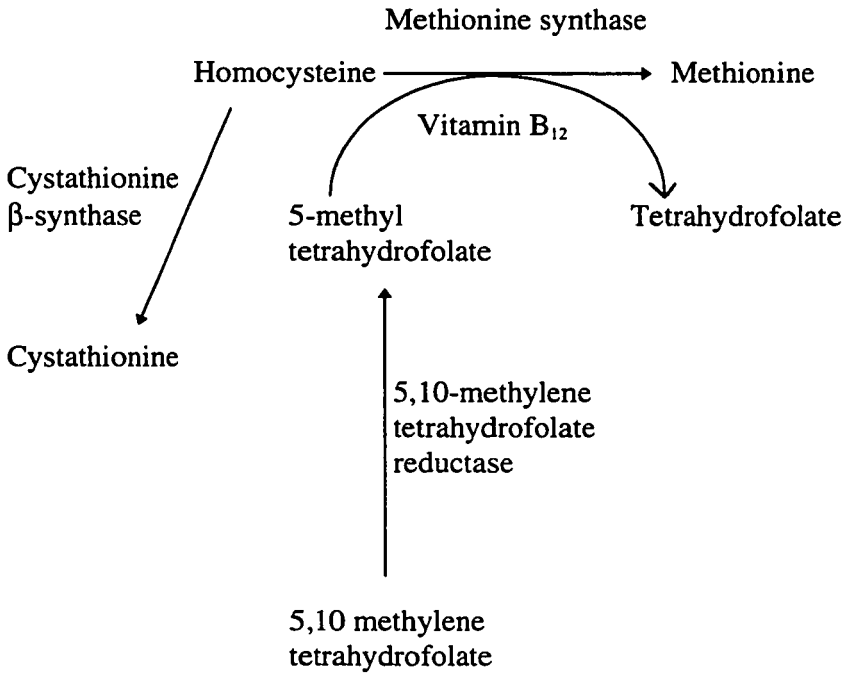

Figure 1.3 Homocysteine metabolic pathway
Methylation of homocysteine to form methionine requires a methyl group from 5-methyl THF and vitamin B_{12} as a cofactor (Mills and Conley, 1996)

involved in the etiology of NTD. Steegers-Theunissen et al., (1994) and Mills et al., (1995) concluded from their observations that mothers with neonates having NTD metabolize homocysteine less effectively than mothers with normal neonates. Thus, if homocysteine is involved in defective fetal neural tube closure, the protection conferred by folic acid supplementation may be explained by its homocysteine lowering effect (Ubbink et al., 1994).

In 1992, the U.S. Public Health Service (PHS) made a recommendation that all women of childbearing age should consume 0.4 mg of folic acid daily to prevent NTD (Centers for Disease Control and Prevention, 1992). As many pregnancies are unplanned, adequate folate must be present early in pregnancy when the fetal nervous system is developing, a time before most women are aware they are pregnant. The PHS recommendations suggest increasing folate consumption via three routes: consuming foods rich in naturally occurring folates, vitamin supplements and fortification of a food staple.

The Food and Drug Administration (FDA, 1993) proposed that cereal grains be fortified at a level of 140 *u*g of folic acid/100 g of grain, a level of fortification that would provide an average woman of reproductive age with an additional 0.1 mg of folic acid/day. Whereas, The Centers for Disease Control Folic Acid Working Group (1991) recommended fortification at 2.5 times this level (350 *ug*/100 g) to provide prevention to a more substantial number of women. The primary reason that FDA proposed fortification at a lower level was due to concern for people with untreated vitamin B_{12} deficiency (e.g. elderly). Folic acid in large doses has been observed to prevent anemia from vitamin B_{12} deficiency in some cases, but not the accompanying neuropathy. The concern is that the presence of additional folic acid in fortified food may delay the diagnosis of vitamin B_{12} deficiency and as result, the neurological abnormalities may become irreversible, even when a correct diagnosis is made (Wald and Bower, 1994).

Research has shown that people who do not take folic acid supplements are at an increased risk for deficiency, which in turn has been proven to cause NTD and which has also been associated with an increased risk for occlusive cardiovascular disease. Thus, a good policy option would be to require all folic acid supplements to contain 0.4 mg of vitamin B_{12} (Oakley et al., 1996).

Currently, there is an absence of a consensus definition for hyperhomocysteinemia. Some studies have employed percentile values from control subjects to identify elevated homocysteine levels in patients with vascular disease. For example, Stampfer et al., (1992) defined an elevated homocysteine concentration as one that exceeds 15.8 nmol/ml (95th percentile for healthy control subjects). Whereas, Joosten et al., (1993) defined an elevated homocysteine concentration as one that exceeded 13.9 nmol/ml (mean value plus 2 standard deviation among healthy young controls). Thus, homocysteinemia has been classified into moderate, intermediate and severe forms by plasma homocysteine concentrations between 16 and 30, between 31 and 100, and above 100 nmol/ml, respectively (Table 1.3).

Table 1.3 Classification of hyperhomocysteinemia

Plasma homocysteine (nmol/ml)		Etiology
Severe form	>100	CBS deficiency MTHFR deficiency Nutritional inadequacy with or without minor genetic defect
Intermediate form	31 - 100	Methionine synthase deficiency due to defect in cobalamin metabolism Heterozygosity of MTHFR Interallelic combination of genetic defects Nutritional inadequacy with or without genetic defect
Moderate form	16-30	Interallelic combination of genetic defects Nutritional inadequacy with or without genetic defect

From Kang, 1996

As various forms of hyperhomocysteinemia are caused either by genetic or nongenetic defect(s), or by the combination of both (Table 1.4), the treatment is approached on the basis of its etiology and severity of defect(s). In nutritional cases of nongenetic hyperhomocysteinemia, the correction of nutrient inadequacy with supplements is the most effective method of treatment.

Table 1.4 Etiology of genetic and nongenetic hyperhomocysteinemia

A. Genetic hyperhomocysteinemia

- CBS activity deficiency: homozygous and heterozygous mutations
- MTHFR deficiency: homozygous and heterozygous mutations and thermolabile MTHFR
- Methionine synthase deficiency due to methylcobalamin depletion

B. Nongenetic (nutritional) hyperhomocysteinemia

- Decreased CBS activity due to low serum pyridoxine or serine
- Decreased MTHFR activity due to low serum folate or low THF
- Decreased methionine synthase due to low serum B_{12}
- Decreased betaine-homocysteine methyltransferase due to low choline
- Increased synthesis of homocysteine due to high methionine intake

From Kang, 1996

1.6 OBJECTIVES OF THE PRESENT STUDY

Niacin has increasingly been the choice of lipid-lowering agent by many physicians and patients. However, the potential risks of using high doses of the vitamin have not been critically considered in the same way as other lipid-lowering drugs. Niacin is excreted in the form of methylated pyridones. This methylation is achieved by a methyl transfer, which depends on SAM as a methyl donor. Since niacin excretion depends on methionine, which is an essential amino acid, an excess intake of the vitamin may affect methionine metabolism. Furthermore, niacin-associated hyperhomocysteinemia could have serious toxicological implications, especially on a long-term basis. Further research is

warranted to explore the role that niacin in its pharmacologic dose levels may have on methionine metabolism and its consequences.

It is hypothesized that niacin in its pharmacologic dose levels adversely affects the metabolism of sulfur amino acids, and subsequently the metabolic availability of vitamin B_{12}, folic acid and vitamin B_6. These effects result in hyperhomocysteinemia and hepatic hypertriglyceridemia. The hypothesis was tested in rats with the following objectives:

1. To modify a High Performance Liquid Chromatography (HPLC) method for quantifying total methionine, homocysteine and cysteine simultaneously.

2. Using the modified method, to examine the effects of niacin on plasma and urinary levels of sulfur amino acids.

3. To determine short and long term effects of niacin administration on vitamin B_{12}, folic acid, and vitamin B_6 status.

4. To investigate the influence of pharmacologic dose of niacin on selected liver enzymes and lipids, signifying hepatic function.

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METHIONINE, HOMOCYSTEINE AND CYSTEINE SIMULTANEOUSLY IN PLASMA AND URINE

The link between vascular disease and hyperhomocysteinemia has been recognized for more than 30 years (Ueland et al., 1992; Selhub et al., 1995). Homocysteine, a sulfur containing amino acid is formed by the demethylation of dietary methionine. It reacts with serine to form cystathionine which is catalyzed by a PLP-dependent enzyme, CBS. Cystathionine is subsequently cleaved by another PLP-dependent enzyme, γ-cystathionase, to form cysteine and α-ketobutyrate. Homocysteine can be remethylated to methionine which requires the enzymes methionine synthase, MTHFR and betaine-homocysteine methyltransferase, as well as, vitamin B_{12}, folic acid, betaine and choline as cofactors. Thus, a deficiency of these vitamins and/or enzymes can result in an abnormal accumulation of homocysteine (Masser et al., 1994).

In normal plasma, approximately 70% of homocysteine (Refsum et al., 1985) and 30% of cysteine (Malloy et al., 1981) is bound to protein. Storage of plasma causes redistribution of plasma thiols causing the protein-bound fraction to increase at the expense of the free fraction (Fiskerstrand et al., 1993). However, the total amount of homocysteine remains unchanged. Therefore, it is more significant to measure total homocysteine than free homocysteine. Total homocysteine comprises of homocysteine, homocystine (homocysteine-homocysteine disulfide), homocysteine-cysteine mixed disulfide, and protein-bound homocysteine. Whereas, total cysteine comprises of cystine (cysteine-cysteine disulfide), homocysteine-cysteine mixed disulfide, and protein-bound cysteine (Stabler et al., 1987). Assays measuring total homocysteine and cysteine require the liberation of protein-bound homocysteine and cysteine by cleavage of the disulfide bridge in the presence of a reducing agent. Therefore, reduction with a suitable sulfhydryl compound such as dithiothreitol, 2-mercaptoethanol,

borohydride, or tributylphosphine is essential for measuring total homocysteine and cysteine.

Since Kang et al., (1979) reported the presence of protein-bound homocysteine in normal individuals, total homocysteine has since been measured by many researchers. Refsum et al., (1985) used a radioenzymic method to measure total homocysteine in plasma and urine, that was quantified by High Performance Liquid Chromatography (HPLC) and Liquid Scintillation Counting. This method was sensitive and selective, but laborious and complicated for clinical purpose. Subsequently, Kang et al., (1982) measured protein-bound homocysteine and cysteine that were quantified on an amino acid analyzer. Araki and Sako (1987) and Jacobsen et al., (1989) measured total homocysteine and cysteine in human plasma by HPLC with fluorescence detection. Fiskerstrand et al., (1993) developed an automated column-switching, reverse-phase HPLC method for determining total plasma homocysteine. Even though these methods can be used in clinical applications, they only measure total homocysteine and/or cysteine levels.

Stabler et al., (1987) extracted total homocysteine, cysteine and methionine from serum and urine by cation-exchange and anion-exchange chromatography. Derivatives were prepared and analyzed using capillary gas chromatography-mass spectrometry. This method was of suitable sensitivity and reliability, but was complex and required expensive equipment. Brattstrom et al., (1988) measured total homocysteine, cysteine and methionine in human plasma using an automatic amino acid analyzer. This procedure was however, less sensitive and required 1.0 ml of plasma.

As it is of significant value to simultaneously quantify the total amount of sulfur containing amino acids involved in the homocysteine metabolic pathway, the present method measures total methionine, homocysteine and cysteine. The

butylphosphine, deproteinization with trichloroacetic acid, oxidation with performic acid, pre-column derivatization with o-phthaldialdehyde and separation and quantitation by reverse-phase HPLC and fluorescence detection.

