University of Alberta

Homocysteine Status in BB Rats

by

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ABSTRACT

Hyperhomocysteinemia is an independent risk factor for coronary heart disease, which is one of the prevalent secondary complications in diabetes mellitus (DM). The present study was undertaken to test the hypothesis that elevated homocysteine levels are a metabolic disorder destined to type 1 diabetes. Using diabetes-prone BB rats (BBdp) and their age-matched control counter parts (diabetes-resistant BB rats, BBn), the plasma concentrations of homocysteine and cysteine, their metabolic cofactors (folate, vitamin B_{12} and vitamin B_6), and cholesterol and triglycerides, were determined at 30 and 50 days of age (pre-diabetic age) and at the onset of diabetes. This study was further extended to examine the modifying effects of the vitamin supplementation on homocysteine and cholesterol status. The plasma homocysteine and cysteine levels were significantly elevated in BBdp compared to BBn rats at both 30 and 50 d ($p<0.0001$); the magnitude of elevation was age-related ($p<0.0016$). These differences, however, disappeared at the onset of diabetes. The plasma total cholesterol levels were not significantly different between strains during the pre-diabetic stage, but were elevated in diabetic rats (BBd) compared to BBn rats at the onset of diabetes. Feeding a diet supplemented with folate, vitamin B_6, and vitamin B_{12} resulted in a significant decrease in plasma homocysteine levels in BBn rats at the onset of diabetes ($p=0.04$), and in plasma cysteine levels in BBdp rats at 50 days of age ($p<0.0001$) and in BBn rats at the onset of diabetes ($p<0.0001$). Interestingly, the vitamin supplementation resulted in a significantly increased total plasma cholesterol level in both BBd and BBn rats. These results suggest that in BBdp rats, there exists a metabolic derangement of homocysteine before the onset

of diabetes, and that such defect appears to be ameliorated with the disappearance of insulin secretions.

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
ANOVA	Analysis of variance
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
BB	Bio-Breeding
BBd	Diabetic Bio-Breeding
BBdp	Diabetes-prone Bio-Breeding
BBn	Normal Bio-Breeding
BHMT	Betaine:homocysteine methyltransferase
BMI	Body mass index
CBS	Cystathionine beta-synthase
COMT	Catechol-O-methyltransferase
CVD	Cardiovascular disease
Cys	Cysteine
d	Days
DRI	Dietary Reference Intake
EDTA	Ethylenediaminetetraacetic acid
ESRD	End-stage renal disease
GNMT	Glycine-M-methyltransferase
Hb_{AIC}	Glycosylated hemoglobin

Hcys	Homocysteine
HDL	High-density lipoprotein
HPLC	High-performance liquid chromatography
IDDM	Insulin-dependent diabetes mellitus
LDL	Low-density lipoprotein
MS	Methionine synthase
MTHFR	Methylenetetrahydrofolate reductase
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (reduced form)
NIDDM	Non-insulin-dependent diabetes mellitus
NIH	National Institute of Health
NO	Nitric oxide
NOD	Non-obese diabetic
NTD	Neural tube defects
OGTT	Oral glucose tolerance test
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SEM	Standard error of the mean
Suppl.	Supplemented
TC	Total cholesterol
TCEP	Tris(2-carboxyl-ethyl)phosphine
TG	Triglycerides
THF	Tetrahydrofolate

tRNA	Transfer ribonucleic acid
VLDL	Very low-density lipoprotein

CHAPTER 1

INTRODUCTION

1.1. *Diabetes*

1.1.1. *Diabetes Epidemiology*

Diabetes mellitus is a disease of defective insulin action, secretion, or both. In 2000, approximately 150 million people worldwide were affected (Donovan, 2002). Of these, 90% have type 2, while approximately 10% have type 1 diabetes. Although less prevalent, type 1 diabetes is the more severe form, as it results in a life-long dependence on insulin injections for survival. It is most prevalent in the North American and European regions, and least prevalent in the African and Western Pacific regions (Donovan, 2002). It appears that incidence rates are currently rising by approximately 2.5% per year, with the highest incidence rates presently in Finland (35 cases per 100,000 people per year). Type 1 diabetes (insulin dependent diabetes mellitus, IDDM) is characterized by autoimmune destruction of the ß-cells of the Islets of Langerhans in the pancreas, which are responsible for insulin secretion, leading to insulin deficiency (often an absolute deficiency) and consequent hyperglycemia. Type 2 diabetes (non-insulin-dependent diabetes mellitus, NIDDM) is characterized by resistance to insulin action, resulting in hyperglycemia and often elevated insulin concentrations. The terms IDDM and NIDDM have been eliminated due to the tendency of classifying patients based on treatment rather than etiology of the disease (Committee Report, 2002). Another major difference between the two types is the propensity of patients with type 1 diabetes to be

prone to ketoacidosis, while type 2 diabetes patients do not appear to follow the same trend.

Several genetic factors have been identified that are associated with incidence of type 1 diabetes. The HLA gene is the most prominent and strongly linked gene identified thus far (Lernmark, 1999), however studies in identical twins have shown concordance rates below 50%, indicating that environmental factors play a significant role in the development of diabetes (Knip & Akerblom, 1999).

1.1.2. *Animal Models of Type 1 Diabetes*

Animal models have played an important role in helping to develop the current theories of diabetes development, progression, and treatment. Several models of type 1 diabetes exist, mainly in the rodent species. Selective destruction of rodent ß-cells by chemicals is commonly done using injections of either streptozotocin or alloxan, but these models are controversial because, although convenient, the procedure often results in damage to other organs such as the liver. Other rodent models develop diabetes spontaneously through autoimmune destruction of the pancreatic ß-cells and more closely resemble the human disease in terms of development and consequences of diabetes. The non-obese diabetic (NOD) mouse and the Bio-Breeding (BB) rat are models of this type. The BB rat exhibits diabetes equally in both sexes, while in the NOD mouse, the females have a significantly higher incidence (Field & Butler, 1999). Furthermore, due to size, the use of BB rats allows for larger sample sizes, particularly of blood and plasma. BB rats develop diabetes between the ages of 60 and 100 days with incidence varying

between 30-90% (Field & Butler, 1999). Although the BB rats have significant genetic homogeneity, the varying incidence suggests that, as in humans, BB rats are susceptible to environmental factors.

1.1.3. *Diagnosis of Diabetes*

Diabetes is diagnosed by a casual plasma glucose concentration greater than, or equal to, 200 mg/dL (11.1 mmol/L) along with other symptoms of diabetes or a fasting plasma glucose concentration greater than, or equal to, 126 mg/dL (7.0 mmol/L) or a 2-hour plasma glucose greater than, or equal to, 200 mg/dL (11.1 mmol/L) during an OGTT (oral glucose tolerance test) using a glucose load of 75g (Committee Report, 2002). Symptoms of diabetes include polyuria, polydypsia, and unexplained weight loss, often accompanied by hyperphagia.

1.1.4. *Secondary Complications of Diabetes*

The major cause of mortality in both type 1 (44% of deaths) and type 2 (52% of deaths) diabetes is cardiovascular disease (CVD) (Morrish et al, 2001). Other complications such as nephropathy, retinopathy, and peripheral neuropathy can also lead to significant morbidity. People with diabetes have a 2.5 fold greater risk of developing CVD than do non-diabetics (Tavani et al, 2002). CVD appears to affect patients with diabetes at a younger age. Thus, 25% of deaths in type 1 diabetes patients aged 30-55 years are caused by CVD compared to only 6% in the same age group in the non-diabetic

population (Idzior-Walus et al, 2001). According to the American Heart Association (2002), 75% of people with diabetes (types 1 and 2) will eventually die from some sort of CVD. Complications in type 2 diabetes are more often studied, most likely because of the larger population, but the rates of CVD are approximately the same in type 1 and type 2 diabetes.

1.1.5. *Causes of Progression to Secondary Complications*

The causes of progression to secondary complications in patients with diabetes are not completely understood. It is generally accepted that poor glycemic control (as evaluated by glycosylated hemoglobin concentration, Hb_{A1c}) is associated with the development of complications, but it appears that this association is more related to microvascular complications such as retinopathy and nephropathy, while the development of macrovascular complications (i.e. CVD) is more multi-factorial and not completely understood. Lipoprotein disorders such as diabetic dyslipidemia (characterized by elevated triglycerides, reduced HDL, and smaller, more dense LDL) are common in patients with diabetes, especially type 2 diabetes (Goldberg, 2000; Verges 1999). Hyperlipidemia and hypercholesterolemia are known to be highly prevalent among type 1 diabetic patients (Idzior-Walus, 2001). Elevated cholesterol concentrations are associated with both glycemic control and age in patients with type 1 diabetes (Idzior-Walus, 2001). An inverse association between lipid and insulin concentrations has been reported in poorly-controlled type 1 diabetes patients, although the lipid abnormalities can generally be corrected with the administration of insulin (Goldberg, 2000). One

potential mechanism by which lipids are elevated in diabetes is an alteration in the production of lipoprotein lipase, an enzyme involved in the degradation of triglycerides contained in circulating chylomicrons and VLDL molecules (Goldberg, 2000). The administration of insulin stimulates lipoprotein lipase synthesis, which helps to explain why lipid abnormalities are often corrected with insulin. Increases in total cholesterol concentrations are related to increased incidence of CVD in diabetic populations (Steiner, 2000).

A newly emerging hypothesis of development of macrovascular complications in diabetes involves homocysteine, a non-proteogenic sulfur-containing amino acid. Homocysteine is often elevated in patients with CVD and is considered an independent risk factor for CVD (Bostom et al, 1999; Stampfer, 1992).

1.2. ***Homocysteine***

1.2.1. *Homocysteine Epidemiology*

The association between homocysteine and atherosclerosis was first identified in subjects with homocystinuria, an inborn error of homocysteine metabolism (Brattstrom & Wilcken, 2000; Finkelstein, 2000). It has since been found that 20-30% of patients with CVD have at least mildly elevated plasma homocysteine concentrations (>15μmol/L) (Brattstrom & Wilcken, 2000), and that hyperhomocysteinemia is associated with increased risk of myocardial infarction, independent of other conventional risk factors such as cholesterol or blood pressure (Bostom et al. 1999; Stampfer et al. 1992).

Plasma homocysteine concentrations increase with age and are higher in men than in women (Jacques et al, 2001; McQuillan, 1999), although this difference is attenuated in post-menopausal age (Krishnaswamy & Lakshmi, 2002). Genetics are another factor that play a role in determining homocysteine status. Mutations exist in the enzymes involved in both the remethylation and transsulfuration pathways of homocysteine metabolism (Figure 1-1). Several mutations exist to the methylene tetrahydrofolate reductase (MTHFR) gene, the product of which is responsible for the synthesis of 5-methyltetrahydrofolate, the methyl donor for the remethylation of homocysteine to methionine. The most common MTHFR defect is a C to T mutation at nucleotide 677 (referred to as MTHFR C677→T), which results in a substitution of valine for alanine, rendering the enzyme less functionally active (Rozen, 2000). Individuals with a defect in this gene have mild to moderate hyperhomocysteinemia, as they are unable to complete the remethylation cycle, resulting in elevated homocysteine and methionine. This defect is more prominent in Caucasian and Asian populations, but has very low incidence rates in African-American populations (Bailey & Gregory, 1999). Deficiency of the cystathionine beta-synthase (CBS) enzyme, involved in the transsulfuration reaction where homocysteine in converted to cysteine, results in homocystinuria (Brattstrom & Wilcken, 2000; Finkelstein, 2000), along with elevated plasma concentrations of homocysteine and methionine.

1.2.2. *Homocysteine Metabolism*

Homocysteine is present in circulation either as free or protein-bound homocysteine. Free homocysteine can either be oxidized with itself (homocysteine-homocysteine disulfide, or homocystine), or with cysteine (cysteine-homocysteine mixed disulfide) or it can be reduced. The oxidized forms account for 30% of the body's homocysteine pool, while the reduced forms account for approximately 1%. The remaining 70% is found bound to albumin (Stamm & Reynolds, 1999). Plasma homocysteine is normally measured and reported as total homocysteine, which accounts for all forms of free and protein-bound homocysteine (Chambers et al, 2001). Inside the cells, homocysteine is present as the reduced form ("homocysteine") but when it is released into circulation it is quickly oxidized (Jacobsen, 2000).