2.1 MATERIALS AND METHODS

Material: Standard amino acids, ascorbic acid, N,N-dimethylformamide and o-phthaldialdehyde were purchased from Sigma Chemical Co. (St. Louis, MO). Tri-*n*-butylphosphine was purchased from Fisher (Ottawa, ON). Water and methanol were from BDH Inc. (Toronto, ON). The guard column and the analytical column were purchased from Supelco (Oakville, ON). All other chemicals were of analytical grade.

Method: The method of Sedgwick et al., (1991) was utilized with the following changes to the mobile phase. The mobile phase consisted of two solvents with a total flow rate of 1.5 ml/min. It contained 0.1 M sodium acetate adjusted to a pH between 7.0 to 7.2 with acetic acid, and 0.1% (w/v) ascorbic acid. The column was developed with solvent B (methanol) as follows: 0 min, 11% B; 0.1 min, 11% B; 10 min, 11% B; 10.5 min, 21% B; 31 min, 21% B; 35 min, 100% B; 39 min, 100% B and 40 min, 11% B. The total run time was 45 minutes.

Individual amino acids were quantified using a Varian 5000 high performance liquid chromatograph and a Varian 2070 Spectrofluorometer detector. The chromatograph was equipped with a 4.6 cm X 50 mm guard column containing Supelco LC-18 reverse phase packing (20-40 μm). Amino acids were separated on a 15 cm X 4.6 mm Supelcosil 3 micron LC-18 reverse phase analytical column. The fluorescence detector operated at an excitation wavelength of 340 nm and an emission wavelength of 450 nm. Chromatographic peaks were recorded and integrated using the Shimadzu Ezchrom Chromatography Data System (Kyoto, Japan).

Sample preparation: Whole blood and 24-hour urine samples were collected from six 12-hour fasted, healthy, male Sprague-Dawley rats weighing 250-350 g. Blood was collected in heparinized tubes by cardiac puncture and centrifuged to obtain plasma. Separated plasma and urine samples were stored at -40°C for later analysis. To 100 *u*l of sample or standard, 50 *u*l of 100 nmol/ml internal standard (L-α-amino-β-guandinopropionic acid [AGPA]) and 25 *u*l of 10% (v/v) tri-*n*-butylphosphine in dimethylformamide were added. Tubes were kept in an icebath for 30 minutes. One hundred microliters of chilled 10% (w/v) trichloroacetic acid was added to all tubes, which were then vortexed and centrifuged at 3000 RPM for 15 minutes. To 150 *u*l of supernatant, 100 *u*l of performic acid (containing hydrogen peroxide: formic acid at a ratio of 1:9 v/v) was added and tubes were left overnight at 4° C. On the following day, 50 *u*l of 0.3362 g/ml sodium metabisulfite was added to all tubes and vortexed vigorously. Samples were vacuum dried and the precipitate was reconstituted with 600 *u*l of saturated potassium borate. In chromatographic vials, 300 *u*l of sample and 100 *u*l of water were added. The pH of the samples was 9.5.

Samples were analyzed by the method of Jones and Gilligan (1983), which uses o-phthaldialdehyde (OPA) as a pre-column derivatizing agent. The fluoraldehyde reagent was prepared by dissolving 2 g of o-phthaldialdehyde in 6 ml methanol, followed by the addition of 56 ml of 0.04 M sodium borate buffer (pH=9.5), 2 ml of 2-mercaptoethanol, and 2 ml of Brij 35. Aliquots of the standard and samples were mixed at a ratio of 2:1 (v/v) with the fluoraldehyde reagent prior to injection. The injection volume was set at 75 *u*l for plasma samples and 50 *u*l for urine samples.

Statistics: Results are expressed as mean and standard error of mean (SEM). Coefficient of variation (C.V) was calculated as standard deviation expressed as a percentage of the mean.

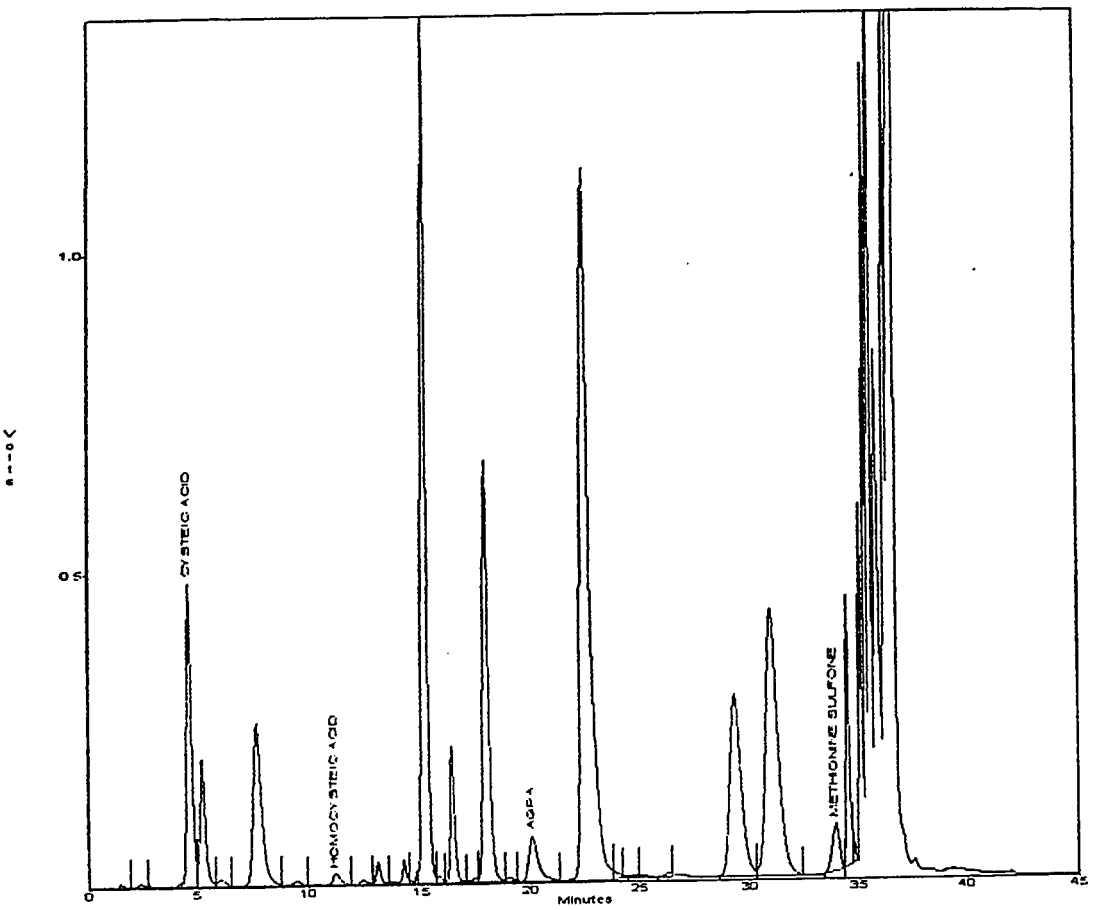
2.2 RESULTS AND DISCUSSION

The present method allows the simultaneous measurement of sulfur containing amino acids involved in the metabolism of homocysteine present in plasma and urine. Fig. 1 shows a chromatogram of rat plasma. The retention times for cysteic acid, homocysteic acid, and methionine sulfone were approximately 4.7, 11.3, and 34.8 minutes, respectively. The retention times varied slightly with changes in the pH of the mobile phase. All peaks could be separated to the baseline.

Standard curves were prepared by adding known concentrations (0-100 nmol/ml) of cysteic acid, homocysteic acid, AGPA and methionine sulfone to water. The standard curve was linear from 0 to 100 nmol/ml in concentration.

To determine within-day precision, 10 replicates of a plasma sample were assayed in one run. The coefficient of variation (C.V) values for total methionine, homocysteine and cysteine were 3.69%, 3.52% and 1.88%, respectively. Between-day precision was obtained by measuring the same plasma sample on ten different days. The C.V values for total methionine, homocysteine and cysteine were 4.24%, 3.91% and 2.32%, respectively. These results indicate that the method is reproducible.

In order to determine the validity, this method was employed to determine total methionine, homocysteine and cysteine in rat plasma and urine. Plasma and 24-hour urine samples were collected from six 12-hour fasted, normal, male Sprague-Dawley rats weighing 250-350 g. The concentrations of total methionine, homocysteine and cysteine in plasma (mean ± SEM) were 43.01 ± 1.41 nmol/ml,


1.0
0.5
0
CYSTEIC ACID
HOMOCYSTEIC ACID
AOPA
METHIONINE SULFONE
5
10
15
20
25
30
35
40
45
Minutes

comparable to those obtained by Stabler et al., (1987) who measured these amino acids using capillary gas chromatography-mass spectrometry (Table 2.1). Total methionine, homocysteine and cysteine were also determined in rat urine (mean ± SEM) and were 2480 ± 225 nmol/24 hr, 344 ± 21 nmol/24 hr and 991 ± 105 nmol/24 hr, respectively. These values are unique since no published data for the rat urinary amino acids could be found.

Table 2.1 Comparison of plasma amino acid values

Amino acids (nmol/ml)	Present Method	Stabler et al., (1987)
Methionine	43.01 ± 1.41	56.00 ± 1.91
Homocysteine	5.81 ± 0.12	5.60 ± 0.28
Cysteine	252.17 ± 4.64	190.00 ± 6.58

Values expressed as mean ± SEM

The current method modified to simultaneously measure total methionine, homocysteine and cysteine in plasma and urine is sensitive and reproducible. The essential steps in this assay include reduction, deproteinization, oxidation and pre-column derivatization for separation on HPLC. Determination of total homocysteine and cysteine requires the reduction of homocysteine and cysteine disulfides in biological samples. Tri-*n*-butylphosphine was used as reducing agent in this procedure, followed by deproteinization with trichloroacetic acid. Performic acid oxidation was carried out to oxidize methionine, homocysteine and cysteine to methionine sulfone, homocysteic acid and cysteic acid, respectively. It is these oxidized compounds that are eluted in the chromatogram. The purpose of adding sodium metabisulfite was to decompose performic acid. Vacuum drying samples increased the column life due to evaporation of acids (e.g. trichloroacetic

acid, formic acid) and prevention of salt buildup on to the column. Addition of ascorbic acid to solvent A was mainly done to prevent microbial growth. Coincidentally, it also improved the resolution by making the peaks well separated, and thus making integration easier.

The HPLC method described here has the following advantages. First, with this method total methionine, homocysteine and cysteine can all be measured simultaneously using just 100 *u*l of sample. Whereas, the method described by Brattstrom et al., (1988) requires 1.0 ml of sample to measure these amino acids, and the method described by Stabler et al., (1987) is complex and requires expensive equipment. Thus, a simple micro-method is more practical for research and clinical application. Second, the derivatizing agent used is inexpensive compared to the one used by Araki and Sako (1987) to measure total homocysteine and cysteine. One gram of o-phthaldialdehyde (OPA) costs approximately $75, whereas, 1 g of ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate (SBD-F) costs approximately $4000. This makes the present method cost effective. Third, with the autosampler used in this method, it is possible to run 50 samples in duplicates unattended for about 80 hours. However, like other analytical methods, the current method also has a few drawbacks. It is time consuming due to the numerous steps involved in the assay, but the utilization of an internal standard ensures good precision and accuracy. It is also necessary to have a HPLC machine equipped with a fluorescence detector and an autosampler. The autosampler is required due to the instability of the derivatizing agent (OPA).

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SULFUR AMINO ACID STATUS

Niacin is a water soluble vitamin; it is not stored in the body beyond its tissue saturation level. Hence, when taken in excess amounts it is excreted in urine. The predominant metabolic excretory products of niacin are methylated pyridones. Pyridone methylation involves a methyl transfer reaction in which SAM donates methyl groups endogenously (Shibata and Matsuo, 1989). Since niacin excretion is dependent on methionine, it is possible that an excess intake of the vitamin may affect the metabolism of this dietary essential amino acid.