Homocysteine has three metabolic fates in the body: 1) to be remethylated to methionine, 2) to be involved in the transsulfuration pathway of cysteine synthesis, or 3) to be released into extracellular fluid (Medina et al, 2001). In the remethylation pathway, the substrate 5-methyltetrahydrofolate (produced by MTHFR), allows for the methylation of homocysteine to form methionine, catalyzed by methionine synthase (MS, a vitamin B_{12}-dependent enzyme) (Figure 1-1). Methionine then undergoes an irreversible transferase reaction with the adenosyl moiety of ATP to form S-adenosylmethionine (SAM), a methyl donor in many of the body's reactions. As SAM is involved in methyl transfer reactions, S-adenosylhomocysteine (SAH) is produced, which is then reversibly hydrolyzed to homocysteine by SAH hydrolase. The latter reaction is thermodynamically favoured, meaning that the products must be metabolized for the production of homocysteine. Hyperhomocysteinemia results in accumulation of SAH and subsequent end-product inhibition of SAM-dependent methylations. Homocysteine can also be

remethylated by a vitamin B_{12}- and folate-independent mechanism involving the enzyme betaine:homocysteine methyltransferase (BHMT), but this pathway has very limited tissue distribution (mainly found in the liver). In the transsulfuration pathway, homocysteine is condensed with serine to form cystathionine; this irreversible reaction occurs in the

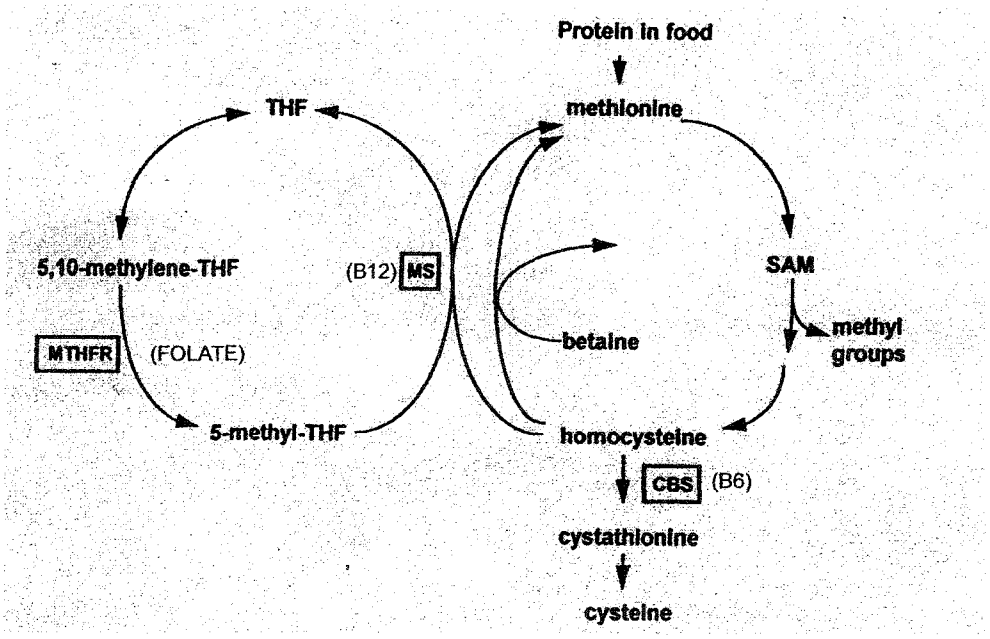


Figure 1-1. Metabolism of homocysteine.
(Adapted from Wilcken, 2002).

presence of vitamin B_6-dependent CBS (Medina, 2001). Cystathionine is then cleaved by the vitamin B_6-dependent enzyme cystathionine gamma-lyase to form cysteine and 2-ketobutyrate (Jacobsen, 2000). Homocysteine is structurally similar to both cysteine and methionine (Figure 1-2).

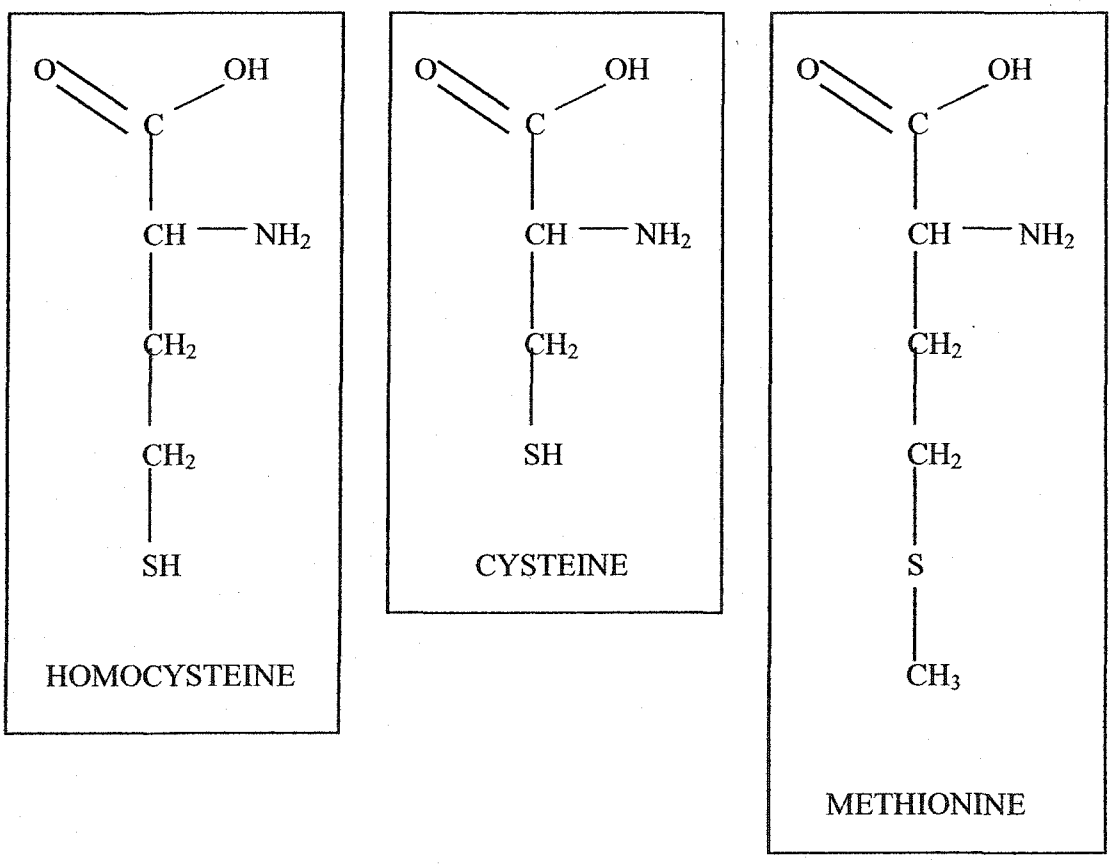


Figure 1-2. Structures of homocysteine, cysteine, and methionine.

In order for the metabolism of homocysteine to be successful, adequate vitamin status is essential. Vitamins B_{12} and B_6 are involved as cofactors for MS and CBS respectively, and folate is a substrate in the remethylation of homocysteine to methionine. Elevated plasma homocysteine concentrations have been reported with deficiency of vitamin B_6 (Martinez et al, 2000), folate (O'Leary et al, 2001), and vitamin B_{12} (Stangl et al, 2000). Plasma homocysteine concentrations are inversely related to concentrations of these vitamins (Stampfer et al, 1992; Jacques et al, 2001), of which folate appears to have the strongest association (Homocysteine Lowering Trialists' Collaboration, 1998; Selhub, 1999). It is therefore important to consider vitamin supplementation as a relevant method of reducing homocysteine concentrations.

1.2.3. *Biochemical Basis for Vitamin Supplementation*

1.2.3.1. *Folate/Folic Acid*

The theory of folate status having the strongest effect on homocysteine concentrations is supported biochemically, as explained by Selhub (1999). Glycine is methylated by SAM to form sarcosine, using the enzyme glycine-N-methyltransferase (GNMT). This enzyme is strongly inhibited by methyltetrahydrofolate polyglutamates, the products of MTHFR, whereas MTHFR activity is inhibited by SAM (Figure 1-3). Hence in the presence of either folate deficiency or if there is a lack of MTHFR activity, the production of SAM will be affected. Furthermore, as MTHFR activity is depressed, fewer methyl-THF polyglutamates will be formed, leading to less inhibition of the GNMT enzyme, and consequently increased use of SAM for the GNMT reaction (Selhub,

1999). The lack of SAM is also thought to inhibit CBS activity, involved in the transsulfuration pathway of methionine metabolism. Hyperhomocysteinemia results because the remethylation pathway is not fully active due to the folate deficiency or MTHFR defect, and the transsulfuration pathway cannot handle the excess homocysteine as CBS is not fully activated (Selhub, 1999).

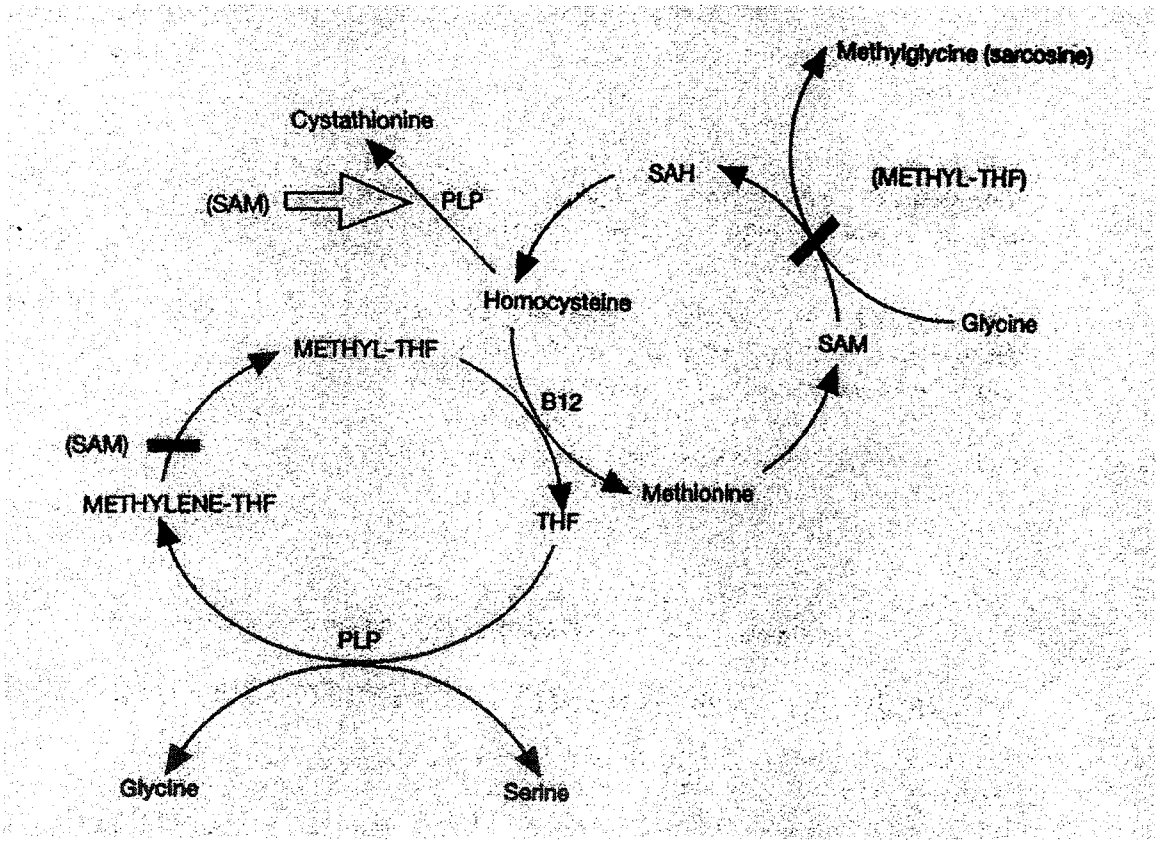


Figure 1-3. Regulation of homocysteine metabolism by SAM, involving B-vitamins. (Adapted from Selhub, 1999).

1.2.3.2. *Vitamin B_{12}*

In B_{12} deficiency, the resulting hyperhomocysteinemia is not as severe because although there is decreased production of SAM (due to decreased activity of MS, which is B_{12}-dependent), methyl-THF polyglutamates are still being formed from MTHFR and these will inhibit the utilization of SAM by GNMT, resulting in less SAM being converted to homocysteine (Figure 1-3). Therefore, not only is less SAM being converted to homocysteine, but there is SAM present to activate CBS, allowing disposal of homocysteine through the transsulfuration pathway (Selhub, 1999).

1.2.3.3. *Vitamin B_6*

In vitamin B_6 deficiency, the remethylation pathway is fully active and there is residual transsulfuration pathway activity. Therefore, homocysteine metabolism functions normally assuming the homocysteine burden is low, such as in fasting conditions or with a low intake of methionine (Selhub, 1999). However, if a methionine load is given, there will be increased SAM production, which will result in decreased activity of MTHFR, and consequently low methyl-THF production, increased activity of GNMT because of the lack of inhibition by the methyl-THF polyglutamates, and increased homocysteine generation (Figure 1-3). Furthermore, since there is depressed activity of CBS due to vitamin B_6 deficiency, hyperhomocysteinemia will result. This is the basis for the methionine-load test, and the finding that vitamin B_6 supplementation is

not effective in reducing baseline homocysteine concentrations, but is effective in reducing post-methionine load homocysteine concentrations (Selhub, 1999).

1.2.4. *Vitamin Supplementation Studies*

Supplementation studies have been done in healthy and elderly populations, and in renal and CVD populations. A meta-analysis (Homocysteine Lowering Trialists' Collaboration, 1998) found that folic acid supplementation of 0.5-5mg daily was associated with a 25% decrease in fasting plasma homocysteine concentrations, and that supplementation of a mean 0.5mg daily of vitamin B_{12} was associated with an additional decrease of 7% in plasma homocysteine concentrations. Supplementation of 16.5mg vitamin B_6 had no additional effect on homocysteine concentrations, although this could be due to the exclusion of studies looking at post-methionine load homocysteine concentrations, which are thought to be more affected by B_6 status (Selhub, 1999). However, another study found that supplementation of vitamin B_6 (120 mg daily) resulted in decreased fasting plasma homocysteine to a concentration not significantly different from that achieved by supplementation of folate or folate plus B_6 (Mansoor et al, 1999).

1.2.4.1. *Studies in Elderly Populations*

Elderly people are particularly at risk for hyperhomocysteinemia, not only because homocysteine concentrations are known to increase with age, but also because

vitamin B_{12} deficiency occurs relatively frequently in elderly populations, due to lack of intrinsic factor, gastric atrophy, or the presence of other disorders affecting the ileum (Basu & Dickerson, 1996). Supplementation studies in the elderly have focused on improving vitamin B_{12} status, in addition to provisions of folic acid and vitamin B_6. Intramuscular injections were used in studies which found that serum vitamin concentrations were increased after treatment with 1mg B_{12}, 1.1mg folate, and 5mg vitamin B_6 but returned to baseline afterwards, while homocysteine concentrations returned to normal in 92% of the treatment group during the study and remained below baseline concentrations after the study was completed (Henning et al, 2001; Naurath et al, 1995).