Niacin consumed at pharmacologic dose levels (100-200 x physiological requirement) is reported to have a hypocholesterolemic effect (Illingworth, 1987; Keenan et al., 1991). Despite its therapeutic usage, the relationship between niacin and methionine has not been adequately studied. In a recent study (Basu and Mann, 1997), rats treated with niacin (400 or 4000 mg/kg diet) for three weeks exhibited hyperhomocysteinemia in plasma and urine. In view of the fact that homocysteine is considered to be an independent risk factor for arterial occlusive diseases, these results have an important toxicologic implication. However, this study measured only the free homocysteine levels, which may not reflect the true biochemical status of the amino acid. Storage of plasma causes redistribution of thiols resulting in an increase of the protein-bound fraction at the expense of the free form (Ueland and Refsum, 1989). It is therefore important that total homocysteine (free plus protein-bound) status is determined in the presence of large dose levels of niacin.

The present study was undertaken to examine the effect of pharmacolgic dose levels of niacin on plasma and urinary levels of total homocysteine, and its precursor methionine, as well as, its catabolic end-product, cysteine.

3.1 MATERIALS AND METHODS

3.1.1 Animals and diets

Male Sprague-Dawley rats (University of Alberta), weighing 150-200 g, were used throughout the study. Animals were housed in plastic shoe box cages, kept in a temperature and humidity controlled room, and maintained on a 12-hour light/dark cycle. The animal protocol of the study was reviewed and approved by the University of Alberta Animal Welfare Committee.

Rats were fed a standard pellet diet (Laboratory Rodent diet # 5001, PMI Feeds, Inc. Richmond, Ontario) for a week before being fed an experimental semi-synthetic diet (Table 3.1). Six animals were randomly assigned to one of the five diet groups. The control group was fed the semi-synthetic diet containing physiologic levels of niacin and methionine (Diet A, Table 3.1). Groups 2 and 3 were fed the semi-synthetic diet supplemented with 400 mg (Diet B) and 1000 mg (Diet C) niacin/kg diet, respectively. Group 4 and 5 were fed the semi-synthetic diet supplemented with 10 g methionine/kg diet either alone (Diet D) or in combination with 1000 mg niacin/kg diet (Diet E). These dose levels of niacin are equivalent to those consumed by humans on body weight basis for its hypolipidemic action. All animals were allowed free access to water and their respective diets for a period of one and three months, respectively (Figure 3.1).

Sample collection: Body weights were recorded throughout the study. One week prior to the end of the appropriate experimental period, rats were transferred from plastic shoe box cages and housed in stainless steel metabolic cages. Daily food intake was recorded and two 24-hour urine samples were collected during that week. At the end of the appropriate experimental period, animals were fasted for 12-hours, and euthanized with CO_2 gas. Blood was collected through cardiac puncture in heparinized tubes, and plasma was separated by centrifugation (3500 RPM for 10 min at room temperature). Livers were removed, excised, weighed

samples were stored at -40°C until analysis.

Table 3.1 Composition of the semi-synthetic diet[1]

Ingredient	g / Kg	Percent (%)
Casein (vitamin free)[2]	200	20
Corn starch	648	64.8
AIN Vitamin mixture[3]	10	1
AIN Mineral mixture[4]	30	3
Cellulose	50	5
DL-Methionine	2	0.2
Corn oil	60	6

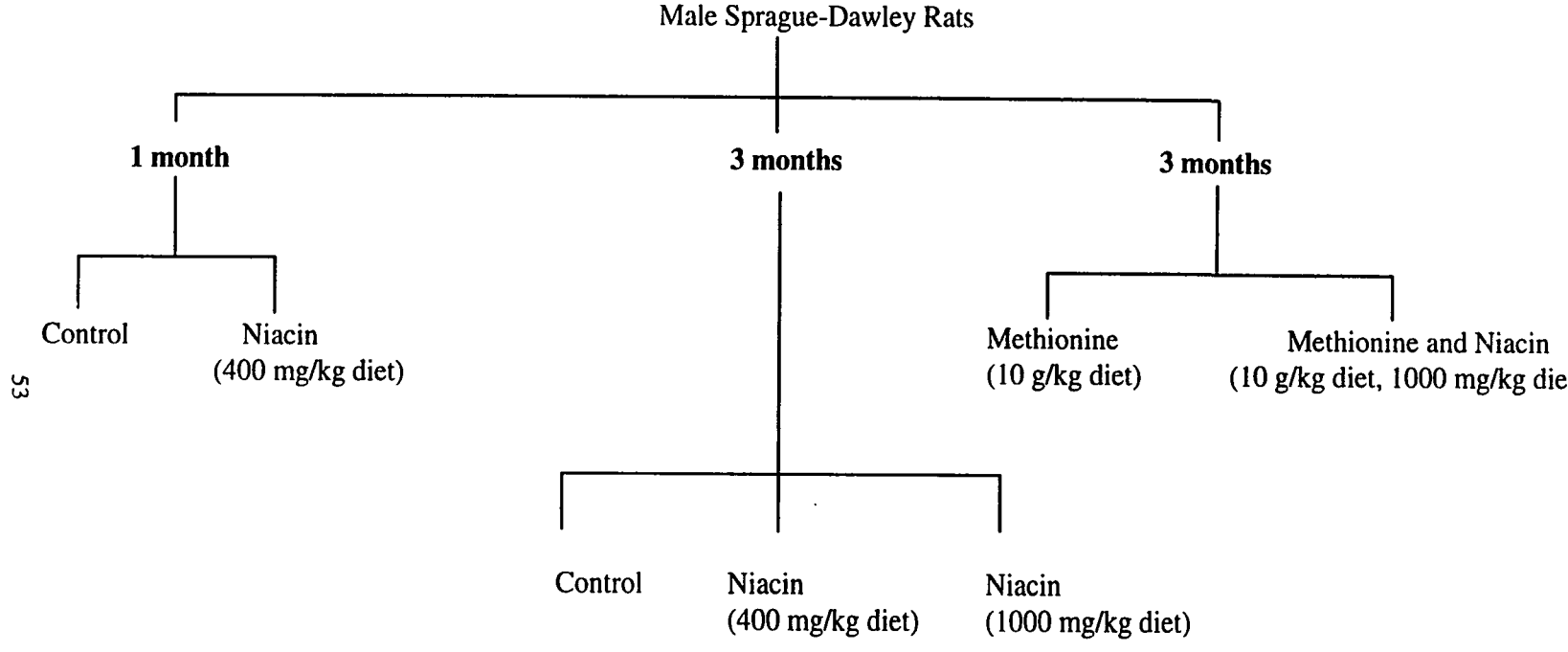
[1]Supplied by ICN Biomedicals, Cleveland, Ohio

[2]Casein (mg/kg diet)
Choline chloride <10

[3]Vitamin Mix (mg/kg diet)
Thiamin hydrochloride 6, riboflavin 6, pyridoxine hydrochloride 7, nicotinic acid 30, D-calcium pantothenate 16, folic acid 2, D-biotin 0.2, cyanocobalamin (Vitamin B_{12}) 0.01, retinyl palmitate (Vitamin A) 16, DL-α-tocopherol acetate 200, cholecalciferol (Vitamin D_3) 2.5, menaquinone (Vitamin K_2) 0.05, sucrose 9729

[4]Mineral Mix (g/kg diet)
Calcium phosphate dibasic 15, sodium chloride 2.22, potassium citrate monohydrate 6.6, potassium sulfate 1.56, magnesium oxide 0.72, manganese carbonate 0.105, ferric citrate 0.18, zinc carbonate 0.048, cupric carbonate 0.009, potassium iodate 0.0003, sodium selenite 0.0003, chromium potassium sulfate 0.0165, sucrose 3.54

Figure 3.1 Experimental design



3.1.2 Amino acid analysis

Plasma and urinary concentrations of total (free and protein-bound) methionine, homocysteine and cysteine were analyzed using the method described in Chapter 2.

Statistical analysis: Means and standard error of means (SEM) were determined for all groups of animals. Data was analyzed by using one-way analysis of variance (ANOVA). Significant differences were analyzed by a protected least significance difference (LSD) test. In the present study, the level of significance (α) considered was 0.05.

3.2 RESULTS

Feeding rats a semi-synthetic diet (Table 3.1) containing 400 mg niacin/kg diet for one month did not affect their food intake, growth rate or liver weight (Table 3.2).

Table 3.2 Effect of feeding a diet supplemented with niacin (400 mg/kg) for one month on food intake and growth of animals

	Control	Niacin
Food intake (g/day)	14.6 ± 0.1a	14.4 ± 0.2a
Body wt gain (g)	172.5 ± 5.9a	161.0 ± 2.8a
Liver wt (g)	11.4 ± 0.3a	11.7 ± 0.5a

Each value is the mean ± SEM of 6 rats. In each row, values with different letter are significantly different at $P<0.05$.

The plasma and 24-hour urinary levels of total methionine, homocysteine and cysteine of these animals also remained unaffected when compared with the corresponding control rats fed a semi-synthetic diet containing no supplemental niacin (Table 3.3).

Table 3.3 Effect of feeding a diet supplemented with niacin (400 mg/kg) for one month on plasma and urinary total methionine, homocysteine and cysteine levels

	Control	Niacin
Plasma (nmol/ml)		
Methionine	43.01 ± 1.41a	42.12 ± 2.87a
Homocysteine	5.81 ± 0.12a	6.60 ± 0.35a
Cysteine	252 ± 5a	271 ± 15a
Urinary (nmol/24 hr)		
Methionine	2480 ± 225a	3811 ± 748a
Homocysteine	344 ± 20a	352 ± 19a
Cysteine	991 ± 105a	1048 ± 94a

Each value is the mean ± SEM of 6 rats. In each row, values with different letter are significantly different at $P<0.05$.

Table 3.4 shows the effect of feeding rats a semi-synthetic diet containing niacin at two dose levels (400 and 1000 mg/kg diet) for three months on daily food intake and body weight gain. Neither the food intake nor the body weight were affected by the supplemental use of niacin at a level of 400 mg/kg diet. Both the parameters were however, significantly increased when dietary supplemental level of the vitamin was increased to 1000 mg/kg diet. These increases were in parallel with an increase in liver weight.

While the supplemental intake of 400 mg niacin/kg diet for three months had no effect on either the food intake or the body weight gain, the plasma levels of amino acids including total methionine, homocysteine and cysteine were significantly elevated in these animals (Table 3.5). Urinary concentrations of these amino acids were also higher in the niacin treated group compared with unsupplemented control rats; the differences however were not statistically significant. The magnitude of elevations in both plasma and urinary levels of homocysteine and cysteine in particular, was exacerbated when the supplemental level of niacin was increased to 1000 mg/kg diet. Thus, the degree of increase in urinary concentrations of the amino acids was changed from a trend at 400 mg of niacin (per kg diet) to a level of statistical significance ($P<0.05$) at 1000 mg of niacin (per kg diet).

Table 3.6 shows that feeding a diet supplemented with either 400 or 1000 mg niacin/kg diet for three months resulted in a significant increase in plasma homocysteine levels in relation to methionine, as well as, to cysteine levels. The diet containing 1000 mg niacin/kg diet exhibited a significantly higher plasma homocysteine:methionine and homocysteine:cysteine ratios than the diet containing 400 mg niacin/kg diet indicating a dose response effect (Table 3.6). Feeding the niacin supplemented diet at both dose levels for three months had no effect on urinary homocysteine:methionine and homocysteine:cysteine ratios when compared to the control diet.

Table 3.4 Effect of feeding a diet supplemented with niacin (400 or 1000 mg/kg) for three months on food intake and growth animals

	Control	Niacin (400 mg/kg)	Niacin (1000 mg/kg)
Food intake (g/day)	19.9 ± 0.5b	20.2 ± 0.4b	22.6 ± 1.0a
Body wt gain (g)	326.8 ± 7.8b	330.0 ± 5.7b	416.0 ± 21.1a
Liver wt (g)	12.7 ± 0.3b	13.9 ± 0.2b	18.0 ± 1.3a

Each value is the mean ± SEM of 6 rats. In each row, values with different letter are significantly different at $P<0.05$.

Table 3.5 Effect of feeding a diet supplemented with niacin (400 or 1000 mg/kg) for three months on plasma and urinary total methionine, homocysteine and cysteine levels

	Control	Niacin (400 mg/kg)	Niacin (1000 mg/kg)
Plasma (nmol/ml)			
Methionine	27.25 ± 0.24c	42.17 ± 1.35a	38.61 ± 0.74b
Homocysteine	3.58 ± 0.17c	7.39 ± 0.44b	9.52 ± 0.56a
Cysteine	247 ± 10c	300 ± 7b	415 ± 11a
Urinary (nmol/24hr)			
Methionine	1993 ± 433b	2077 ± 410b	3869 ± 421a
Homocysteine	242 ± 15b	275 ± 25b	451 ± 64a
Cysteine	950 ± 78b	1137 ± 103b	1791 ± 229a

Each value is the mean ± SEM of 6 rats. In each row, values with different letter are significantly different at $P<0.05$.

Table 3.6 Effect of feeding a diet supplemented with niacin (400 or 1000 mg/kg) for three months on plasma and urinary ratio of amino acids

Ratio	Control	Niacin (400 mg/kg)	Niacin (1000 mg/kg)
Plasma			
Homocysteine:Methionine	0.1314 ± 0.0062c	0.1746 ± 0.0051b	0.2474 ± 0.0167a
Homocysteine:Cysteine	0.0147 ± 0.0010b	0.0247 ± 0.0014a	0.0229 ± 0.0010a
Urine			
Homocysteine:Methionine	0.1424 ± 0.0210a	0.1559 ± 0.0294a	0.1151 ± 0.0048a
Homocysteine:Cysteine	0.2576 ± 0.0099a	0.2420 ± 0.0059a	0.2508 ± 0.0063a

Each value is the mean ± SEM of 6 rats. In each row, values with different letter are significantly different, $P<0.05$.

The niacin-induced hyperhomocysteinemia is an important toxicologic observation. In order to provide further confirmation of this effect, a methionine loading experiment was designed in which rats were fed a semi-synthetic diet (Table 3.1) containing 10 g methionine/kg diet either alone or in combination with 1000 mg niacin/kg diet for three months. No significant differences in food intake, growth rate or liver weight were observed between the two groups (Table 3.7). There was however, a significant increase in plasma homocysteine, a significant decrease in plasma cysteine and no change in plasma methionine levels in animals fed the niacin supplemented diet compared to the group fed the diet without niacin (Table 3.8). Urinary concentrations of these amino acids, on the other hand, were all significantly higher in the methionine plus niacin group compared to the methionine only group.

Table 3.7 Effect of feeding a diet supplemented with methionine (10 g/kg) and niacin (1000 mg/kg) for three months on food intake and growth of animals

	Methionine	Methionine & Niacin
Food intake (g/day)	22.8 ± 0.4a	21.5 ± 0.5a
Body wt gain (g)	386.7 ± 24.2a	364.5 ± 11.2a
Liver wt (g)	15.3 ±1.1a	14.6 ± 0.4a

Each value is the mean ± SEM of 6 rats. In each row, values with different letter are significantly different, $P<0.05$.

The diet containing both methionine and niacin caused a significant increase in plasma homocysteine:methionine and homocysteine:cysteine ratios compared to the diet containing only methionine (Table 3.9). However, no significant difference on urinary homocysteine:methionine and homocysteine:cysteine ratios was seen between the two groups.

Table 3.8 Effect of feeding a diet supplemented with methionine (10 g/kg) and niacin (1000 mg/kg) for three months on plası and urinary total methionine, homocysteine and cysteine levels

	Methionine	Methionine & Niacin
Plasma (nmol/ml)		
Methionine	40.57 ± 1.27a	43.57 ± 2.35a
Homocysteine	7.53 ± 0.41b	10.56 ± 0.55a
Cysteine	394 ± 8a	360 ± 12b
Urinary (nmol/24 hr)		
Methionine	5632 ± 1384b	16483 ± 4273a
Homocysteine	443 ± 55b	806 ± 127a
Cysteine	1030 ± 146b	2015 ± 195a

Each value is the mean ± SEM of 6 rats. In each row, values with different letter are significantly different, $P<0.05$.

Table 3.9 Effect of feeding a diet supplemented with methionine (10 g/kg) and niacin (1000 mg/kg) for three months on plasm and urinary ratio of amino acids

Ratio	Methionine	Methionine & Niacin
Plasma		
Homocysteine:Methionine	0.1866 ± 0.0118b	0.2441 ± 0.0134a
Homocysteine:Cysteine	0.0191 ± 0.0008b	0.0295 ± 0.0018a
Urine		
Homocysteine:Methionine	0.1096 ± 0.0318a	0.0640 ± 0.0214a
Homocysteine:Cysteine	0.4477 ± 0.0464a	0.3935 ± 0.0427a

Each value is the mean ± SEM of 6 rats. In each row, values with different letter are significantly different, $P<0.05$.

An overall analysis of rats' responses to large doses of niacin, in terms of methionine metabolism is summarized in Table 3.10. Niacin induced accumulation of homocysteine in relation to either methionine or cysteine was primarily observed in plasma and not in urine.

Table 3.10 Effect of niacin (1000 mg/kg diet) intake for three months on the percent change of plasma and urinary homocysteine in relation to methionine and cysteine levels

	Niacin/ Control	Methionine & Niacin/ Methionine
Plasma		
Homocysteine:Methionine	+ 88	+ 30
Homocysteine:Cysteine	+ 56	+ 54
Urine		
Homocysteine:Methionine	- 19	- 42
Homocysteine:Cysteine	- 3	- 12

The metabolism of niacin in pharmacologic dose levels requires methionine as a methyl donor in order to be excreted as methylated pyridones (Shibata and Matsuo, 1989). The present study examined the relationship between niacin in pharmacologic dose levels and the metabolism of methionine, particularly its conversion to cysteine via homocysteine.

Supplementation of 1000 mg niacin/kg diet to male Sprague-Dawley rats for three months resulted in a significant increase of both plasma and urinary total methionine, homocysteine and cysteine levels. The increase in plasma and urinary homocysteine levels was further accentuated by loading the diets with methionine. It was noteworthy, that the ratio between homocysteine and methionine and between homocysteine and cysteine at three months was significantly higher in plasma, but not in urine of the niacin treated animals. This was true at both dose levels of niacin (400 and 1000 mg/kg diet). These effects were further confirmed by loading methionine in the diet. Overall, these results suggest that pharmacologic doses of niacin can induce hyper(total)homocysteinemia.

The results found in the present study are in agreement with others who showed a hyperhomocysteinemic effect of niacin in rats when fed a diet containing either 400 or 4000 mg niacin/kg for a period of three weeks (Basu and Mann, 1997). The only flaw in this study was that they measured free homocysteine levels in plasma and urine, which make up only a small percentage of total homocysteine. Approximately 80% of homocysteine in blood is bound to protein by a disulfide linkage (Mansoor et al., 1992). Storage causes redistribution of thiols resulting in an increase in the protein-bound fraction at the expense of the free form (Ueland and Refsum, 1989). Therefore, it is important to measure total (free plus protein-bound) homocysteine, as this value remains constant regardless of storage time. The present study measured total homocysteine status, and the results obtained are in parallel with that of the earlier study by Basu and Mann (1997). These studies

clearly indicate that, niacin when used in pharmacologic dose levels could be potentially hyperhomocysteinemic.

Significant research activity has resulted in the development of the 'Homocysteine Theory of Atherosclerosis', and the realization that elevated plasma homocysteine levels is an independent risk factor for cardiovascular disease (Malinow and Stampfer, 1994; Boushey et al., 1995). Both retrospective (Ueland et al., 1992; Kang et al., 1992) and prospective (Stampfer et al., 1992; Arnesen et al., 1995) studies have demonstrated a relation between moderate hyperhomocysteinemia and premature vascular disease in coronary, cerebral and peripheral arteries. Accumulation of homocysteine in plasma can damage endothelial cells and promote atherosclerosis (Starkebaum and Harlan, 1986).

Homocysteine is an intermediate formed during the metabolism of methionine, an essential sulfur-containing amino acid supplied from dietary proteins. It is metabolized via two pathways: 1) the transsulfuration of homocysteine through cystathionine synthesis, a catabolic pathway, and 2) the remethylation of homocysteine to resynthesize methionine, a recycling pathway (Miller et al., 1994). Experimental evidence indicates that the transsulfuration and remethylation pathways are coordinated; reduced activity in one pathway will lead to a more effective use of homocysteine by the other pathway (Selhub and Miller, 1992).

Methionine is activated in an ATP-dependent reaction to form SAM. SAM is demethylated to form SAH, which is then hydrolyzed to adenosine and homocysteine. As observed in the present study, the conversion of methionine to homocysteine was affected in the presence of pharmacologic dose levels of niacin. As SAM is required as a methyl donor for the excretion of niacin, it demethylates to form SAH, which consequently hydrolyzes to form homocysteine.

pathway. In the transsulfuration pathway, homocysteine condenses with serine to form cystathionine in an irreversible reaction catalyzed by a PLP-dependent enzyme, CBS. Cystathionine is hydrolyzed by a second PLP-dependent enzyme, γ-cystathionase, to form cysteine and α-ketobutyrate (Ubbink et al., 1994). By excluding possible synthesis and degradation, the ratio between homocysteine to cysteine can be used as an indication for vitamin B_6 deficiency, and it may reflect the activity of the transsulfuration pathway from homocysteine to cysteine (Araki and Sako, 1987).

In the present study, the amount of homocysteine in relation to cysteine was significantly higher in the niacin treated group compared to the control group. This difference in the ratio means that the breakdown of homocysteine to cysteine is being affected in the presence of supplemental niacin. As vitamin B_6 in its coenzyme form PLP is involved in the transsulfuration of homocysteine, niacin in pharmacologic dose levels may be affecting the biochemical status of this cofactor. In a situation of excess methionine, the transsulfuration pathway is generally favored by up-regulation of CBS and down-regulation of the remethylation pathway (Finkelstein and Martin, 1986). The cysteine that is formed from homocysteine is either oxidized to taurine and inorganic sulfates or excreted in the urine. This means that methionine loading stresses the metabolic pathway responsible for the irreversible degradation of homocysteine to cysteine. It is noteworthy, that niacin in the presence of a methionine load, significantly lowered plasma cysteine levels and increased urinary cysteine levels. This further confirms that niacin in pharmacologic dose levels causes a disturbance in the transsulfuration of homocysteine to cysteine.

In the remethylation pathway, homocysteine is recycled to methionine by two different reactions. The first requires the presence of the enzyme methionine synthase. Methylcobalamin and 5-methyl THF serve as cofactor and cosubstrate

for this enzyme. The ratio between homocysteine to methionine can be an indication of vitamin B_{12} and/or folate deficiency. This ratio was significantly higher in the plasma of the niacin treated animals compared to their controls, implying a disturbance in the remethylation of homocysteine to methionine. Therefore, the accumulation of homocysteine in plasma and urine may be a reflection of a derangement in the resynthesis of methionine from homocysteine in the presence of pharmacologic dose levels of niacin.