1.2.4.2. *Studies in CVD Patients*

CVD patients are known to have elevated homocysteine concentrations, and supplementation of vitamins has been shown to decrease homocysteine concentrations, both in CVD patients, and people at risk of developing CVD. In patients with peripheral vascular disease, administration of 5 or 10mg folic acid for 3 months resulted in a significant decrease in homocysteine concentrations, although no difference was found between the two dose concentrations (Mayer et al, 2002). In the healthy siblings of patients with CVD, supplementation of folic acid (5mg) and vitamin B_6 (250mg) daily for 2 years resulted in decreased homocysteine concentrations, and decreased risk of abnormal exercise electrocardiographs (a marker of subclinical atherosclerosis) (Vermeulen et al, 2000). Administration of the lipid-lowering drug fenofibrate has

recently been shown to increase plasma homocysteine concentrations by 40% (Dierkes et al, 1999), which would counteract the desired reduction in cardiovascular risk. However, supplementation of folic acid (650μg), vitamin B_{12} (50μg), and vitamin B_6 (5mg) in patients receiving fenofibrate resulted in a significantly smaller increase in plasma homocysteine concentrations than when no vitamins were administered (Dierkes et al, 2001).

1.2.4.3. *Studies in Renal Disease Patients*

Patients with renal disease commonly have elevated plasma homocysteine concentrations, possibly due to reduced elimination by renal routes, or vitamin deficiencies (Robinson & Dennis, 2002). Renal transplant patients receiving multivitamin preparations containing normal (0.4mg/d) or supraphysiological (2.4mg/d) dosages of folic acid, and hemodialysis patients receiving double the normal concentration of folate (0.8mg/d), along with vitamins B_{12} and B_6, found a dose-response reduction in plasma homocysteine concentrations due to folate supplementations (Beaulieu et al, 1999; Dierkes et al, 2001); lower folate concentrations (160μg/d), however, did not decrease plasma homocysteine concentrations differently from placebo (Dierkes et al, 2001). Pharmacological doses of folic acid, vitamin B_6 and vitamin B_{12} administered to chronic renal failure patients on dialysis resulted in significant decreases in plasma homocysteine concentrations, but concentrations increased to pre-supplementation concentrations 6 months after the vitamins were discontinued (Naruszewicz et al, 2001). Folate appears to be the most effective vitamin in reducing

plasma homocysteine concentrations, although higher concentrations than normal are often needed to produce results in this population (Robinson & Dennis, 2002).

1.2.4.4. *Folate Fortification of Grain Products*

The fortification of grain products with folate has been mandated in the United States since 1997 and in Canada since 1998. Since that time, enriched flour and flour-products have been fortified with 140μg folate per 100g in the United States (Jacques et al, 1999) and 0.15mg/100g flour and 0.27mg/100g pasta in Canada (Health Canada, 2002). These regulations were proposed to improve the folate status of the women of child-bearing age in order to decrease the prevalence of neural tube defects. These levels of fortification are meant to improve folate status by providing 0.1-0.2 mg per day of dietary folate without being detrimental to the health of the general population (Ray et al, 2002). In Canada, a decrease in prevalence of NTDs since the inception of folate fortification has been confirmed, from 1.13 per 1000 pregnancies before fortification to 0.58 per 1000 pregnancies after fortification (Ray et al, 2002). Another benefit of folate fortification is the population-wide improvement in folate status and concurrent decrease in plasma homocysteine concentrations. In the United States, an increase in plasma folate concentrations from 11 to 23 nmol/L and a decrease in plasma homocysteine concentrations from 10.1 to 9.4 μmol/L were seen after fortification was implemented (Jacques et al, 1999). However, in coronary heart disease patients, a supplementation of 127μg daily was not sufficient to produce a significantly decreased homocysteine concentration, while daily supplementation of 499-665μg folic acid resulted in decreases

in plasma homocysteine concentrations of 11.0% and 14.0% respectively (Malinow et al, 1998). A higher level of folic acid fortification has been suggested to be necessary to reduce plasma homocysteine concentrations.

1.2.5. *Is Homocysteine an Indicator of CVD Risk?*

An elevated plasma homocysteine concentration has been suggested to be either a marker of unhealthy lifestyles (Cleophas et al, 2000) or a result rather than a cause of CVD (Brattstrom & Wilcken, 2000; Christen et al, 2000). Through meta-analysis, Christen et al (2000) suggest that homocysteine may be an acute-phase reactant and hence a marker of atherosclerosis among those in the preliminary stages of CVD. Another meta-analysis by Brattstrom & Wilcken (2000) has suggested that mild hyperhomocysteinemia is associated with the decline in renal function commonly seen in CVD patients, and would therefore be an effect rather than a cause of the atherosclerotic disease. It is also possible that elevated homocysteine is a potential marker of an increased risk from other compounds, such as SAH (Kerins et al, 2001) or cysteine (El-Khairy et al, 1999). Kerins et al (2001) found statistically significant differences in plasma SAH concentrations but not in plasma homocysteine concentrations between CVD patients and control groups, and that statistical significance could be reached with smaller study populations. S-adenosylhomocysteine has been suggested to be a more sensitive indicator of risk of CVD than homocysteine because of its strong correlations with plasma homocysteine concentrations, smaller concentrations (present in nmol/L concentrations in plasma; homocysteine present in μmol/L) and smaller body pool size

(approximately 500 times smaller than the body homocysteine pool). Plasma SAH concentrations presumably increase proportionately with plasma homocysteine concentrations through reversal of the enzyme SAH hydrolase. Furthermore, there is often overlap of homocysteine concentrations between patients and controls, which necessitates the use of large populations to achieve significance (Kerins et al, 2001). Cysteine has also been suggested to be a more potent risk factor for CVD than homocysteine because of its similar reactive aminothiol group and because of its presence in plasma at a 20-fold greater concentration than homocysteine (El-Khairy et al, 1999). Several factors have been associated with elevated plasma cysteine concentrations, such as cholesterol concentrations, blood pressure, and BMI, which are also predisposing factors in the development of CVD, suggesting that cysteine may be related to CVD risk, although few studies have examined this relation (El-Khairy et al, 1999).

1.2.6. *Mechanisms of Homocysteine Pathology*

The mechanisms by which homocysteine exerts its effects on the cardiovascular system are not clear. Many possibilities have been suggested, but the most promising theories focus on oxidative stress induced by elevated circulating plasma homocysteine concentrations (Exner et al, 2002; Hanratty et al. 2001; Hirano et al, 1994; Mujumdar et al, 2001; Symons et al, 2002; Weiss et al, 2001; Zappacosta et al, 2001), and on damage to proteins caused by homocysteinylation of lysine residues (Jakubowski, 2000a; Jakubowski, 2000b). Other theories include effects on nitric oxide (NO) production (Chow et al, 1999; Ikeda et al, 1999; Mujumdar et al, 2001; Weiss et al, 2001; Zhang et al,

2000), effects on catechol concentrations (Zhu, 2002), and promotion of vascular smooth muscle cell growth by homocysteine (Tsai et al, 1994).

1.2.6.1. *Oxidation of LDL*

Studies of lipid peroxidation and oxidation of LDL particles in the presence of homocysteine have been reported (Exner et al, 2002; Hirano et al, 1994). The presence of metal cations such as copper or iron, as well as homocysteine, appears to be necessary for this oxidation to occur. Under this theory, the Cu^{2+} of ceruloplasmin is reduced by homocysteine, thereby increasing the oxidizing potential of this protein. Ceruloplasmin has 7 copper ions, of which one is responsible for the oxidizing potential of the protein. Chelation eliminates the oxidizing ability of the protein, but the addition of homocysteine stimulates some LDL oxidation, although not to the same level as in the non-chelated state (Exner et al, 2002). These results indicate that although the oxidation of LDL by the ceruloplasmin protein is dependent on the presence of a reduced copper ion, homocysteine has the ability to reduce the remaining copper ions that are not normally involved in LDL oxidase activity of the protein. Homocysteine is involved in the modification of LDL through oxidation by the prevention of this mechanism through the addition of antioxidants such as alpha-tocopherol (Hirano et al, 1994). Oxidation of homocysteine upon entering circulation is thought to be responsible for reduction in endothelial function through the production of the free radical hydrogen peroxide (Jacobsen, 2000). However, co-administration of the antioxidant ascorbic acid with a

methionine load was not capable of altering the decrease in endothelial function seen in a normal methionine-loading test (Hanratty et al, 2001), suggesting that it is not an oxidative mechanism. Furthermore, it was found that the oxidation of homocysteine produces only a negligible amount of hydrogen peroxide, and then only in the presence of metal ions (Zappacosta et al, 2001).

1.2.6.2. *Protein Homocysteinylation*

One of the largest emerging theories of homocysteine pathology is that of protein homocysteinylation. Although not normally incorporated into proteins, the structural similarity of homocysteine with methionine, isoleucine, and leucine allows homocysteine to enter the first steps of protein synthesis. The error-editing mechanisms of Met-, Ile-, and Leu-tRNA synthetases, which activate homocysteine in these first steps, are capable of converting misactivated homocysteine into another molecule called homocysteine thiolactone (Jakubowski, 2000b).This molecule is formed when the homocysteine molecule is not found to have efficient side chain interaction with the specificity subsite of the synthetase enzyme (Jakubowski, 2000b). The thiolactone molecule post-translationally modifies the lysine residues of proteins through protein homocysteinylation, where the thiolactone molecule is added on to the amino group of lysine residues. This mechanism occurs proportionally to the lysine content of the protein and results in loss of protein function (Jakubowski, 2000a). Homocysteine thiolactone can also be incorporated translationally into proteins if the side-chain of homocysteine is reversibly modified with a small molecule to increase the efficiency of

the interaction with the specificity subsite of the synthetases (Jakubowski, 2000b). However, because these studies were done *in vitro*, and because concentrations of homocysteine thiolactone are regulated by the enzyme homocysteine thiolactonase (Jakubowski, 2000a) and by other enzymes with esterase-like activities (Jacobsen, 2000), it is unclear whether concentrations of homocysteine thiolactone can actually accumulate *in vivo*.

1.2.6.3. *Nitric Oxide*

Nitric oxide is a vasodilator and is responsible for the endothelium-dependent vasodilation in vivo (Duell & Malinow, 2000). Nitric oxide is capable of reacting with homocysteine to form S-nitrosohomocysteine, which has a lower toxicity than homocysteine, does not produce hydrogen peroxide, and cannot convert to homocysteine thiolactone, but is still a bioactive form of NO as it can act as a nitric oxide donor (Eberhardt & Loscalzo, 2000). However, homocysteine is also capable of oxidatively inactivating NO (Mujumdar et al, 2001), although this can be attenuated by increased expression of glutathione peroxidase (Weiss et al, 2001). Homocysteine has been found both to decrease (Chow et al, 1999; Zhang et al, 2000) and to increase (Ikeda et al, 1999) NO production. It has been suggested that brief exposure of endothelial cells to homocysteine may increase NO production, while over longer periods of time, the dysfunctional endothelium is unable to sustain NO production (Eberhardt & Loscalzo, 2000), but the reports mentioned above do not support this theory.

1.2.6.4. *Other Mechanisms*

Other theories of homocysteine pathology include effects on smooth muscle cell proliferation, and effects on catecholamine metabolism. The former theory is based on the finding of increased smooth muscle cells in young homocystinuric patients (Finkelstein, 2000). This theory has been further explored by *in vitro* studies, which found that homocysteine promoted proliferation of rat aortic smooth muscle cells and inhibited endothelial cell growth, although very high concentrations of homocysteine were used (0.1-1mM) (Tsai et al, 1994). The latter theory suggests that hyperhomocysteinemia results in accumulation of SAH, which is an inhibitor of catechol-O-methyltransferase (COMT) mediated methylation in the metabolism of catechol substrates (Zhu, 2002). Inhibition of this methylation will result in elevated concentrations of catecholamines which could over-stimulate the cardiovascular system, leading to endothelial cell damage. Further research is required to confirm this theory.

*1.3. **Homocysteine in Diabetes***

1.3.1. Plasma Homocysteine Concentrations in Type 1 and Type 2 Diabetes

As mentioned previously, CVD is a major complication for people with diabetes. Type 1 and type 2 diabetes have approximately equivalent rates of CVD (Morrish et al, 2001), although type 2 diabetes is more often studied. Studies looking at plasma homocysteine concentrations in diabetes have been minimal, despite the impact of the presence of diabetes on CVD development. Reports of plasma homocysteine

concentrations in diabetes have so far been inconsistent, finding increased (Chiarelli et al, 2000; Drzewoski et al, 2000; Hofman et al, 1997), decreased (Cronin et al, 1998), and unchanged (Cronin et al, 1998; Lanfredini et al, 1998; Pavia et al, 2000) concentrations in diabetic patients compared to controls. Possible reasons for these inconsistencies include differences between the studies in terms of type of diabetes, time of onset of the disease, glycemic control, and more importantly plasma insulin concentrations and the presence of complications.

1.3.2. *Homocysteine and Glycemic Control in Diabetes*

The association between glycemic control and plasma homocysteine concentrations is unclear. Studies have reported inverse (Drzewoski et al, 2000; Kark et al, 1999) and no association (Pavia et al, 2000) between plasma glucose concentrations and plasma homocysteine concentrations. It appears that plasma homocysteine concentrations are higher in adults with poorly-controlled diabetes (i.e. high Hb_{A1c} levels), although this has not been found to be the case in adolescent type 1 diabetes patients (Pavia et al, 2000). It is possible that the association between glycosylated hemoglobin (Hb_{A1c}, a marker of glucose control) and homocysteine is related to the association between homocysteine and insulin.

1.3.3. *Homocysteine and Insulin Concentrations in Diabetes*

The association between plasma insulin and homocysteine concentrations in diabetes is also unclear. An inverse association has been reported in patients with diabetes (Drzewoski et al, 2000) and in healthy subjects (Rosolova et al, 2002). In non-diabetic, insulin-resistant subjects, positive (De Pergola et al, 2001) and non-significant (Godsland et al, 2001) associations between plasma insulin and homocysteine concentrations have been reported.