The alternative route of homocysteine methylation is through a transfer of a methyl group from betaine, which is catalyzed by the enzyme betaine-homocysteine methyltransferase. This enzyme catalyzes the conversion of betaine and homocysteine to dimethylglycine and methionine, respectively (Finkelstein, 1990). This reaction is not dependent on either cobalamin or folate. The remethylation of homocysteine via 5-methyl THF occurs in all tissues, while the reaction via betaine is confined mainly to the liver and kidneys (Finkelstein et al., 1971) and depends on dietary choline. It has been suggested that due to limited tissue availability, betaine-homocysteine methyltransferase is not capable of handling excessive homocysteine accumulation. As a result, in congenital and acquired defects affecting vitamin B_{12} and folate-dependent remethylation pathway, the alternative route for conversion of homocysteine is unable to compensate sufficiently and causes hyperhomocysteinemia (Mason and Miller, 1992).

The results of the present study clearly suggest that both the remethylation and transsulfuration of homocysteine are impeded in the presence of pharmacologic levels of niacin, and subsequently cause an accumulation of homocysteine in both plasma and urine. Therefore, the next step would be to examine the effect niacin supplementation on the blood levels of nutrients that modulate total homocysteine (folate, cobalamin and PLP).

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4. EFFECT OF PHARMACOLOGIC DOSES OF NIACIN ON VITAMINS B_6, B_{12} AND FOLATE LEVELS

Administration of niacin in pharmacologic dose levels (400 and 1000 mg/kg diet) for three months have been shown to elevate plasma and urinary total methionine, homocysteine and cysteine levels (Chapter 3). These results have an important toxicologic implication, as elevated plasma homocysteine levels is considered to be an independent risk factor for arterial occlusive diseases.

Vitamin B_6, vitamin B_{12} and folate are involved as cofactors in the metabolism of homocysteine. The catabolism of homocysteine to cysteine via cystathionine requires the enzymes CBS and γ-cystathionase, which are dependent on vitamin B_6 in the form of PLP (Mudd and Levy, 1983). The remethylation of homocysteine to methionine via the enzyme methionine synthase requires both vitamin B_{12} in the form of methylcobalamin and folate in the form of 5-methyl THF (Ueland et al., 1992).

Niacin is excreted as methylated pyridones requiring methionine as a methyl donor (Shibata and Matsuo, 1989), and possibly vitamin B_{12} and folate as methyl carriers. It is therefore plausible that niacin, when taken in non-physiologic dose levels may affect the status of these vitamins. This may in turn be contributing to the hyperhomocysteinemia reported in the previous chapter. The latter condition may further affect vitamin B_6 status since this vitamin is an important cofactor in the metabolism of homocysteine (Masser et al., 1994).

The present study was undertaken to investigate the short and long term effects of pharmacologic dose levels of niacin on the biochemical status of vitamin B_6 and vitamin B_{12}, as well as, folic acid in rats.

4.1.1 Animals and diets

Six male Sprague-Dawley rats were randomly assigned to one of the five diet groups. The control group was fed the semi-synthetic diet containing physiologic levels of niacin and methionine (Diet A, Table 3.1). Groups 2 and 3 were fed the semi-synthetic diet supplemented with 400 mg (Diet B) and 1000 mg (Diet C) niacin/kg diet, respectively. Group 4 and 5 were fed the semi-synthetic diet supplemented with 10 g methionine/kg diet either alone (Diet D) or in combination with 1000 mg niacin/kg diet (Diet E). All animals were allowed free access to water and their respective diets for a period of one and three months, respectively (Figure 3.1).

Sample collection: At the end of the appropriate experimental period, animals were sacrificed and blood was collected as described in Chapter 3. The separated plasma was protected from light and stored at -40^{0}C until analysis.

4.1.2 Vitamin analysis

1. Determination of vitamin B_{12} and folic acid

Plasma vitamin B_{12} and folic acid were determined using a commercially available Solid Phase No Boil Dualcount kit (Intermedico, Markham, Ontario). Vitamin B_{12} and folic acid present in the sample were released from their carrier proteins by incubation at an elevated pH (>12) in the presence of dithiothreitol and potassium cyanide. This inactivates intrinsic factor antibodies and vitamin B_{12} transport proteins. Purified porcine intrinsic factor and folate binding protein were employed as binders for vitamin B_{12} and folic acid, respectively. The unlabeled vitamin B_{12} and folate compete with its labeled species for unlimited number of available binding sites on its specific binder, thus reducing the amount of labeled vitamin B_{12} and bound folate. Vitamin B_{12} analogs do not interfere as the binder is

free of R-protein. Moreover, the reaction takes place at a pH where the intrinsic factor is fully active and the folic acid binder has equal affinity for methyl THF, the predominant form of folic acid in circulation. After an adequate incubation period, isolation of the bound fraction was achieved by centrifugation and aspiration. Counts in the precipitate obtained using a gamma counter were transferred by comparison with a calibration curve into vitamin B_{12} and folic acid concentrations. The level of radioactivity (^{57}Co and ^{125}I) bound was inversely related to the concentration in the sample and standard.

2. Determination of vitamin B_6

Plasma PLP was determined using a commercially available radioassay kit (Dimensions Lab Inc., Mississauga, Ontario) as a modification of the method described by Shin et al., (1983). The principle of the assay involved the decarboxylation of ^3H-tyrosine to ^3H-tyramine by the vitamin B_6-dependent enzyme, tyrosine apodecarboxylase. The activity of tyrosine apodecarboxylase is quantitatively dependent on the amount of vitamin B_6 (PLP) in the reaction mixture. The ^3H-tyramine resulting from this reaction was extracted in the scintillation cocktail and measured by liquid scintillation counting. Counts in the scintillation cocktail obtained using a beta counter were transferred by comparison with a calibration curve into vitamin B_6 concentrations. The level of radioactivity (^3H) was directly related to the concentration in the sample and standard.

Statistical analysis: Means and standard error of means (SEM) were determined for all groups of animals. Data was analyzed by using one-way analysis of variance (ANOVA). Significant differences were analyzed by a protected least significance difference (LSD) test. In the present study, the level of significance (α) considered was 0.05.

Feeding rats a semi-synthetic diet (Table 3.1) containing 400 mg niacin/kg diet for one month did not affect their plasma vitamin B_{12}, folic acid or PLP levels (Table 4.1).

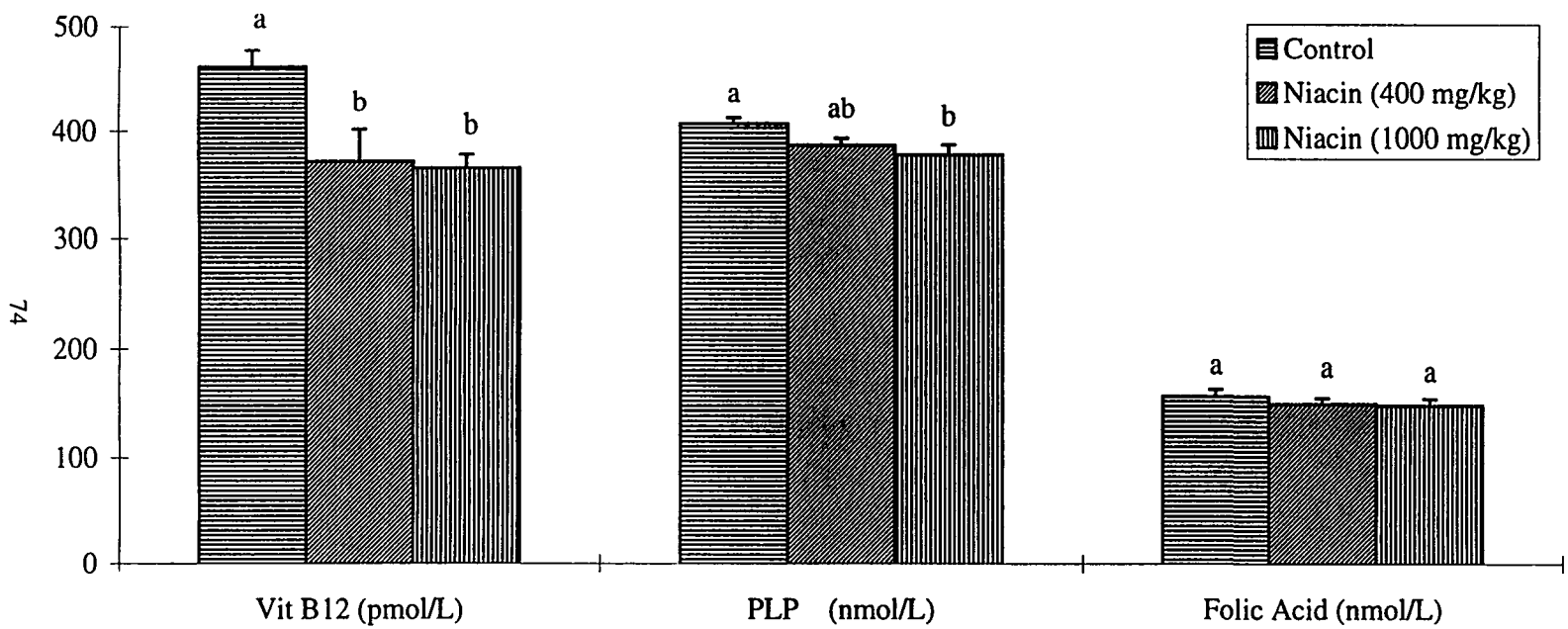
Table 4.1 Effect of feeding a diet supplemented with 400 mg niacin/kg for one month on vitamin status

	Control	Niacin
Vitamin B_{12} (pmol/L)	264 ± 41a	252 ± 16a
Folic acid (nmol/L)	151.4 ± 4.3a	144.3 ± 2.9a
PLP (nmol/L)	751.51 ± 45.18a	828.19 ± 45.17a

Each value is the mean ± SEM of 6 rats. In each row, values with different letter are significantly different at $P<0.05$.

Figure 4.1 shows the effect of feeding rats a semi-synthetic diet containing niacin (400 and 1000 mg/kg diet) for three months on plasma vitamin B_6, vitamin B_{12} and folate levels. While the supplemental intake of 400 mg niacin/kg diet for one month had no effect on plasma vitamin B_{12} (Table 4.1), the same level of niacin supplementation for three months caused a significant reduction in plasma vitamin B_{12}. The magnitude of decrease in plasma vitamin B_{12} levels remained the same when dietary niacin levels were increased to 1000 mg/kg diet and fed for three months. Unlike vitamin B_{12}, plasma folic acid remained unchanged in the animals fed niacin at all dose levels, even after three months of niacin administration. The response of plasma PLP level to niacin supplementation, on the other hand, was similar to that of vitamin B_{12}. The degree of reduction in

Figure 4.1 Effect of feeding large doses of niacin for three months on vitamin status



of statistical significance ($P<0.05$) at 1000 mg niacin (per kg diet) when fed for three months.

To further confirm the niacin associated reduction in plasma vitamin B_{12} and PLP levels, a methionine loading experiment was designed. Animals were fed a semi-synthetic diet (Table 3.1) containing 10 g methionine/kg diet either alone or in combination with 1000 mg niacin/kg diet for three months. This study revealed results that were similar to those found in animals supplemented with niacin (1000 mg/kg diet) alone for three months. Thus, niacin supplementation resulted in a significant reduction in plasma vitamin B_{12} and PLP levels, while no effect was seen on plasma folic acid concentration (Table 4.2).