Studies supporting a positive association at the cellular level have been published. Dicker-Brown et al (2001) found that the activity of MTHFR and CBS enzymes in mouse hepatocytes was significantly decreased in the presence of elevated insulin in culture. This decrease in the activity of the enzymes involved in the metabolism of homocysteine in the presence of elevated insulin would lead to increased plasma homocysteine concentrations, and hence a positive association between plasma insulin and homocysteine concentrations. Furthermore, Jacobs et al (1998) found that the activities of the hepatic transsulfuration enzymes (e.g. CBS) were increased in rats with streptozotocin-induced but untreated diabetes, and that the enzyme activities returned to normal when insulin was administered. Similarly, plasma homocysteine concentrations were decreased in induced but untreated diabetic rats compared to treated and healthy controls, and these levels returned to normal when insulin was administered (Jacobs et al, 1998). Based on these findings, it appears that insulin plays a role in the regulation of plasma homocysteine concentrations through effects on the enzymes involved in its metabolism, although additional research is required to further elucidate the mechanisms behind this role.

1.3.4. *Homocysteine and Renal Function in Diabetes*

Nephropathy is a common complication of diabetes, with greater than 50% of all incidence of end-stage renal disease (ESRD) coming from diabetic patients (Rosenstock & Appel, 2002). The prevalence of nephropathy is higher in type 1 compared to type 2 diabetes, with 25-40% of type 1 diabetes patients developing nephropathy, of which most will develop ESRD (Rosenstock & Appel, 2002). The earliest signs of renal dysfunction are glomerular hyperfiltration, which is associated with decreased plasma homocysteine concentrations (Wollesen et al, 1999), and increased albumin excretion, which is associated with increased plasma homocysteine concentrations (Chiarelli et al, 2000; Lanfredini et al, 1998; Stabler et al, 1999; Vaccaro et al, 2000). Further progression of the disease leads to decreased glomerular filtration rate, which is associated with increased plasma homocysteine concentrations (Hovind et al, 2001; Wollesen et al, 1999), although this could be due to loss of kidney function and therefore increased plasma homocysteine concentrations, as the kidney is one of the major sites of homocysteine metabolism. It appears that plasma homocysteine concentrations in diabetes are largely dependent on renal function, although the evidence does not show that homocysteine is a contributing factor in the development of renal complications (Hovind et al, 2001).

1.4. Conclusions

Macrovascular complications in diabetes are common, and altered lipid metabolism and hyperglycemia do not adequately explain the elevated prevalence of CVD in this

patient population. Homocysteine has been shown to be an independent risk factor for CVD, and it is possible that homocysteine has a role in the development of cardiovascular complications in patients with diabetes. However, plasma homocysteine concentrations in diabetes are largely inconsistent, and vitamin supplementation studies to reduce plasma homocysteine concentrations have not been attempted in diabetic populations. Furthermore, plasma homocysteine concentrations and metabolism have not been fully elucidated in animal models of diabetes, particularly bio-breeding (BB) rats, in which diabetes mellitus resembling human type 1 diabetes develops spontaneously. Additionally, little is known of the metabolism of homocysteine, and plasma homocysteine concentrations before the onset of diabetes, as this is difficult to predict in humans. In order to further understand the role of homocysteine in diabetes, the present study was undertaken to investigate homocysteine status before and after onset of diabetes in BB rats.

1.5.Hypothesis and Objectives

It is hypothesized that diabetes-prone Bio-breeding (BBdp) rats are associated with elevated homocysteine levels before and after the onset of diabetes, and that the homocysteine status is improved by supplementing with folate and vitamins B_6 and B_{12}. BB rats generally develop diabetes between the ages of 60 and 100 days (Field & Butler, 1999). This hypothesis was tested with the following objectives:

1. To determine the plasma concentrations of homocysteine and its metabolic product cysteine during prediabetic stages (e.g. 30 and 50 days old) and at the onset of diabetes
2. To reveal the relationship between homocysteine and cholesterol (also a risk factor for CVD) in BB rats before and at the onset of diabetes
3. To investigate the impact that the supplementations of vitamins B_6, B_{12}, and folate have on homocysteine status before and at the onset of diabetes.

CHAPTER 2

METHODS

2.1. *Animals*

Male (out bred) Bio-Breeding (BB) diabetes-prone (BBdp) and normal (BBn) Wistar rats were obtained from the Department of Agricultural, Food and Nutritional Science breeding colony at weaning. The stock from this colony originated from the Sir Frederick Banting Research Centre (Health Products and Food Branch, Health Canada, Ottawa ON, Canada). Animals were individually housed in stainless steel hanging metabolic cages for the duration of the study, and were in a well-ventilated, temperature- and humidity-controlled windowless room on a 12-hour light/dark cycle. The study was conducted in accordance with the Canadian Council on Animal Care guidelines (Olfert et al, 1993) and the protocol was approved by the Faculty of Agriculture, Forestry and Home Economics Animal Policy and Welfare Committee (protocol number 2002-18C).

2.2. *Experimental Design*

To meet the objectives of the study, three experiments were performed. The first experiment investigated homocysteine metabolism in the prediabetic stages of BB rats. At weaning (21 days), the animals were randomly divided into two groups: group A was kept until 30 days of age while group B was kept until 50 days of age (Figure 2-1). Both groups received NIH-07 meal diet (Ziegler Brothers, Gardners, Pennsylvania). The amino acid and vitamin contents of the diet are shown in Table 2-1.

The second experiment examined the effects of the diabetic state on homocysteine metabolism in BB rats. Animals were kept from weaning (21 days) until the onset of diabetes, and received the NIH-07 meal diet (Figure 2-2).

The third experiment investigated the effects of supplementing vitamins B_6, B_{12}, and folate on homocysteine metabolism in BB rats at pre- and post-diabetes stages (Figure 2-3). These supplemented groups received NIH-07 meal diet supplemented with 10 times the amount of pyridoxine (vitamin B_6), cobalamin (vitamin B_{12}), and folic acid (all from ICN Biomedicals, Aurora, Ohio) of the control diet. The supplementary vitamins were added in our laboratory. The amino acid and vitamin contents of the diets are shown in Table 2-1.

All animals had *ad libitum* access to water and their respective diets throughout the study periods. Body weight and food intake were recorded once a week and three times per week, respectively. BBdp rats older than 50 days of age were tested three times per week for glycosuria with ChemStrip uG/K (Roche, Laval, Quebec). Glucose concentrations were determined in blood samples taken from the tail vein, using One Touch Ultra Glucometer and strips (Lifescan Inc., Milpitas, California). Diabetes was diagnosed by a urinary glucose concentration of >2+ and subsequent hyperglycemia (blood glucose >13mmol/L). These animals were then described as BBd (diabetic) rats.

Within 24 hours of onset of diabetes, the rats (BBd), along with their age-matched counterparts (BBn) were killed. All animals were fasted overnight and were anaesthetized by 5% v/v Halothane inhalation anesthetic (Halocarbon Laboratories, River

Edge, NJ, USA). Blood was collected by cardiac puncture into heparinized tubes and kept on ice until centrifugation (3000 rpm for 10 minutes at 4°C). Animals were then killed by carbon dioxide. The separated plasma samples were stored at -80°C until analyses. By 120 days, BBdp rats that had not developed diabetes were killed by carbon dioxide.

Table 2-1. Amino acid and vitamin concentrations of NIH-07 diet.*

Amino Acid	*g/Kg diet*
Arginine	13.0
Lysine	12.8
Methionine	4.8
Cystine	3.3
Tryptophan	2.6
Histidine	5.4
Leucine	19.6
Isoleucine	10.7
Phenylalanine	10.6
Tyrosine	8.0
Threonine	9.0
Valine	11.9

Vitamin	*unit/Kg diet*	*[supplemented]*
Vitamin A	8770 IU	
Vitamin D3	5000 IU	
Alpha-tocopherol	51000 IU	
Thiamine	17.50 mg	
Riboflavin	7.00 mg	
Niacin	77.00 mg	
Pantothenic acid	33.70 mg	
Choline	1957.00 mg	
Pyridoxine	10.00 mg	[100.00 mg]
Folic acid	3.00 mg	[30.00 mg]
Biotin	0.40 mg	
Vitamin B12	47.90 µg	[479.00 µg]
Vitamin K	3.00 mg	

* NIH-07 diet contains (% diet): protein minimum 22.5, fat minimum 5.0, fibre maximum 4.5, ash maximum 7.0; gross energy 4.08 kcal/g diet.

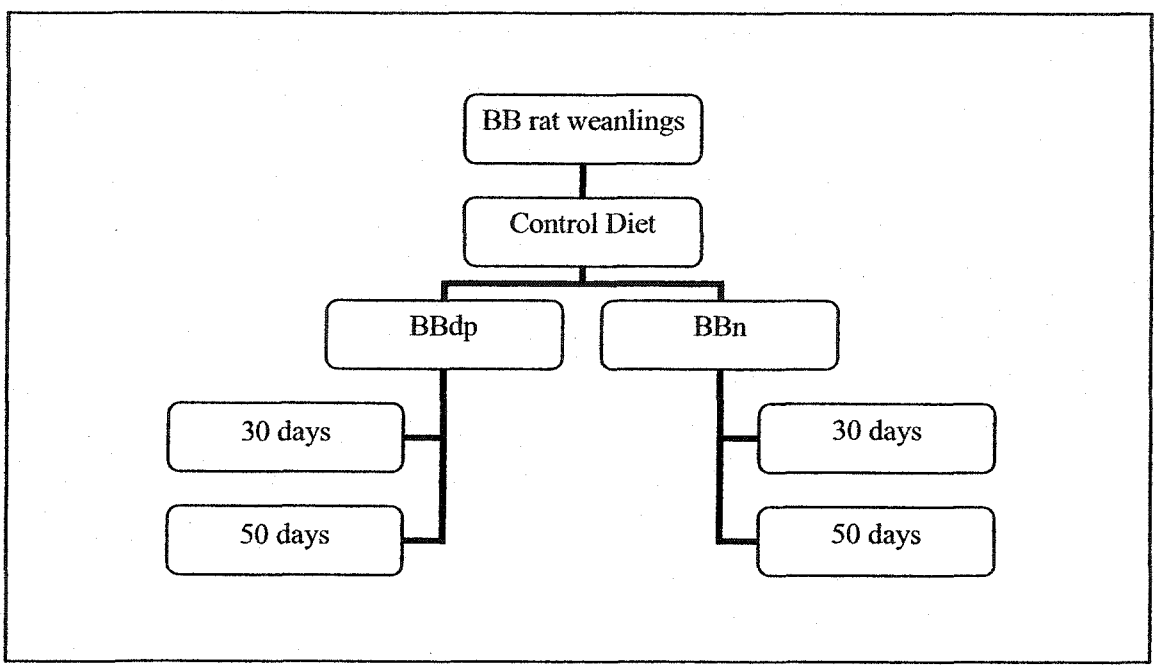


Figure 2-1. Experimental design – Experiment 1.

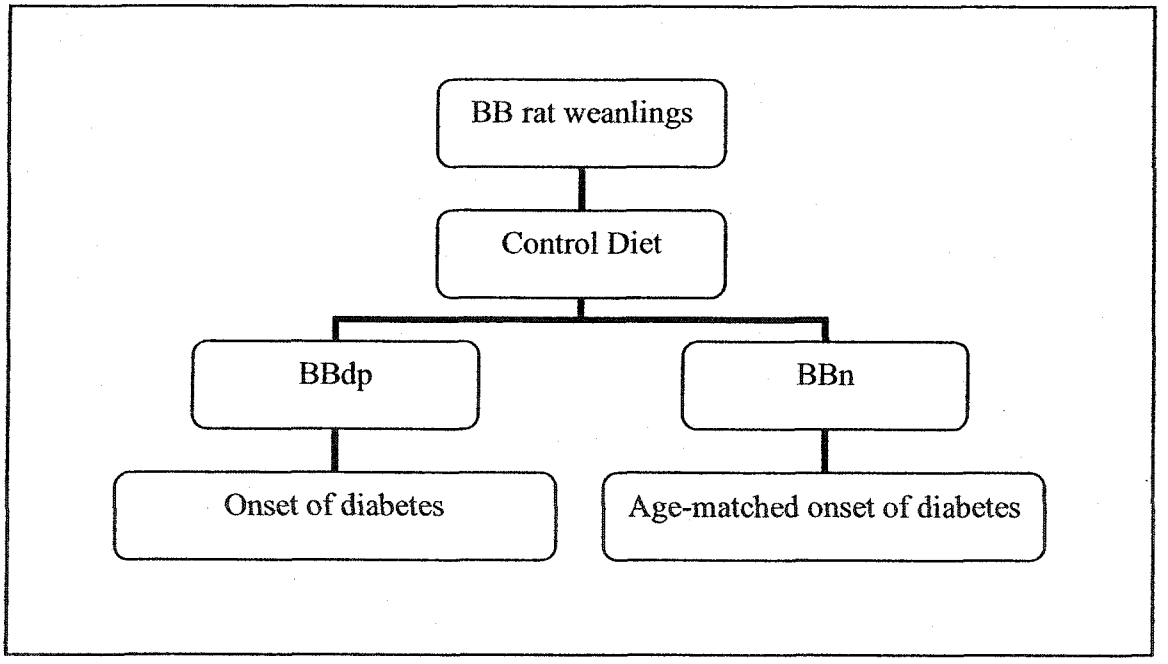


Figure 2-2. Experimental design – Experiment 2.

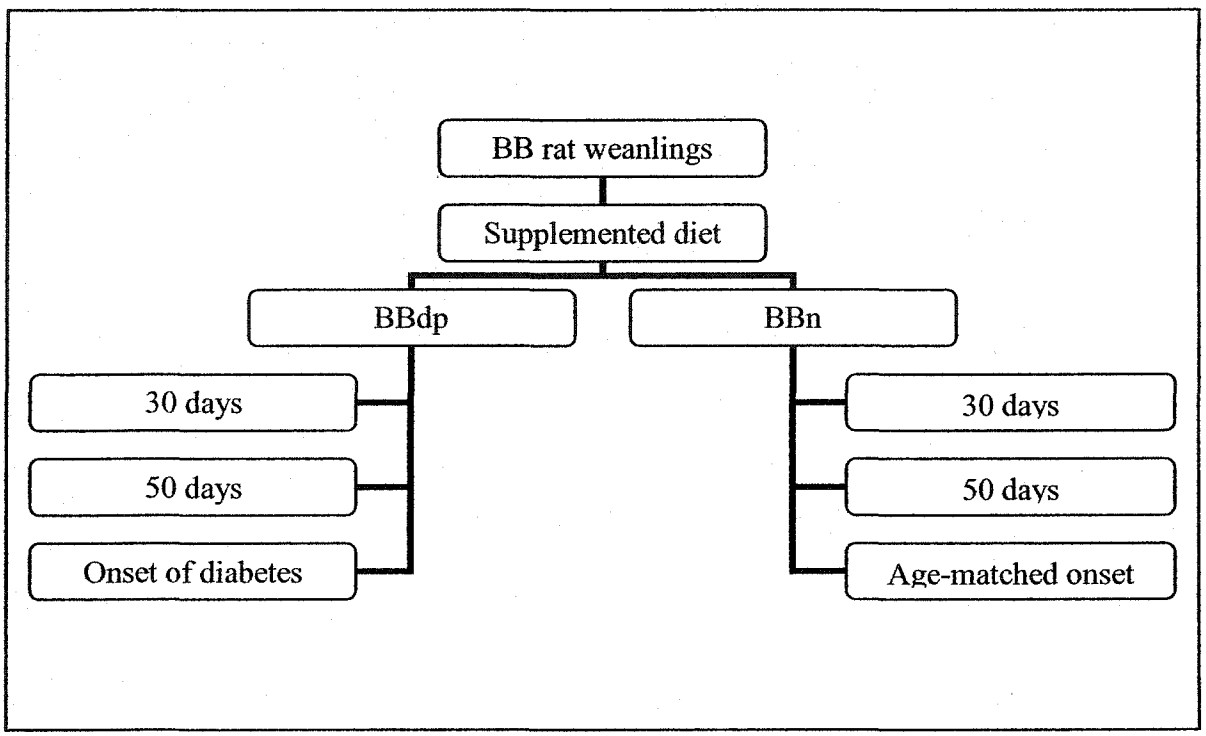


Figure 2-3. Experimental design – Experiment 3.

2.3. ***Determination of Plasma Homocysteine and Cysteine***

Plasma homocysteine and cysteine were analyzed by reverse phase, high-performance liquid chromatography using a Shimadzu HPLC system complete with a SCL 10A system controller, a DGU-14A in-line degasser, a LC-10AD VP liquid chromatograph, a SIL-10 A sample preparation and auto injection unit, and a RF-10AXL fluorescence detector (Mantech, Guelph, ON). Resolution of amino acids was accomplished using a 5 μm Hypersil ODS column, 4.6 X 150 mm column (Mandel Scientific, Guelph, ON). Plasma homocysteine and cysteine were analyzed according to the method of Araki and Sako (1987) with modifications as suggested by Gilfix et al (1997). Homocysteine and homocysteine-mixed disulfides present in the samples were reduced by the addition of tris(2-carboxyl-ethyl)phosphine (TCEP) and subsequently derivatized with ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBD-F) in order to obtain a fluorescent product. A 150 μL aliquot of plasma was mixed with 20 μL of 100g/L TCEP, mixed on a vortex mixer and incubated at room temperature for 30 minutes, in order to reduce all oxidized thiols, including those bound to proteins. After the incubation period, 125 μL of 0.6 M perchloric acid was added to the mixture for deproteinization of the plasma. This mixture was then vortexed and centrifuged at 10,000g for 10 minutes. Following centrifugation, 0.2mL of 2.5M sodium borate buffer (pH 10.5) with 4mM sodium EDTA was mixed with 0.1mL of SBD-F (1.0mg/mL) in 2.5M sodium borate buffer (pH 9.5) and 0.1mL of the supernatant. This mixture was incubated, protected from light, in a 60°C shaking water bath for 60 minutes. The mixture was then cooled on crushed ice and centrifuged at 10,000g for 5 minutes. Aliquots of 10μL were injected onto the HPLC systems for analysis. Plasma thiols were resolved using isocratic elution with 0.1M

acetate buffer (pH 5.5) with 2% methanol (v/v), with a flow rate of 1mL/minute. Fluorescence detection was used for the thiols, with excitation and emission wavelengths established at 385 and 515 nm, respectively. Peaks were identified with reference to external amino acid standards, and quantified using the Class VP integration software (Mantech, Guelph, ON). Average retention times of homocysteine and cysteine were 3.9 and 3.0 minutes, respectively. A chromatogram of external amino acid standard peaks is shown in Figure 2-2.

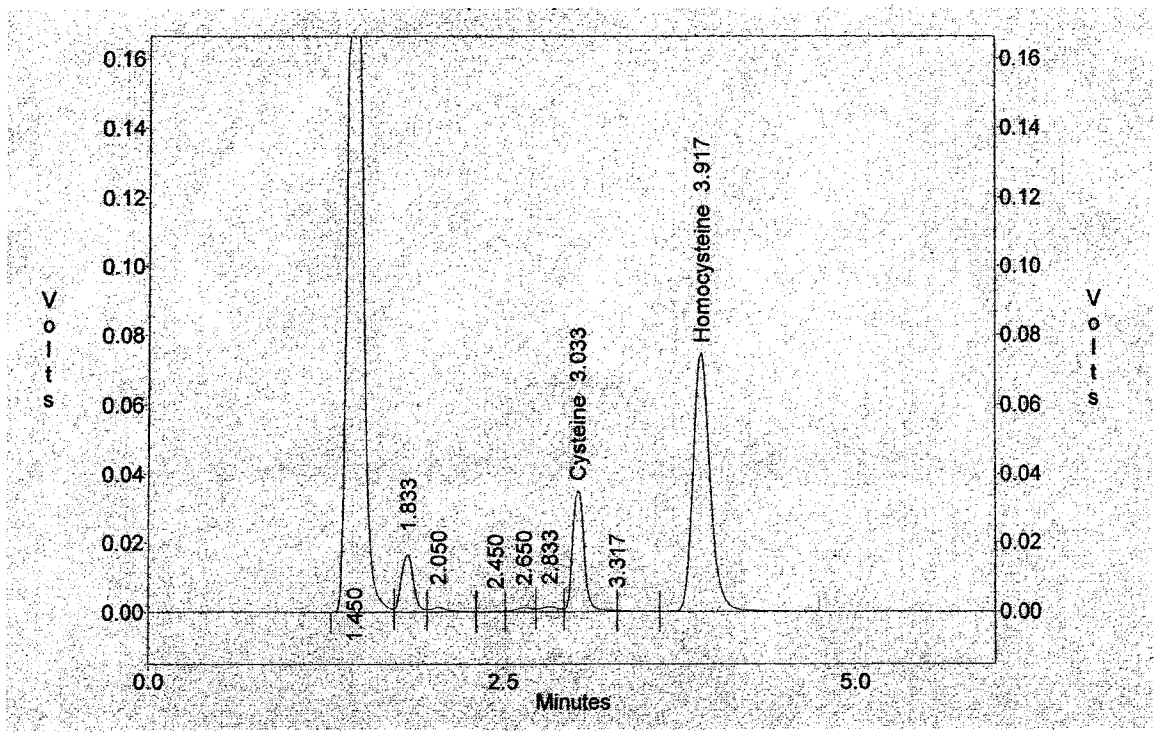
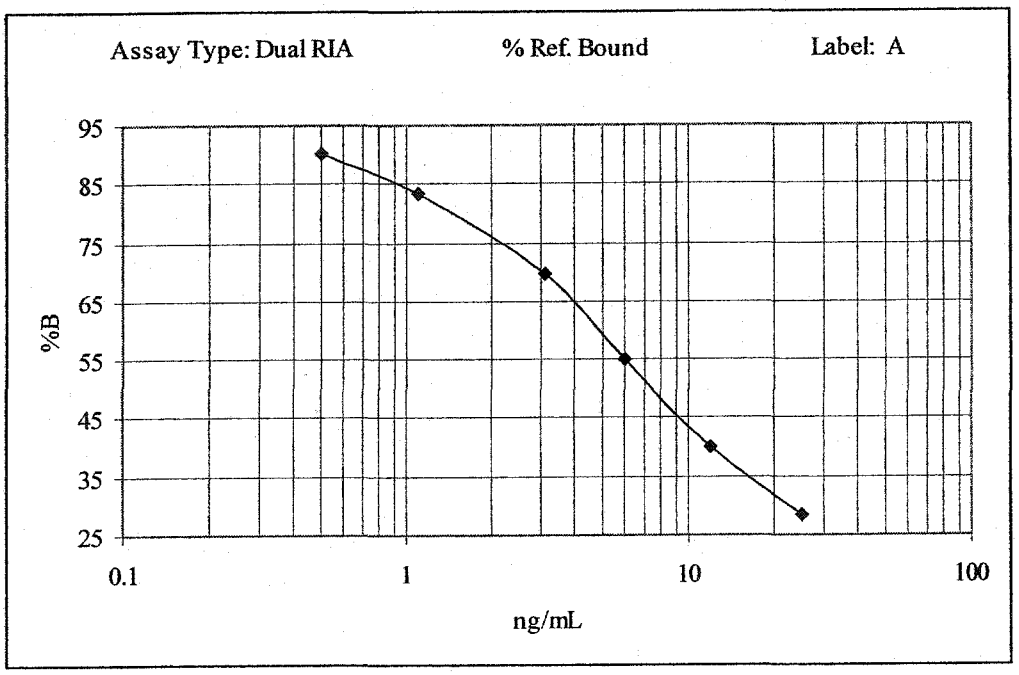


Figure 2-4. Chromatogram of amino acid external standards.

2.4. ***Determination of Plasma Vitamin B_{12} and Folic Acid***

Plasma vitamin B_{12} and folic acid concentrations were determined using a commercially available radioimmunoassay Dualcount Solid Phase No Boil Assay kit (Intermedico, Markham, Ontario). Samples were mixed with labeled tracer (containing ^{57}Co-vitamin B_{12} and ^{125}I-folic acid) and the pH was increased to 9.3 by the addition of potassium cyanide-sodium hydroxide. This elevated pH allows for the complete inactivation of intrinsic factor antibodies and vitamin B_{12} transport proteins. The addition of a binder solution (containing purified hog intrinsic factor and folate binding protein) causes competition between the B_{12} and folate of the sample, and the labeled tracers for the binding sites on their respective binders, thereby reducing the amount of labeled tracer that can be bound. The amount of tracer bound, and conversely the counts, is inversely related to the concentrations of folic acid and vitamin B_{12} in the samples. The bound fraction was then isolated through centrifugation (3000 rpm at 22°C for 35 minutes) and subsequent aspiration of the supernatant. The remaining precipitate was counted using a Cobra Auto-Gamma® Counter (Canberra Packard Canada Ltd, Mississauga, Ontario) and compared to the curve of standards from the same run to obtain vitamin B_{12} and folic acid concentrations. The standard curves for vitamin B_{12} and folate using this method are found in Figure 2-5.

(A)



(B)

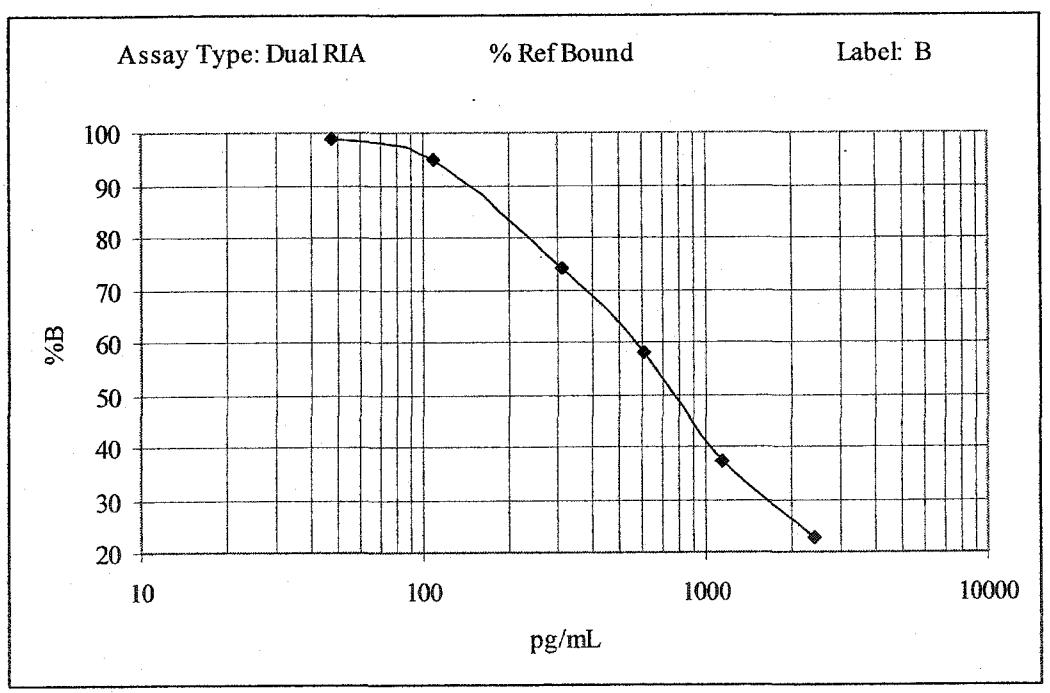


Figure 2-5. Standard curves for folate (A) and vitamin B_{12} (B) using the method described above.

2.5. ***Determination of Plasma Vitamin B_6***

Relative plasma vitamin B_6 concentrations were determined by measurement of the vitamin B6-dependent enzyme, aspartate aminotransferase (AST). The enzyme activity was determined by UV spectrophotometry using Sigma Diagnostics kit number 122-UV (INFINITY™ AST Reagent). 100µl of sample was added to a cuvette containing 1.0 mL of reagent at 37°C, gently mixed, and incubated for exactly 1 minute at 37°C. The absorbance at 340nm was then read by Cary 1E UV-Visible Spectrophotometer with Cary Temperature Controller (Varian Inc., Palo Alto, California, USA), and continually recorded at 30-second intervals for 1.5 minutes, maintaining temperature.