Table 4.2 Effect of feeding a diet supplemented with methionine (10 g/kg) and niacin (1000 mg/kg) for three months on vitamin status

	Methionine	Methionine & Niacin
Vitamin B_{12} (pmol/L)	410 ± 31a	327 ± 10b
Folic acid (nmol/L)	149.3 ± 4.3a	141.6 ± 5.7a
PLP (nmol/L)	441.81 ± 11.82a	388.91 ± 13.84b

Each value is the mean ± SEM of 6 rats. In each row, values with different letter are significantly different, $P<0.05$.

4.3 DISCUSSION

The excretion of niacin in the form of methylated pyridones is dependent on the essential amino acid, methionine (Shibata and Matsuo, 1989). As reported in the previous chapter, niacin in pharmacologic dose levels caused an elevation in plasma and urinary total methionine, homocysteine and cysteine concentrations. This niacin-induced hyperhomocysteinemia is an important observation, as

arterial occlusive diseases.

The metabolism of homocysteine lies at the intersection of two pathways: remethylation and transsulfuration. In the remethylation pathway, 5-methyl THF transfers a methyl group to vitamin B_{12}, which subsequently transfers it to homocysteine and converts it to methionine. The present study examined the effect of niacin using non-physiologic levels on vitamin B_{12} and folate status in rats. According to this study, supplementation of 1000 mg niacin/kg diet either alone or in combination with methionine for 90 days did not have an affect on folate status, but resulted in a significant reduction in plasma vitamin B_{12} levels. This niacin-associated decline in plasma vitamin B_{12} level was in parallel with a significant increase in plasma and urinary total homocysteine concentrations.

These results do not agree with Basu and Mann (1997) who failed to show any biochemical evidence of a reduction in vitamin B_{12} status in rats fed a semi-synthetic diet supplemented with either 400 or 4000 mg niacin/kg diet for a period of three weeks. The biological half-life of vitamin B_{12} is estimated to be more than 480 days (Herbert, 1987); this is longer than any other water soluble vitamin. It is therefore possible that the difference in the response of plasma vitamin B_{12} to niacin treatment between the present study and that reported by Basu and Mann (1997) is caused by the difference in length of the experiments (i.e. 21 days vs 90 days).

In the remethylation pathway, homocysteine acquires a methyl group to form methionine. In one *de novo* route 5,10-methylene THF formed from THF is reduced to 5-methyl THF in an irreversible reaction catalyzed by MTHFR. The methyl group of 5-methyl THF is transferred to homocysteine in a reaction that is catalyzed by a vitamin B_{12}-dependent enzyme, methionine synthase (D'Angelo et al., 1997). Thus, both vitamin B_{12} and folate are important factors involved in the

metabolism of homocysteine. Unlike the effect of niacin on vitamin B_{12} level, plasma folic acid remained unaffected in the presence of supplemental niacin.

Adequate vitamin B_{12} must be present for the activity of methionine synthase, the enzyme that removes the methyl group from 5-methyl THF and delivers it to homocysteine, thereby converting homocysteine to methionine and regenerating free THF. As 5-methyl THF may only return to the body's folate pool via a vitamin B_{12}-dependent step, a deficiency of vitamin B_{12} traps body folate in the methyl form (Herbert and Coleman, 1988). It is plausible that the niacin-induced vitamin B_{12} decrease may have resulted in less utilization of folate, and this may explain for the folate status being unaffected.

In the transsulfuration pathway, homocysteine condenses with serine to form cystathionine in an irreversible reaction. CBS catalyzes the first step in the transsulfuration pathway with vitamin B_6 in its active form, PLP, as a cofactor. Cystathionine is then hydrolyzed by a second PLP-dependent enzyme, γ-cystathionase, to form cysteine and α-ketobutyrate. There is currently no consensus on the best biochemical marker for vitamin B_6 status in humans. Plasma PLP is most often studied and is a reasonable indicator of human vitamin B_6 status (Leklem, 1990). However, some researchers suggest that plasma PLP is a good indicator of body store, whereas pyridoxic acid is a better indicator of intake (Merrill and Henderson, 1987). As rats in the present study were given the same semi-synthetic diet with the exception of niacin, the variation in vitamin B_6 intake was not a concern. Therefore, plasma PLP was measured to determine if niacin affects vitamin B_6 status.

Supplementation of 1000 mg niacin/kg diet to rats for three months resulted in a significant lower plasma PLP level compared to the control rats. This niacin-associated decline in plasma PLP level was in parallel with a significantly higher plasma and urinary total homocysteine concentration. These results suggest that

the niacin-induced hyperhomocysteinemia.

As homocysteine is a branch point metabolite, its biologic fate is linked to vitamin B_{12}, folic acid and vitamin B_6. The results from the present study clearly suggest that the biochemical status of vitamin B_{12} and vitamin B_6 is affected in the presence of pharmacologic dose levels of niacin, and consequently causes hyperhomocysteinemia. These data suggest an important role for nutritional status in homocysteine metabolism.

In recent years, the vitamin-homocysteine relationship has been explored by numerous researchers. Homocysteine levels can be markedly elevated in deficiencies of the essential cofactors vitamins B_{12} and B_6, and essential cosubstrate folic acid. Brattstrom et al., (1988) reported that isolated vitamin B_{12} deficiency could cause a moderate to intermediate fasting hyperhomocysteinemia. Selhub and Miller (1991) presented a scheme linking vitamin status with plasma homocysteine levels, particularly vitamin B_6, vitamin B_{12} and folate. There are now numerous studies that document lowering of plasma homocysteine levels with vitamin intervention therapy (Wilken et al., 1988; Ubbink et al., 1994; Van der Berg et al., 1994).

The treatment of hyperhomocysteinemia is approached on the basis of its etiology and severity of defect(s). In nutritional cases of nongenetic hyperhomocysteinemia, the correction of nutrient inadequacy with supplements is the most effective method of treatment. It is therefore important to maintain folic acid, vitamin B_{12} and vitamin B_6 levels above the low normal serum limit (Kang, 1996). Advising concurrent supplementation of vitamin B_{12} and vitamin B_6 to patients taking niacin for its hypolipidemic effect, may be beneficial in preventing the development of niacin-induced hyperhomocysteinemia.

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LIVER FUNCTION

Niacin in non-physiologic dose levels caused an elevation in plasma and urinary total methionine and its metabolic products, homocysteine and cysteine levels (Chapter 3). These abnormalities in the sulfur amino acid status were accompanied by a significant reduction in both plasma vitamin B_{12} and PLP concentrations (Chapter 4).

Methionine is a dietary essential sulfur-containing amino acid and a precursor of cysteine. The latter is formed via the transsulfuration of homocysteine. Although, methionine is an essential amino acid, it can be resynthesized from homocysteine subject to the availability of methyl donors, such as choline and 5-methyl THF and methyl carriers, such as vitamin B_{12}. Furthermore, vitamin B_{12} is also involved in the *de novo* synthesis of choline from its precursor, serine, via ethanolamine. Thus methionine, choline and vitamin B_{12} are interrelated (Davis, 1984). Methionine and choline are both considered to be lipotropic factors. Thus, diets deficient in methionine and choline have been shown to produce triglyceridemic fatty liver in rats (Griffith and Dyer, 1968).

Hepatotoxity, a rare complication of niacin therapy has gained more attention with its increased use. In most cases, patients have jaundice, pruritis, and mild to moderate elevations in serum bilirubin, alkaline phosphatase and alanine aminotransferase levels, although, in some instances, more severe hepatocellular injury has occurred (Mullin et al., 1989).

The present study was undertaken to investigate the influence of pharmacologic dose levels of niacin on selected liver enzymes and lipids, signifying hepatic function.

5.1.1 Animals and diets

Six male Sprague-Dawley rats were randomly assigned to one of the five diet groups. The control group was fed the semi-synthetic diet containing physiologic levels of niacin and methionine (Diet A, Table 3.1). Groups 2 and 3 were fed the semi-synthetic diet supplemented with 400 mg (Diet B) and 1000 mg (Diet C) niacin/kg diet, respectively. Group 4 and 5 were fed the semi-synthetic diet supplemented with 10 g methionine/kg diet either alone (Diet D) or in combination with 1000 mg niacin/kg diet (Diet E). All animals were allowed free access to water and their respective diets for a period of one and three months, respectively (Figure 3.1).

Sample collection: Body weights were recorded throughout the study. At the end of the appropriate experimental periods, animals were sacrificed and blood was collected as described in Chapter 3. Livers were removed, excised, weighed and frozen immediately in liquid nitrogen. The separated plasma and liver samples were stored at -40^0 C until analysis.

5.1.2 Liver function analysis

1. Determination of liver function enzymes

Clinical chemistry on plasma samples was performed at the Pathology Department (University of Alberta). Plasma bilirubin and alanine aminotransferase levels were measured using a Kodak 700XR clinical analyzer. Commercially available controls were run daily on the analyzer and had to be within the ranges specified by the manufacturer prior to any specimens being run. All clinical chemistry results were reviewed by a Board Certified Veterinary Pathologist prior to release.

2. Liver lipids

a) Extraction of liver lipids

Liver lipids were extracted from approximately 100 mg portions as described by Folch et al., (1957). Liver samples were homogenized with 50 mM NaCl and chloroform:methanol mixture (2:1 v/v). The homogenate was centrifuged and the upper layer was removed by aspiration. The lower layer containing the lipids was collected, dried under nitrogen and reconstituted with isopropanol.

b) Determination of liver triglyceride

Using Sigma Diagnostics kit # 336 (St. Louis, MO), liver triglyceride (TG) was measured enzymatically based on a method by Bucola and David (1973). TG was first hydrolyzed by lipoprotein lipase to glycerol and free fatty acids. Glycerol was then phosphorylated by adenosine-5-triphosphate (ATP), to form glycerol-1-phosphate (G-1-P) and adenosine-5-diphosphate (ADP) in a reaction catalyzed by glycerol kinase (GK). The G-1-P was oxidized to dihydroxyacetone phosphate (DAP) with the concomitant reduction of nicotinamide adenine dinucleotide (NAD) to NADH in a reaction catalyzed by glycerol-1-phosphate dehydrogenase (G-1-PDH).

In a subsequent step, NADH was oxidized with a simultaneous reduction of 2-(p-iodophenyl)-3-p-nitrophenyl-5-phenyltetrazolium (INT) to INTH (formazan) in the presence of diaphorase. The resulting formazan was highly colored and had an absorbance maximum at 500 nm. The intensity of color produced was directly proportional to TG concentration in the sample. Enzymatic reactions involved in the procedure are as follows:

Triglycerides ⟶ Glycerol + Fatty acids

$$\text{Glycerol + ATP} \xrightarrow{\text{GK}} \text{G-1-P + ADP}$$

$$\text{G-1-P + NAD} \xrightarrow{\text{G-1-PDH}} \text{DAP + NADH}$$

$$\text{NADH + INT} \xrightarrow{\text{Diaphorase}} \text{Formazan + NAD}$$

c) Determination of liver total cholesterol

Using Sigma Diagnostics kit # 352 (St. Louis, MO), liver total cholesterol was measured enzymatically based on a method by Allain et al., (1974). Cholesterol esters were first hydrolyzed by cholesterol esterase to free cholesterol. The free cholesterol produced by hydrolysis was oxidized by cholesterol oxidase to cholest-4-en-3-one and hydrogen peroxide. Hydrogen peroxide was then coupled with chromogens, 4-aminoantipyrine and p-hydroxybenzenesulfonate in the presence of peroxidase to yield a quinoneimine dye which had a maximum absorbance at 500 nm. The intensity of color produced was directly proportional to the total cholesterol concentration in the sample. Enzymatic reactions involved in the procedure are as follows:

$$\text{Cholesterol Esters + } H_2O \xrightarrow{\text{Cholesterol Esterase}} \text{Cholesterol + Fatty Acids}$$

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

$$2H_2O_2 + \text{4-Aminoantipyrine + p-Hydroxybenzenesulfonate} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine Dye + } 4H_2O$$

Statistical analysis: Means and standard error of means (SEM) were determined for all groups of animals. Data was analyzed by using one-way analysis of variance (ANOVA). Significant differences were analyzed by a protected least significance difference (LSD) test. In the present study, the level of significance (α) considered was 0.05.

5.2 RESULTS

Feeding rats a semi-synthetic diet (Table 3.1) containing 400 mg niacin/kg diet for one month did not affect their liver weight relative to body weight when compared with animals without niacin supplementation (Table 5.1). Bilirubin and aminotransferase levels in plasma are thought to reflect liver functions and thus were measured in these animals. The plasma level of bilirubin was significantly elevated, while alanine aminotransferase remained unaffected in the niacin treated animals compared to their control counterparts (Table 5.1).

Table 5.1 Effect of feeding a diet supplemented with 400 mg niacin/kg diet for one month on liver function

	Control	Niacin
Liver Weight / Body Weight (ratio)	3.28 ± 0.05a	3.40 ± 0.11a
Total bilirubin (*u*mol/L)	11.8 ± 1.1b	19.3 ± 2.1a
Alanine aminotransferase (IU/L)	53 ± 6a	54 ± 4a

Each value is the mean ± SEM of 6 rats. In each row, values with different letter are significantly different at $P<0.05$.

These niacin responses remained unchanged even when the duration of the vitamin supplementation at the level of 400 mg/kg diet was extended to three months (Table 5.2). It was thus, only the bilirubin concentration in plasma and not aminotransferase nor liver weight to body weight ratio that was significantly increased in the presence of niacin. These parameters, however, were all significantly elevated when the supplemental level of niacin was increased to 1000 mg/kg diet and fed for three months. The higher liver weight in relation to the body weight in the niacin supplemented rats was in parallel with a higher food intake. The response of plasma bilirubin to niacin was dose-related, and was more sensitive than either aminotransferase level or the liver weight to body weight ratio.

Subsequently, a methionine loading experiment was designed in which rats were fed a semi-synthetic diet (Table 3.1) containing 10 g methionine/kg diet either alone or in combination with 1000 mg niacin/kg diet for three months. The results from this experiment were in parallel with the results obtained in the absence of a methionine load. Both plasma bilirubin and alanine aminotransferase were significantly higher in the methionine plus niacin group than those in the methionine only group (Table 5.3).

Table 5.4 shows the effect of niacin on hepatic triglyceride and total cholesterol concentrations. Supplementation of 1000 mg niacin/kg for three months caused a significant increase of 77% in hepatic triglyceride levels compared to the control group. A significant increase of 73% in hepatic triglyceride levels was also seen in the methionine plus niacin treated group compared to the methionine only group. Unlike triglyceride, the hepatic concentrations of total cholesterol remained unaffected in these animals.

Table 5.2 Effect of feeding a diet supplemented with niacin (400 or 1000 mg/kg diet) for three months on liver function

	Control	Niacin (400 mg/kg)	Niacin (1000 mg/kg)
Liver Weight / Body Weight (ratio)	2.59 ± 0.07b	2.73 ± 0.04ab	2.95 ± 0.12a
Total bilirubin (*u*mol/L)	5.2 ± 0.3c	10.3 ± 0.7b	23.5 ± 1.6a
Alanine aminotransferase (IU/L)	45 ± 6b	49 ± 6ab	84 ± 20a

Each value is the mean ± SEM of 6 rats. In each row, values with different letter are significantly different at $P<0.05$.

Table 5.3 Effect of feeding a diet supplemented with methionine (10 g/kg) and niacin (1000 mg/kg) for three months on liver function

	Methionine	Methionine & Niacin
Liver Weight / Body Weight (ratio)	2.56 ± 0.08a	2.69 ± 0.06a
Total bilirubin (*u*mol/L)	14.2 ± 0.4b	17.8 ± 1.1a
Alanine aminotransferase (IU/L)	38 ± 3b	63 ± 9a

Each value is the mean ± SEM of 6 rats. In each row, values with different letter are significantly different at P<0.05.

Table 5.4 Effect of feeding a diet supplemented with 1000 mg niacin/kg diet either alone or in combination with 10 g methionine/kg diet for three months on liver lipids

Treatment		Liver lipids	
Niacin	Methionine	Triglyceride (mg/g liver)	Total cholesterol (mg/g liver)
–	–	40.63 ± 3.43b	19.67 ± 1.80a
+	–	71.83 ± 3.86a	17.67 ± 1.45a
–	+	43.13 ± 2.93b	15.66 ± 2.26a
+	+	74.47 ± 2.65a	13.12 ± 2.77a

Each value is the mean ± SEM of 6 rats. In each column, values with different letter are significantly different at P<0.05.

5.3 DISCUSSION

In recent years, niacin-induced hepatotoxicity has gained more attention due to its increased therapeutic use as a hypocholesterolemic agent (Henkin et al., 1990). The niacin-associated increase in plasma bilirubin and alanine aminotransferase

seen in the present study are indicative of hepatic dysfunction. These results are in agreement with numerous other studies that report an increase in serum bilirubin and aminotransferase during niacin therapy (Patterson et al., 1983; Clementz and Holmes, 1987; Ferenchick and Rovner, 1989; Reimund and Ramos, 1994).

Niacin is available in regular and sustained-release (SR) preparations; the latter are associated with a lower incidence of flushing but, are more hepatotoxic than the regular preparations (McKenney et al., 1994). Rare cases of severe hepatic injury have been attributed to niacin therapy. Mullin et al., (1989) reported the case of a patient who tolerated 6 grams of regular niacin daily but in whom fulminant hepatic failure developed three days after an unintentional switch to an SR preparation; the patient required liver transplantation. Hodis (1990) reported fulminant hepatic failure in an otherwise healthy patient taking 500 mg of SR niacin for eight weeks. Fischer et al., (1991) reported the case of a patient in whom unexplained fulminant hepatic failure developed following treatment with 2 grams of SR niacin per day.

Niacin has been used for many years to treat hyperlipidemia. It reduces plasma total cholesterol, LDL cholesterol and VLDL cholesterol, as well as, TG levels. It is however noteworthy, that niacin treatment did not affect total cholesterol in the liver despite its established hypocholesterolemic effect in plasma. Hepatic storage of cholesterol is essentially in its ester form (Goldstein and Brown, 1984). Unfortunately in the present study, hepatic cholesterol esters were not measured to better characterize the lipid deposition in liver in the presence of supplemental niacin.

The present study has also demonstrated that niacin in its non-physiologic dose levels causes hepatic hypertriglyceridemia. These results provide further evidence supporting some isolated cases that have been reported. Lawrence (1993) reported a case of niacin-induced focal fatty infiltration of the liver that was seen by

taking therapeutic niacin have also shown fatty change (Parsons, 1961; Baggenstoss et al., 1967).

Under normal conditions, the rate of TG synthesis by the liver is balanced by the rate at which they are released into the blood. TG are exported from the liver with protein (β-globulin), phospholipids, and cholesterol as VLDL. Impaired transport of TG into blood can cause the development of fatty livers (Banks et al., 1976). The underlying mechanism for the niacin-induced hepatic hypertriglyceridemia can not be fully elucidated at the present time.

Choline deficiency has been shown in experimental studies to cause an accumulation of hepatic TG, and thereby produce fatty livers (Mookerjea, 1965; Griffith and Dyer, 1968; Reitz, 1979). The increase in the level of hepatic TG in choline deficient rats is believed to be caused by an impaired transport of lipids, as lipoproteins, from the hepatic cells into serum (Chalvardjian, 1970). This has been suggested to be due to the limited synthesis of phosphatidylcholine (PC) (Haines, 1966; Reitz, 1979). As a component of PC, choline is important to the structure of cell membranes and plasma lipoproteins (McMahon, 1987).

The *de novo* synthesis of choline begins with the amino acid serine, which is decarboxylated in a vitamin B_6-dependent reaction to ethanolamine. This latter compound is then progressively methylated to choline. This methylation process, in which methionine acts as a methyl donor, indirectly depends upon vitamin B_{12} and folate for the synthesis of methionine, and hence choline biosynthesis (Tuma et al., 1975). In view of the fact that niacin can potentially affect vitamin B_{12} status (Chapter 4), it is plausible to suggest that the decrease in vitamin B_{12} may account for the hepatic triglyceridemia observed in the niacin treated rats.

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6. GENERAL DISCUSSION AND CONCLUSION

Niacin (nicotinic acid), a water-soluble B vitamin in large doses is widely used in the treatment of hyperlipidemia (Naito, 1987). It is effective in the treatment of all lipoprotein disorders characterized by elevated LDL and VLDL levels. It is often chosen as a hypolipidemic agent because of its ability to increase HDL levels (Crouse, 1996). In evaluating the cost effectiveness of strategies of intervention in atherosclerosis, the relatively low cost of niacin makes it an attractive agent.

The versatile action of niacin on lipoprotein metabolism, as well as its low cost, should make it a drug of choice in patients with hyperlipidemia, but unfortunately these beneficial effects are accompanied by some adverse effects. The most common side effect associated with using therapeutic levels of niacin is cutaneous flushing. This unpleasant side effect can be reduced by initiating therapy with low doses, premedication with aspirin, taking niacin with meals and avoiding hot liquids and alcohol immediately after a dose (Brown, 1995). Other occasional side effects of niacin include pruritus, rash, heartburn, nausea, bloating, diarrhea, hyperglycemia and hyperuricemia (Felicetta, 1994).

Hepatotoxity is a rare but potentially severe adverse effect of niacin therapy. Liver function abnormalities can include hepatitis, jaundice, malaise, hepatomegaly, and mild to moderate elevations of serum bilirubin, alkaline phosphatase and alanine aminotransferase levels (Mullin et al., 1989). Sustained-Release (SR) preparations of niacin were developed to minimize the cutaneous flushing associated with its use. However, unlike the hepatotoxicity seen with immediate-release niacin, which usually occurs at higher dosages and after prolonged usage, toxicity seen with SR niacin can occur within the first seven weeks of therapy and at relatively low dosages (Etchason et al., 1991). The mechanism by which niacin induces liver injury remains unclear. Although, it has been speculated that SR niacin affects the liver enzyme systems for longer periods than regular niacin, resulting in shorter recovery periods and thus more severe toxic effects (Christensen et al., 1961). In

most cases, liver function returns to normal after discontinuation of niacin administration (Fischer et al., 1991).

The potential risks of using non-physiologic dose levels of niacin on a long term basis have not been critically considered in the same way as other lipid-lowering drugs. Apart from the well known side effects of niacin therapy, Basu and Mann (1997) were the first to report altered sulfur amino acid status in the presence of niacin. They showed that administration of large doses of niacin (400 or 4000 mg/kg diet) to rats for three weeks interfered with the metabolism of methionine.

As niacin is a water soluble vitamin, it is not stored in the body beyond its tissue saturation level. Thus when taken in excess amounts, it is excreted in the form of methylated pyridones. This pyridone methylation involves a methyl transfer reaction in which SAM donates methyl groups endogenously (Shibata and Matsuo, 1989). Since the excretion of niacin is dependent on methionine, a dietary essential amino acid, it is possible that an excess intake of niacin may affect the metabolism of methionine. In view of this fact, the present study was undertaken to examine the short and long term effects of pharmacologic dose levels of niacin on plasma and urinary total homocysteine, its precursor methionine, as well as, its catabolic end-product, cysteine.