AST present in the sample transfers the amino group from aspartate to oxoglutarate, forming oxaloacetate and glutamate (reaction 2.1). Plasma AST activity is indicative of plasma vitamin B_6 concentrations as it is a pyridoxal-5-phophate (PLP, active form of vitamin B6) dependent enzyme.

$$\text{L-Aspartate} + \text{2-Oxoglutarate} \xrightarrow{\text{AST}} \text{Oxaloacetate} + \text{L-Glutamate} \qquad \textbf{(Reaction 2.1)}$$

In the presence of NADH and malate dehydrogenase, oxaloacetate is subsequently reduced to malate, oxidizing NADH to NAD (reaction 2.2). The reduction in absorbance of NADH is recorded at 340nm and is a direct measure of AST concentrations, and hence an indirect measure of vitamin B_6 concentrations.

$$\text{Oxaloacetate} + \text{NADH} \xrightarrow[\text{Dehydrogenase}]{\text{Malate}} \text{L-Malate} + \text{NAD} \qquad \textbf{(Reaction 2.2)}$$

AST concentrations of the samples were determined with the following calculations (Sigma Diagnostics, 2001):

$$\text{Units/L} = \left[\frac{\text{change in Absorbance/min} * \text{TV} * 1000}{6.22 * \text{SV} * \text{LP}} \right]$$

Where TV = total reaction volume in mL

SV = sample volume in mL

LP = cuvet path length in cm

6.22 = millimolar absorption coefficient of NADH at 340nm

1000 = Conversion of units/mL to units/L

2.6. *Determination of Plasma Insulin*

Plasma insulin concentrations were determined using a commercially available Rat Insulin Radioimmunoassay kit (Linco Research Inc., St. Charles, Missouri). Samples were mixed with ^{125}I-labeled insulin and rat insulin antibody (guinea pig anti-rat insulin serum) to induce competition between the labeled insulin and the insulin present in the sample for the antigen binding sites on the antibody. The amount of tracer bound to antibody, and conversely the counts, is inversely related to the concentration of insulin in the samples. After overnight incubation at 4°C, a precipitating reagent (goat anti-guinea pig IgG serum) was added and the bound fraction was isolated by centrifugation (3200 rpm at 4°C for 40 minutes) and subsequent aspiration of the supernatant. The precipitate was counted using a Cobra Auto-Gamma® Counter (Canberra Packard Canada Ltd,

Mississauga, Ontario) and compared to the curve of standards from the same run to obtain insulin concentrations (Figure 2-6).

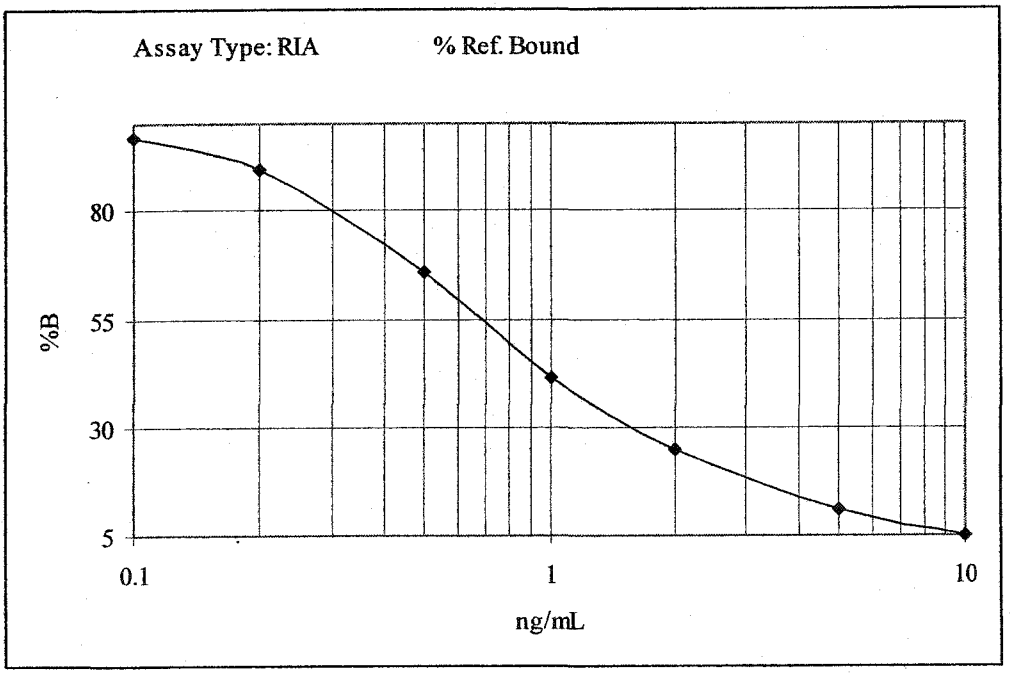


Figure 2-6. Standard curve for insulin using method described above.

2.7. ***Determination of Plasma Glucose***

Plasma glucose concentrations were determined enzymatically using Sigma Diagnostics kit number 315 (Glucose Trinder Reagent). The kit was based on the instructions and specifications of Trinder (1969). At timed intervals, 5µl of water (blank), standard, or sample was added to a cuvette containing room-temperature reagent, gently mixed, and incubated for exactly 18 minutes at room temperature. The absorbance at 505nm was then read by Lambda 3B UV/Vis spectrophotometer (PerkinElmer Instruments, Oak Brook, Illinois, USA).

The first reaction is the oxidation of glucose in the sample to gluconic acid and hydrogen peroxide (reaction 2.3).

$$\text{Glucose} + H_2O + O_2 \xrightarrow{\text{Glucose Oxidase}} \text{Gluconic Acid} + H_2O_2 \qquad \textbf{(Reaction 2.3)}$$

The hydrogen peroxide formed in reaction 2.3 then reacts with 4-aminoantipyridine and p-hydroxybenzene sulfonate to form H_2O and quinoneimine dye, which is maximally absorbed at 505nm (reaction 2.4). The intensity of the colour produced by these reactions is directly proportional to the glucose concentration of the sample.

$$H_2O_2 + \text{4-Aminoantipyridine} + \text{p-Hydroxybenzene Sulfonate} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine Dye} + H_2O \qquad \textbf{(Reaction 2.4)}$$

Glucose concentrations (mg/dL) of the samples were determined by the following calculations (Sigma Diagnostics, 1998):

$$\left[\frac{(\text{Abs. of sample}) - (\text{Abs. of blank})}{(\text{Abs. of standard}) - (\text{Abs. of blank})}\right] * \text{Concentration of standard (mg/dL)}$$

Results were then divided by, the molecular weight of glucose divided by 10 (conversion of dL to L) in order to express values in mmol/L.

2.8. ***Determination of Plasma Total Cholesterol***

Plasma cholesterol was determined enzymatically using Sigma Diagnostics kit number 402 (INFINITY™ Cholesterol Reagent), based on the formulation of Allain et al (1974). Using a 96-well plate, 2µl of water (blank), standard, or sample was added to the appropriate well. Using a multi-channel pipetter, addition of 200µl reagent began the reaction, which proceeded for 300 seconds. The plate was then read using the SpectraMax 190 microplate reader (Molecular Devices Corporation, Sunnyvale, California, USA) at 500nm.

Cholesterol esters present in the samples are hydrolyzed by cholesterol esterase to cholesterol and free fatty acids (reaction 2.5).

$$\text{Cholesterol Esters} \xrightarrow[\text{Esterase}]{\text{Cholesterol}} \text{Cholesterol + Free Fatty Acids} \qquad \textbf{(Reaction 2.5)}$$

The free cholesterol produced by reaction 2.5, as well as that already present in the sample, is then oxidized by cholesterol oxidase to the products seen in reaction 2.6.

$$\text{Cholesterol} + O_2 \xrightarrow[\text{Oxidase}]{\text{Cholesterol}} \text{Cholest-4-en-3-one} + H_2O_2 \quad \textbf{(Reaction 2.6)}$$

A chromophore (quinoneimine dye) is produced by the combination of hydrogen peroxide with hydroxybenzoic acid and 4-aminoantipyridine in the presence of the enzyme peroxidase (reaction 2.7). This chromophore has a maximal absorbance at 500-550nm. The intensity of the colour produced by these reactions is directly proportional to the total cholesterol concentration of the sample (Sigma Diagnostics, 1999).

$$2H_2O_2 + \text{Hydroxybenzoic Acid} + \text{4-Aminoantipyridine} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine Dye} + 4H_2O_2 \quad \textbf{(Reaction 2.7)}$$

Total cholesterol concentrations of the samples were determined by comparison to the curve of standards from the same run (Figure 2-7, A) to obtain total cholesterol concentrations. Results were then divided by, the molecular weight of cholesterol divided by 10 (conversion of dL to L) in order to express values as mmol/L.

2.9. *Determination of Plasma Triglycerides*

Plasma triglyceride concentrations were determined enzymatically using Sigma Diagnostics kit number 344 (INFINITY™ Triglycerides Reagent). The kit was based on reactions suggested by McGowan et al (1983) and Fossati & Prencipe (1982). Using a

96-well plate, 2µl of water (blank), standard, or sample was added to the appropriate well. Addition of 200µl reagent with a multi-channel pipetter began the reaction, which proceeded for 300 seconds. The plate was then read using the SpectraMax 190 microplate reader (Molecular Device Corporation, Sunnyvale, California, USA) at 520nm.

The first reaction is the hydrolysis of triglycerides in the sample to glycerol and free fatty acids (reaction 2.8).

$$\text{Triglycerides} \xrightarrow[\text{Lipase}]{\text{Lipoprotein}} \text{Glycerol + Fatty Acids} \qquad \textbf{(Reaction 2.8)}$$

Glycerol is then phosphorylated by ATP to form glycerol-3-phosphate and ADP. This reaction is catalyzed by the enzyme glycerol kinase (reaction 2.9).

$$\text{Glycerol + ATP} \xrightarrow[\text{Kinase}]{\text{Glycerol}} \text{Glycerol-3-phosphate + ADP} \qquad \textbf{(Reaction 2.9)}$$

Glycerol-3-phosphate is then oxidized to dihydroxyacetone phosphate by the enzyme glycerol phosphate oxidase, producing hydrogen peroxide (reaction 2.10).

$$\text{G-3-P + NAD} \xrightarrow[\text{Oxidase}]{\text{Glycerol Phosphate}} \text{Dihydroxyacetone Phosphate + } H_2O_2 \qquad \textbf{(Reaction 2.10)}$$

The final step involves the reaction of the hydrogen peroxide with 4-aminoantipyridine and 3,5 dichloro-2-hydroxybenzene sulfonate to form a red-coloured dye, which has a

maximal absorbance of 500-550nm (reaction 2.11). The intensity of the colour produced by these reactions is directly proportional to the triglycerides concentration of the sample (Sigma Diagnostics, 1999).

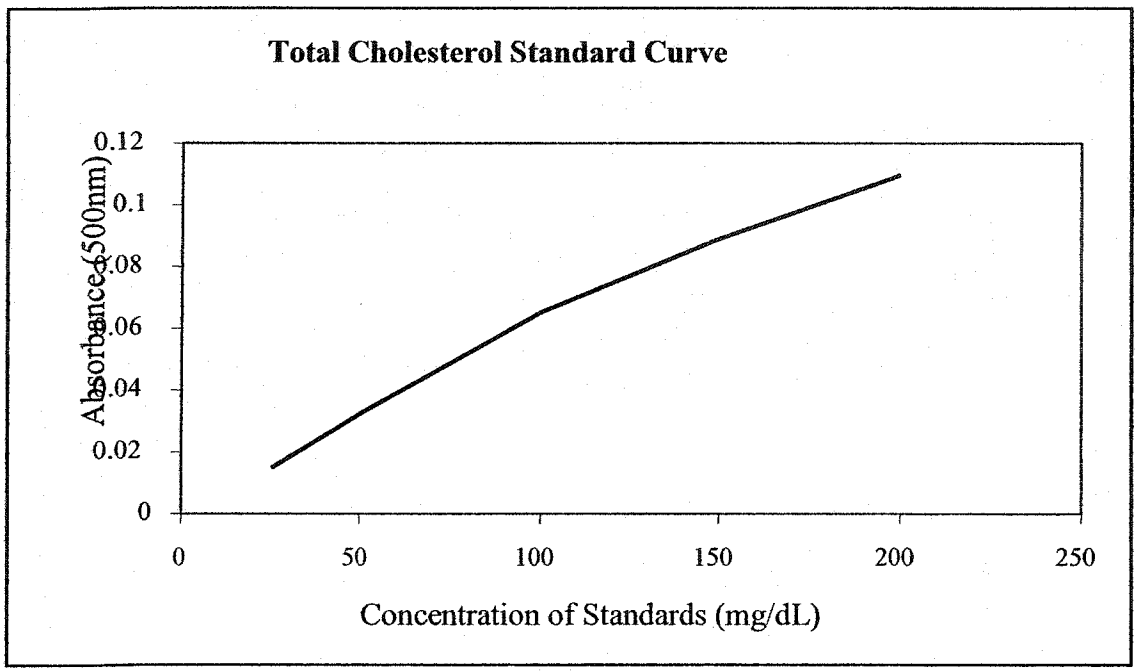
H_2O_2 + 4-aminoantipyridine + 3,5 dichloro-2-hydroxybenzene sulfonate

Peroxidase **(Reaction 2.11)**

$\longrightarrow$ Quinoneimine dye + $2H_2O_2$

Triglyceride concentrations of the samples were determined by comparison to the curve of standards (Figure 2-7, B) from the same run to obtain plasma triglycerides concentrations. Results were then divided by, the molecular weight of trigyceride divided by 10 (conversion of dL to L) in order to express values as mmol/L.