Methionine is activated in an ATP-dependent reaction to form SAM. SAM is demethylated to form SAH, which is then hydrolyzed to form homocysteine. As the excretion of niacin is dependent on the methyl groups from SAM, an excess intake of niacin will cause the demethylation of SAM to SAH, which will subsequently hydrolyze to homocysteine at a higher rate. Thus, administration of non-physiologic doses of niacin as illustrated in the present study, resulted in a significant increase in homocysteine concentrations.

remethylation of homocysteine to reform methionine, a recycling pathway, and 2) the transsulfuration of homocysteine to cysteine via cystathionine synthesis, a catabolic pathway (Miller et al., 1994). Administration of niacin in large doses in the present study not only increased total homocysteine levels, but also increased total methionine and cysteine concentrations in both plasma and urine.

Experimental evidence indicates that the remethylation and transsulfuration pathways are coordinated; reduced activity in one pathway will lead to a more effective use of homocysteine by the other pathway (Selhub and Miller, 1992). Therefore, an impairment in one homocysteine metabolic pathway should not lead to an accumulation of homocysteine, which is solely derived from methionine. As seen in the present study, niacin in pharmacologic dose levels causes an increase in plasma homocysteine in relation to both plasma methionine and cysteine levels. This implies that both remethylation and transsulfuration of homocysteine were impeded in the presence of supplemental niacin.

This niacin-induced hyperhomocysteinemia is an important observation, and may have serious health implications due to the potential toxic effects of homocysteine. An elevation in plasma homocysteine concentration is considered to be an independent risk factor for cardiovascular disease (Stampfer et al., 1992; Boushey et al., 1995). Numerous studies have indicated that elevated plasma homocysteine levels are associated with increased risk of premature occlusive vascular diseases (Genest et al., 1990; Kang et al., 1992; Selhub et al., 1995). A variety of mechanisms have been proposed by which homocysteine may promote atherogenesis. These include endothelial cell damage (Starkebaum and Harlan, 1986), proliferation of smooth muscle cells (Tsai et al., 1994) and altered thrombomodulin expression (Stampfer and Malinow, 1995).

presence of supplemental niacin, the next objective was to examine the effect of niacin supplementation on the vitamins that modulate homocysteine levels. Therefore, the biochemical status of vitamin B_6, vitamin B_{12} and folic acid was assessed.

In the remethylation pathway, homocysteine is recycled to methionine in two different reactions. In the first reaction, homocysteine acquires a methyl group from 5-methyl THF to form methionine. This reaction is catalyzed by a vitamin B_{12}-dependent enzyme, methionine synthase. Thus, the remethylation of homocysteine to methionine requires both vitamin B_{12} in the form of methylcobalamin, and folate in the form of 5-methyl THF (Ueland et al., 1992). Supplementation of large doses of niacin caused a significant decrease in plasma vitamin B_{12} levels. This niacin-associated decline in plasma vitamin B_{12} was in parallel with an increase in plasma homocysteine in relation to methionine in the niacin supplemented animals. However unlike the effect of niacin on vitamin B_{12} levels, plasma folic acid remained unaffected in the presence of niacin. This could be attributed to the decrease in vitamin B_{12}, which is required for the utilization of folic acid in the body. The significant reduction of vitamin B_{12} observed in the present study, can cause an impairment in the remethylation of homocysteine to methionine resulting in an accumulation of homocysteine.

The second reaction by which homocysteine is remethylated to methionine is catalyzed by the enzyme betaine-homocysteine methyltransferase. This enzyme catalyzes the conversion of betaine and homocysteine to dimethylglycine and methionine, respectively. This reaction is neither dependent on cobalamin nor on folate. However, this reaction is confined mainly to the liver and is dependent on dietary choline. It is therefore not capable of handling excess amounts of homocysteine (Finkelstein and Martin, 1984).

In the transsulfuration pathway, homocysteine condenses with serine to form cystathionine in an irreversible reaction. CBS catalyzes the first step in the transsulfuration pathway with vitamin B_6 in its active form, PLP as a cofactor. Cystathionine is subsequently cleaved by a second PLP-dependent enzyme, γ-cystathionase, to form cysteine and α-ketobutyrate (Mudd and Levy, 1983). Supplementation of niacin resulted in a significant decrease in plasma PLP levels. This niacin-associated decline in plasma PLP level was in parallel with an increase in plasma homocysteine in relation to cysteine levels in the niacin supplemented animals. This means that the abnormality in the biochemical status of vitamin B_6 in the presence of large doses of niacin may also be a factor that contributed to an increase in total homocysteine concentration. Overall, the results of the present study clearly suggest that both remethylation and transsulfuration of homocysteine are impaired due to the significant decrease in vitamin B_{12} and vitamin B_6, respectively (Figure 6.1). Thus, the abnormality in the biochemical status of these two vitamins is most likely the cause of the niacin-induced hyperhomocysteinemia.

Hepatic function abnormalities have also been reported with the use of pharmacologic dose levels of niacin (DiPalma and Thayer, 1991; Crouse, 1996). A significant elevation in plasma bilirubin and alanine aminotransferase was observed in the niacin treated animals. Both these parameters are indicative of hepatic dysfunction. The hyperbilirubinaemic effect of niacin is due to the competitive interference between niacin and unconjugated bilirubin at the level of hepatocyte uptake (Gentile et al., 1986). Whereas, the increase in alanine aminotransferase is due to the leakage of liver enzymes into circulation from damaged hepatic cells (Davidson, 1970). The biochemical results of the present study suggest the occurrence of hepatic dysfunction in the presence of pharmacologic dose levels of niacin.

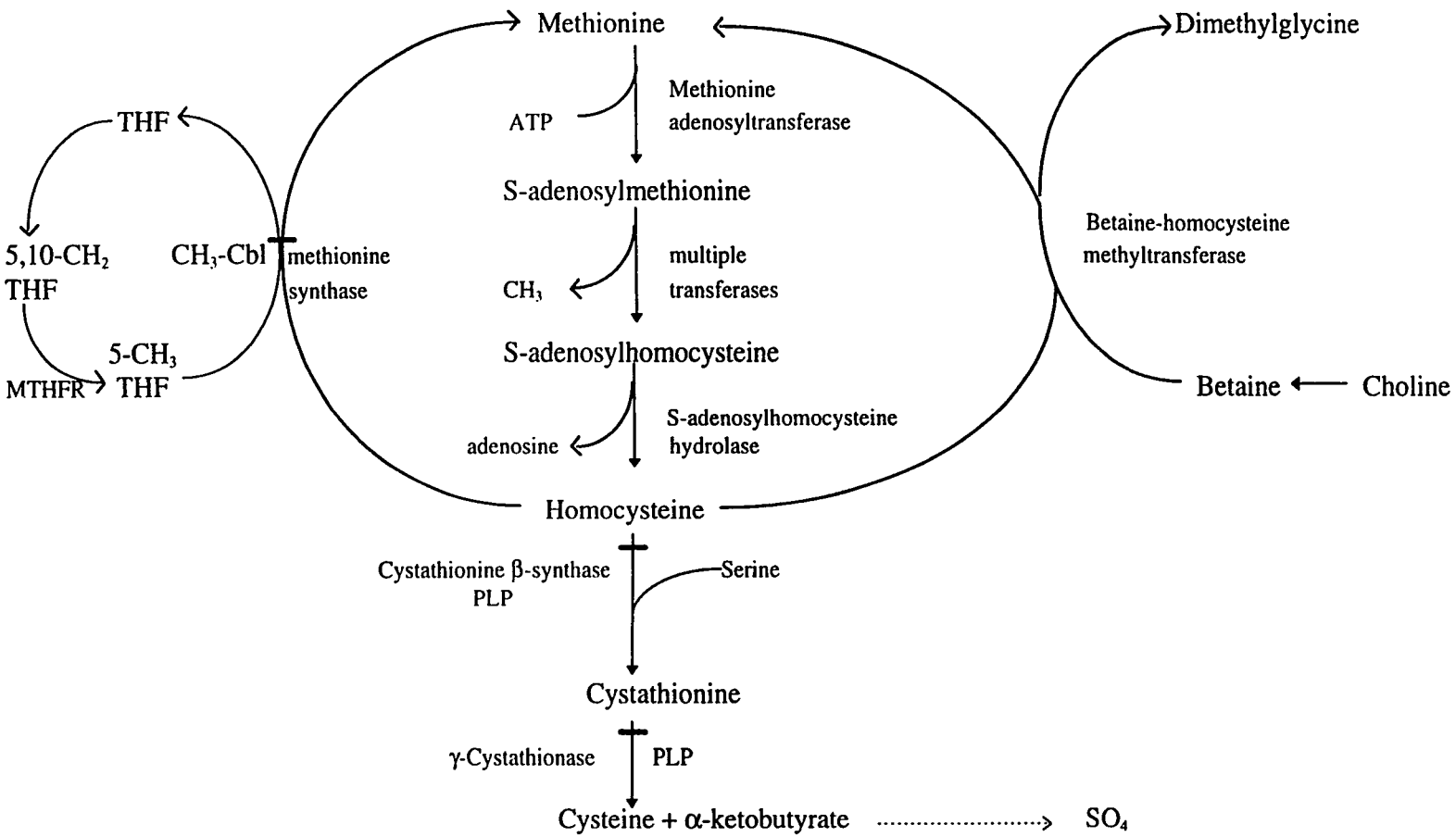

Figure 6.1 Interruption in homocysteine metabolism following treatment with large doses of niacin

metabolism. Feeding diets deficient in methyl donor compounds such as choline and methionine have been shown to produce fatty liver in rats (Griffith and Dyer, 1968). The *de novo* synthesis of choline begins with the amino acid serine which is decarboxylated in a vitamin B_6-dependent reaction to ethanolamine. This latter compound is then progressively methylated to choline. This methylation process, in which methionine acts as a methyl donor is therefore indirectly dependent on vitamin B_{12} and folate for the synthesis of methionine, and also controls the amount of choline available in the body (Basu and Dickerson, 1996). Feeding of diets supplemented with niacin resulted in a significant increase in hepatic triglyceride levels. It is plausible to suggest that this hepatic hypertriglyceridemia was a result of a decrease in vitamin B_{12}.

It can be concluded from the results of the present study that pharmacologic dose levels of niacin causes hyperhomocysteinemia due to a reduction in vitamin B_{12} and vitamin B_6. The decrease in vitamin B_{12}, a lipotropic factor, further caused hepatic hypertriglyceridemia. Due to the occurrence of these untoward effects, it is important that hyperlipidemic subjects are precautioned about taking niacin in large doses.

Clinical trials are now warranted to determine whether the experimental results can be extrapolated to humans. It is also important that future studies examine if concurrent supplementation of niacin with vitamins B_{12} and B_6 counteracts the niacin-associated adverse effects without affecting the hypolipidemic action of niacin.

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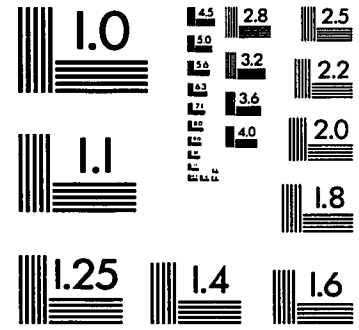

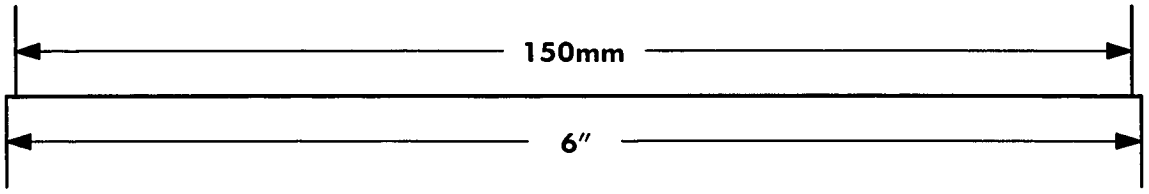

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