A



B

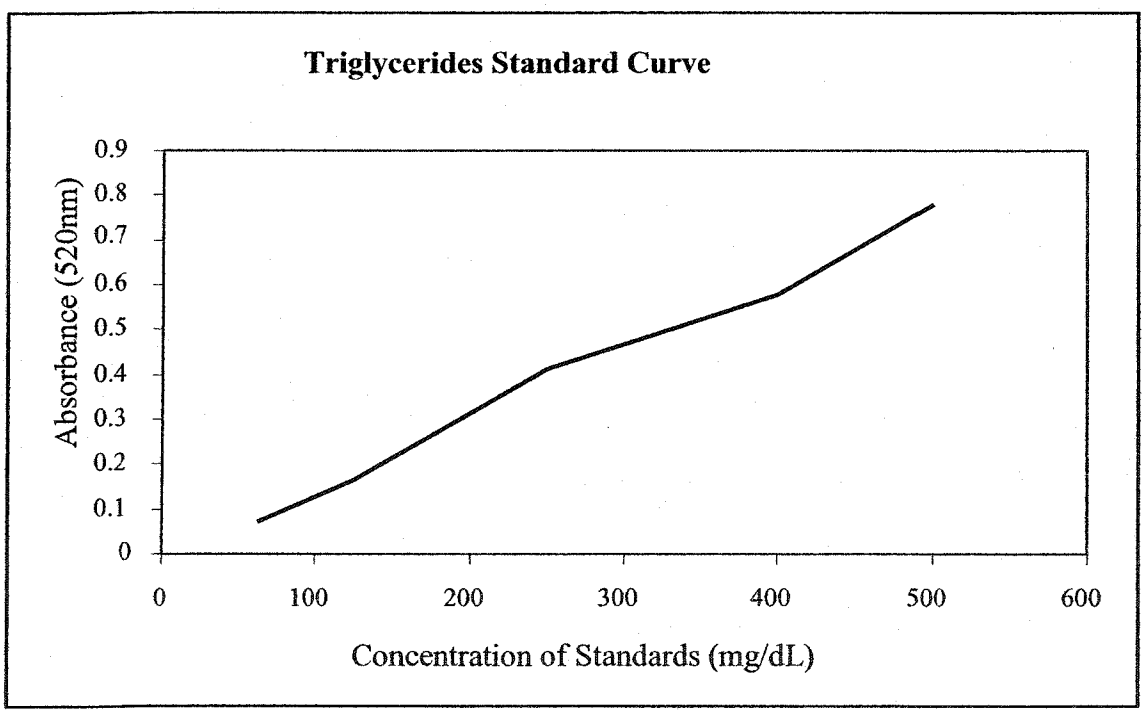


Figure 2-7. Standard curve for total cholesterol (A) and triglycerides (B) using methods described above.

2.10. ***Statistical Analysis***

Means and standard error of the means were determined for values for all groups of animals. A 3-way analysis of variance (ANOVA) was used to analyze the data for effects of age, diet, and strain. When significant differences were detected, the means were compared using least squares means/pdiff function in cases of interactions. A chi-square procedure was used to analyze incidence data. PROC CORR was used to determine correlations. All statistical analyses were completed using commercially available software (SAS Statistical Analysis System version 7.0, SAS Institute Inc., Cary, NC, USA). The significance level for this study was considered to be at $p=0.05$.

CHAPTER 3

RESULTS

3.1. *Pre-Onset of Diabetes*

3.1.1. *Body Weight Gain and Food Intake*

In BBdp rats, the onset of diabetes generally takes place between 60 and 100 days of age. Body weight gain and daily food intake of these rats were determined at 30 and 50 d of age. At these pre-onset (of diabetes) ages, the body weight change was greater in BBdp rats than that of their age-matched control counterparts (i.e. BBn rats), whereas the mean daily food intake was not different between the two groups (Table 3-1). The magnitude of body weight gain in BBdp rats was, however, greater at a statistically significant level only at 30 d of age.

Table 3-1. Body weight gain and daily food intake in BBdp and BBn rats at 30 and 50 d of age.

	Age (d)	Body Weight Gain (g)	Daily Food Intake (g/d)
BBdp	30	46.5 ± 2.4^{a}	15.8 ± 0.8^{a}
BBn	30	35.9 ± 2.4^{b}	16.2 ± 0.8^{a}
BBdp	50	158.9 ± 6.1^{c}	16.9 ± 0.7^{a}
BBn	50	137.2 ± 6.2^{c}	15.0 ± 0.8^{a}

BBdp: Bio-Breeding diabetes-prone; BBn: Bio-Breeding normal rats.
Values are expressed as means ± SEM of at least 6 animals.
Values not sharing common letters within columns are significantly different, $p<0.05$

3.1.2. *Plasma Lipids, Insulin, and Glucose*

At 30 d, the plasma glucose concentration was significantly lower in BBdp than that in BBn rats (Table 3-2). The plasma insulin concentrations in these animals did not differ significantly. At 50 d of age, the plasma glucose concentrations were higher, but there was no difference between BBdp and BBn rats. The plasma insulin concentration in 50-day old BBdp rats was only 50% of the level of that found in age-matched BBn rats.

Table 3-2. Plasma glucose and insulin concentrations in BBdp and BBn rats at 30 and 50 d of age.

	Age (d)	Glucose (mmol/L)	Insulin (ng/mL)
BBdp	30	5.6 ± 0.8^{a}	0.07 ± 0.2^{a}
BBn	30	8.4 ± 0.8^{b}	0.06 ± 0.1^{a}
BBdp	50	9.8 ± 0.6^{c}	0.7 ± 0.2^{b}
BBn	50	9.7 ± 0.6^{bc}	1.5 ± 0.2^{c}

For legend, see Table 3-1.

Plasma total cholesterol concentrations were not significantly different between BBdp and BBn rats at 30 and 50 d of age, although the concentrations were decreased significantly with age (Table 3-3) in both BBdp and BBn rats. Plasma triglyceride concentrations were significantly lower in BBdp than in BBn rats at 30 days; the strain difference disappeared at 50 d of age.

Table 3-3. Plasma total cholesterol and triglyceride concentrations in BBdp and BBn rats at 30 and 50 d of age.

	Age (d)	Total Cholesterol (mmol/L)	Triglycerides (mmol/L)
BBdp	30	3.3 ± 0.3^{a}	0.8 ± 0.3^{a}
BBn	30	3.8 ± 0.3^{a}	1.7 ± 0.3^{b}
BBdp	50	1.8 ± 0.3^{b}	0.7 ± 0.3^{a}
BBn	50	1.5 ± 0.3^{b}	0.9 ± 0.3^{a}

For legend, see Table 3-1.

3.1.3. *Plasma Sulfur Amino Acid Concentrations*

Table 3-4 illustrates the plasma concentrations of the sulfur amino acids, homocysteine and cysteine, in BBdp and BBn rats at 30 and 50 d of age. Both sulfur amino acid plasma concentrations increased with age; the age-associated increase was evident in both BBdp and BBn rats (Table 3-4). It was noteworthy that the plasma concentrations of both homocysteine and cysteine were markedly elevated in BBdp when compared with BBn rats at both 30 and 50 d of age.

There was a significant positive correlation between plasma homocysteine and insulin concentrations in BBdp rats in the pre-onset of diabetes stage (r-value = 0.612; p=0.0261). However, when separated into different age groups, the correlations were no longer significant (Table 3-5).

Table 3-4. Sulfur amino acid concentrations in the plasma of BBdp and BBn rats at 30 and 50 d of age.

	Age (d)	Total Homocysteine (μmol/L)	Cysteine (μmol/L)
BBdp	30	7.7 ± 0.5^{a}	144.2 ± 9.2^{a}
BBn	30	2.5 ± 0.5^{b}	77.0 ± 9.2^{b}
BBdp	50	10.8 ± 0.5^{c}	245.6 ± 8.7^{c}
BBn	50	5.3 ± 0.5^{d}	163.9 ± 8.7^{a}

For legend, see Table 3-1.

Table 3-5. Correlations between plasma homocysteine and insulin concentrations in BBdp and BBn rats at 30 and 50 d of age.

	Age (d)	Homocysteine (mmol/L)	Insulin (ng/mL)	Correlation (r)	Significance (p)
BBdp	30	7.7 ± 0.5	0.07 ± 0.2^{a}	- 0.481	0.2278
BBn	30	2.5 ± 0.5	0.06 ± 0.1^{a}	0.124	0.7515
BBdp	50	10.8 ± 0.5	0.7 ± 0.2^{b}	0.837	0.0771
BBn	50	5.3 ± 0.5	1.5 ± 0.2^{c}	- 0.649	0.5506

For legend, see Table 3-1.

3.1.4. *Correlations Between Plasma Homocysteine and Lipid Concentrations*

As shown in Table 3-6, homocysteine and cholesterol were significantly correlated only in BBn rats at 50 d of age. When considered together, there was a trend towards a significant negative correlation between plasma total cholesterol and homocysteine concentrations in BBdp and BBn rats at 30 and 50 d of age, as the r-value was – 0.353 (p=0.0905). Unlike cholesterol, however, plasma triglyceride concentrations were not significantly correlated with homocysteine at 30 or 50 d, regardless of strain (Table 3-7). When considered as a single group, the correlation of plasma triglycerides and homocysteine in BBdp and BBn rats at 30 and 50 d of age was still non-significant (r-value = - 0.394; p=0.2047).

Table 3-6. Correlations between plasma homocysteine and total cholesterol concentrations in BBdp and BBn rats at 30 and 50 d of age.

	Age (d)	Homocysteine (mmol/L)	TC (mmol/L)	Correlation (r)	Significance (p)
BBdp	30	7.7 ± 0.5	3.3 ± 0.3	- 0.593	0.2143
BBn	30	2.5 ± 0.5	3.8 ± 0.3	0.363	0.4791
BBdp	50	10.8 ± 0.5	1.8 ± 0.3	0.152	0.7732
BBn	50	5.3 ± 0.5	1.5 ± 0.3	- 0.836	0.0381

For legend, see Table 3-1.

Table 3-7. Correlations between plasma homocysteine and triglyceride concentrations in BBdp and BBn rats at 30 and 50 d of age.

	Age (d)	Homocysteine (mmol/L)	TG (mmol/L)	Correlation (r)	Significance (p)
BBdp	30	7.7 ± 0.5	0.8 ± 0.3	0.287	0.5817
BBn	30	2.5 ± 0.5	1.7 ± 0.3	0.115	0.8282
BBdp	50	10.8 ± 0.5	0.7 ± 0.3	0.506	0.3054
BBn	50	5.3 ± 0.5	0.9 ± 0.3	0.217	0.6803

For legend, see Table 3-1.

3.1.5. *Vitamin Status*

Since vitamins B_6, B_{12}, and folate are important cofactors in the metabolism of homocysteine, their biochemical status was determined in BBdp and BBn rats at 30 and 50 d of age. As shown in Table 3-8, plasma folate and vitamin B_{12} concentrations were not significantly different between BBdp rat and their age-matched control counterparts. Aspartate aminotransferase (AST) is a relative measure of vitamin B_6 status. Using this biomarker, vitamin B_6 status appeared to be different in BBdp rats but only at 30 d of age. It was noteworthy, however, that the plasma concentrations of folate, vitamin B_{12}, and AST were all lower in BBdp compared with BBn rats at both 30 and 50 d of age,

although the difference was not statistically significant. It was also of interest that the plasma folate concentration at 50 d of age was 50% of the value that was found at 30 d of age. Such age-associated changes were observed in both BBdp and BBn rats.

Table 3-8. Biochemical status of folate, vitamin B_{12}, and vitamin B_6 in BBdp and BBn rats at 30 and 50 d of age.

	Age (d)	Folate (ng/mL)	Vitamin B_{12} (pg/mL)	AST (units/L)*
BBdp	30 days	116.9 ± 11.2^a	768.1 ± 149.4^a	34.9 ± 6.0^a
BBn	30 days	120.1 ± 11.2^a	973.8 ± 149.4^a	53.4 ± 6.0^b
BBdp	50 days	65.6 ± 11.2^b	601.8 ± 149.4^a	16.6 ± 6.0^c
BBn	50 days	67.3 ± 15.9^b	803.8 ± 211.2^a	20.6 ± 6.0^{ac}

For legend, see Table 3-1.

* AST (aspartate aminotransferase) is a vitamin B_6-dependent enzyme, and is therefore presented as a relative measure of vitamin B_6 status.

3.2. *Effect of Vitamin Supplementation in Pre-Onset of Diabetes*

BBdp rats were fed a diet containing folate, vitamin B_{12}, and vitamin B_6, at 10 times the levels present in the control diet. This supplemented diet was fed from 21 d of age (weaning) until 50 d of age. The plasma concentrations of folate, B_{12}, and AST (a marker of vitamin B_6 status) were markedly elevated in these animals, reflecting their increased intakes of the vitamins (Table 3-9). These increases were accompanied by a decreased plasma concentration of homocysteine and cysteine; only the latter, however, was found to be statistically significant (Table 3-10). It was of importance that the B-vitamin-supplemented BBdp rats had significantly lower plasma glucose concentration (Table 3-11) despite their greater (although non-significant) body weight gain (Table 3-9) than that of the age-matched BBdp rats fed a diet containing only the basal levels of the B-vitamins. Interestingly, plasma total cholesterol concentrations were significantly elevated in supplemented animals at this age, compared to animals on control diet.

Table 3-9. Modifying effects of the supplemental intakes of folate, vitamin B_{12}, and vitamin B_6 on food intake, weight gain, and plasma B-vitamin concentrations in 50-day old BBdp rats.

Suppl.*	Food Intake (g/d)	Body Weight Gain (g)	Folate (ng/mL)	Vitamin B_{12} (pg/mL)	AST (Units/L)
—	16.9 ± 0.7^a	148.8 ± 8.6^a	65.6 ± 11.2^a	601.9 ± 149.4^a	16.6 ± 6.0^a
+	24.6 ± 0.7^b	169.0 ± 8.6^a	118.7 ± 11.2^b	1072.0 ± 149.4^b	28.6 ± 6.0^b

For legend, see Table 3-1.

* See Table 2-1 for supplemental diet details.

Table 3-10. Modifying effects of the supplemental intakes of B-vitamins on the plasma concentrations of homocysteine and cysteine in 50-day old BBdp rats.

Suppl.*	Homocysteine (mmol/L)	Cysteine(mmol/L)
—	10.8 ± 0.5^a	245.6 ± 8.7^a
+	10.1 ± 0.5^a	207.9 ± 8.7^b

For legend, see Table 3-1.

* See Table 2-1 for supplemental diet details.

Table 3-11. Modifying effects of the supplemental intakes of B-vitamins on the plasma concentrations of glucose and lipids in 50-day old BBdp rats.

Suppl.*	Glucose (mmol/L)	Triglycerides (mmol/L)	Total Cholesterol (mmol/L)
—	9.8 ± 0.6^{a}	0.7 ± 0.3^{a}	1.8 ± 0.3^{a}
+	4.4 ± 0.8^{b}	0.9 ± 0.3^{a}	3.6 ± 0.3^{b}

For legend, see Table 3-1.

* See Table 2-1 for supplemental diet details.

3.3. ***Onset of Diabetes***

BBdp rats were maintained on a basal diet until the onset of diabetes, which occurred between 58-108 d of age. Within 24 hours of the onset of diabetes, rats (termed as BBd) were sacrificed along with age-matched controls (BBn). Neither the daily food intake nor the body weight gain was affected in BBd, compared with that of the BBn rats. BBd rats displayed markedly lower insulin and higher glucose concentrations in the plasma than their control counterparts, reflecting the presence of diabetes (Table 3-12). The hyperglycemia, in BBd rats, was accompanied by elevated cholesterol levels. Plasma triglyceride concentration was also found to be higher in BBd than in BBn rats, although not statistically significant. Correlations between plasma concentrations of glucose and triglycerides in BBn and BBd rats were not found to be significant, as shown in Table 3-13.

Table 3-12. Plasma insulin, glucose, and lipid concentrations of BBd and BBn rats at the onset of diabetes.

	Insulin (ng/mL)	Glucose (mmol/L)	Triglycerides (mmol/L)	TC (mmol/L)
BBn	1.5 ± 0.2^a	10.1 ± 0.7^a	1.1 ± 0.3^a	1.8 ± 0.3^a
BBd	0.4 ± 0.1^b	13.7 ± 0.9^b	1.7 ± 0.3^a	3.8 ± 0.3^b

For legend, see Table 3-1.

Table 3-13. Correlations between plasma glucose and triglyceride concentrations at the onset of diabetes.

	Glucose (mmol/L)	Triglycerides (mmol/L)	Correlation (r)	Significance (p)
BBn	10.1 ± 0.7^a	1.1 ± 0.3^a	0.472	0.3441
BBdp	13.7 ± 0.9^b	1.7 ± 0.3^a	- 0.659	0.1549

For legend, see Table 3-1.

All animals at the onset of diabetes: r= - 0.350, p=0.2654

3.3.1. *Plasma Sulfur Amino Acid Concentrations*

Plasma concentrations of homocysteine and cysteine were determined, along with the B-vitamins that are involved as cofactors in the metabolism of the amino acids. Plasma homocysteine as well as folate and vitamin B_{12} concentrations were not significantly different between diabetic and control animals (Table 3-14). However, plasma cysteine concentrations were significantly lower, and plasma AST concentrations significantly higher, in diabetic animals compared to non-diabetic controls of the same age.

There was a significant positive association between plasma homocysteine and insulin concentrations at the onset of diabetes in BBd rats, while no significant correlation was found in control animals at this age (Table 3-15).

Table 3-14. Sulfur amino acid and B-vitamin concentrations in the plasma of diabetic and control animals.

	Homocysteine (mmol/L)	**Cysteine (mmol/L)**	**Folate (ng/mL)**	**Vitamin B_{12} (pg/mL)**	**AST (units/L)**
BBn	6.8 ± 0.6^a	212.5 ± 8.7^a	50.0 ± 11.2^a	740.4 ± 149.4^a	51.0 ± 6.02^a
BBd	6.7 ± 0.5^a	168.4 ± 7.6^b	85.3 ± 11.2^a	615.7 ± 149.4^a	28.1 ± 6.02^b

For legend, see Table 3-1.

Table 3-15. Correlations between plasma homocysteine and insulin concentrations at the onset of diabetes.

	Homocysteine (mmol/L)	Insulin (ng/mL)	Correlation (r)	Significance (p)
BBn	6.8 ± 0.6^{a}	1.5 ± 0.2^{a}	- 0.393	0.4403
BBdp	6.7 ± 0.5^{a}	0.4 ± 0.1^{b}	0.625	0.0223

For legend, see Table 3-1.

All animals at onset of diabetes: r= 0.133 ; p=0.5860

3.3.2. *Correlations Between Plasma Homocysteine and Lipid Concentrations*

As shown in Table 3-16, plasma homocysteine and cholesterol concentrations were not significantly correlated in either control or diabetic animals at the onset of diabetes. When considered as a single group, the correlation between plasma homocysteine and total cholesterol concentrations was still non-significant (r-value= -0.007, p=0.9824). Like cholesterol, plasma triglyceride concentrations were not significantly correlated with homocysteine in either BBd or BBn rats at the onset of diabetes (Table 3-17). When considered as a single group, the correlation of plasma triglyceride and homocysteine concentrations, at the onset of diabetes, was still non-significant (r-value= -0.356, p=0.2561).

Table 3-16. Correlations between plasma homocysteine and cholesterol concentrations at the onset of diabetes.

	Homocysteine (mmol/L)	**Total Cholesterol (mmol/L)**	**Correlation (r)**	**Significance (p)**
BBn	6.8 ± 0.6^{a}	1.8 ± 0.3^{a}	- 0.140	0.7920
BBdp	6.7 ± 0.5^{a}	3.8 ± 0.3^{b}	- 0.002	0.9970

For legend, see Table 3-1.

Table 3-17. Correlations between plasma homocysteine and triglyceride concentrations at the onset of diabetes.

	Homocysteine (mmol/L)	Triglycerides (mmol/L)	Correlation (r)	Significance (p)
BBn	6.8 ± 0.6^{a}	1.1 ± 0.3^{a}	0.031	0.9536
BBdp	6.7 ± 0.5^{a}	1.7 ± 0.3^{a}	- 0.530	0.2794

For legend, see Table 3-1.

3.4. *Effect of Vitamin Supplementation at the Onset of Diabetes*

Table 3-18 shows the effect of a vitamin B_6-, B_{12}-, and folate-supplemented diet on weight gain and food intake at the onset of diabetes in BB rats. No significant effect of the supplementation was observed on either food intake or weight gain from weaning to the onset of diabetes; this was also observed in non-diabetic control rats.

Table 3-18. Effect of vitamin supplementation on body weight gain and daily food intake at the onset of diabetes.

	Diet*	Body Weight Gain (g)	Daily Food Intake (g/d)
BBd	Control	240.4 ± 18.3^a	25.8 ± 1.4^a
BBd	Suppl.	238.2 ± 22.0^a	25.8 ± 1.7^a
BBn	Control	269.3 ± 19.1^a	20.6 ± 2.1^a
BBn	Suppl.	281.9 ± 24.9^a	23.8 ± 1.9^a

For legend, see Table 3-1.

* Suppl. = Supplemented diet

3.4.1. *Plasma Lipids, Insulin, and Glucose*

As expected, the plasma insulin concentration was significantly lower and the plasma glucose concentration significantly higher in BBd rats than that in BBn rats (Table 3-19). Feeding a B-vitamin supplemented diet to these animals had no significant effect on the plasma concentrations of glucose and insulin.

The effect of dietary supplementations of vitamins B_6, B_{12}, and folate on plasma concentrations of total cholesterol and triglycerides in BB rats is shown in Table 3-20. Plasma total cholesterol concentrations were significantly elevated in diabetic rats on the supplemented diet compared to animals on a control diet; this effect was more pronounced in BBn than in BBd rats (Table 3-20). The difference between BBd and BBn rats disappeared, however, following the intake of a diet supplemented with B-vitamins. Plasma triglyceride concentrations at the onset of diabetes were also significantly increased with supplementation, in both BBd and BBn rats. After supplementation, plasma triglyceride concentrations were significantly higher in diabetic animals than in non-diabetic control animals, whereas on the control diet, there was no difference between strains.

Table 3-19. Effect of vitamin supplementation on plasma insulin and glucose concentrations at the onset of diabetes.

	Diet*	Insulin (ng/mL)	Glucose (mmol/L)
BBd	Control	0.4 ± 0.1^{a}	13.6 ± 0.9^{a}
BBd	Suppl.	0.5 ± 0.1^{a}	13.3 ± 0.8^{a}
BBn	Control	1.5 ± 0.2^{b}	10.1 ± 0.7^{b}
BBn	Suppl.	1.3 ± 0.2^{b}	9.3 ± 0.8^{b}

For legend, see Table 3-1.

* Suppl. = Supplemented diet

Table 3-20. Effect of vitamin supplementation on plasma lipid concentrations at the onset of diabetes.

	Diet*	Total Cholesterol (mmol/L)	Triglycerides (mmol/L)
BBd	Control	3.8 ± 0.3^{a}	1.7 ± 0.3^{ac}
BBd	Suppl.	4.7 ± 0.3^{b}	3.5 ± 0.3^{b}
BBn	Control	1.7 ± 0.3^{c}	1.1 ± 0.3^{a}
BBn	Suppl.	4.4 ± 0.3^{ab}	2.0 ± 0.3^{c}

For legend, see Table 3-1.

*Suppl. = Supplemented diet

3.4.2. *Plasma Sulfur Amino Acid Concentrations*

Table 3-21 shows the effect of dietary vitamin supplementation on homocysteine and cysteine concentrations in plasma at the onset of diabetes in BB rats. Supplementation of vitamins B_6, B_{12}, and folate resulted in a significant decrease in plasma homocysteine concentrations in BBn rats, whereas the concentrations remained unchanged in BBd rats. Like homocysteine, plasma cysteine concentrations were significantly decreased in BBn but not in BBd rats after supplementation.

Table 3-21. Effect of vitamin supplementation on plasma homocysteine and cysteine concentrations at the onset of diabetes.

	Diet*	Homocysteine (µmol/L)	Cysteine (µmol/L)
BBd	Control	6.7 ± 0.4^{a}	168.4 ± 7.6^{ab}
BBd	Suppl.	6.4 ± 0.5^{ab}	174.7 ± 9.2^{a}
BBn	Control	6.8 ± 0.5^{a}	212.5 ± 8.7^{c}
BBn	Suppl.	5.1 ± 0.6^{b}	146.2 ± 10.4^{b}

For legend, see Table 3-1.

*Suppl. = Supplemented diet

3.4.3. *Vitamin Status*

Table 3-22 shows the effect of vitamin supplementation on plasma concentrations of vitamin B_{12} and folate in BB rats at the onset of diabetes. No effect of supplementation was seen on plasma folate and AST concentrations in BBd rats. However, the plasma folate concentrations in non-diabetic rats were significantly elevated with supplementation. Contrary to these results, supplementation had no significant effect on plasma vitamin B_{12} concentrations in BBn rats, but did significantly increase the concentrations in diabetic animals at the onset of diabetes.

Table 3-22. Effect of vitamin supplementation on plasma concentrations of folate, vitamin B_{12}, and AST at the onset of diabetes.

	Diet*	**Folate (ng/mL)**	**Vitamin B_{12} (pg/mL)**	**AST 3(units/L)**
BBd	Control	85.3 ± 11.2^{a}	615.7 ± 149.4^{a}	51.0 ± 6.0^{a}
BBd	Suppl.	89.9 ± 11.2^{a}	1695.9 ± 149.4^{b}	32.7 ± 6.0^{b}
BBn	Control	50.0 ± 11.2^{b}	740.4 ± 149.4^{ac}	28.1 ± 6.0^{b}
BBn	Suppl.	112.0 ± 11.2^{a}	1115.6 ± 149.4^{c}	34.1 ± 6.0^{b}

For legend, see Table 3-1.

* Suppl. = Supplemented diet.

3.4.4. *Incidence of Diabetes and Age at Onset*

BBdp rats were maintained either on a control diet or the control diet enriched with vitamins B_6, B_{12}, and folate (10 times the levels present in the control diet) from weaning (21 d) to 120 d of age. During this period, 30% of the supplemented group and 37% of the unsupplemented rats displayed hyperglycemia, as evidenced by elevated concentrations of plasma and urinary glucose (Table 3-23). The ages (d) at which the hyperglycemia manifested in the supplemented and unsupplemented groups were 80 and 77, respectively.

Table 3-23. Effect of vitamin supplementation on the incidence of diabetes and on the age at onset of diabetes.

Diet	Diabetes Incidence (%)	Age at Onset (days)
Control	37[a]	77 ± 4[a]
Supplemented	30[a]	80 ± 5[a]

For legend, see Table 3-1.

3.5. *Effects of Age on Lipid and Amino Acid Parameters*

Both plasma total cholesterol and triglyceride concentrations were similar in BBdp and BBn rats at 30 and 50 days of age, but at the onset of diabetes, plasma lipid concentrations were significantly higher in the diabetic animal. Both plasma total cholesterol and triglyceride concentrations were higher at the onset of diabetes than at 50 days of age in BBdp rats, while concentrations of these plasma lipids were unchanged over this period in BBn rats (Figure 3-1). In contrast, both plasma total cholesterol and triglyceride concentrations were lower in BBn rats at 50 days of age and age-matched to the onset of diabetes, compared to 30 days of age. This same trend was not seen in BBdp rats, indicating a strain-specific effect of age on plasma lipid concentrations in the BB rat. Plasma homocysteine concentrations in BBn rats increased at each time point studied. Although a similar increase from 30 to 50 days of age was observed in BBdp rats, plasma homocysteine concentrations were decreased at the onset of diabetes in this strain (Figure 3-2). Similar age-associated changes were observed with plasma cysteine concentrations (Figure 3-3).

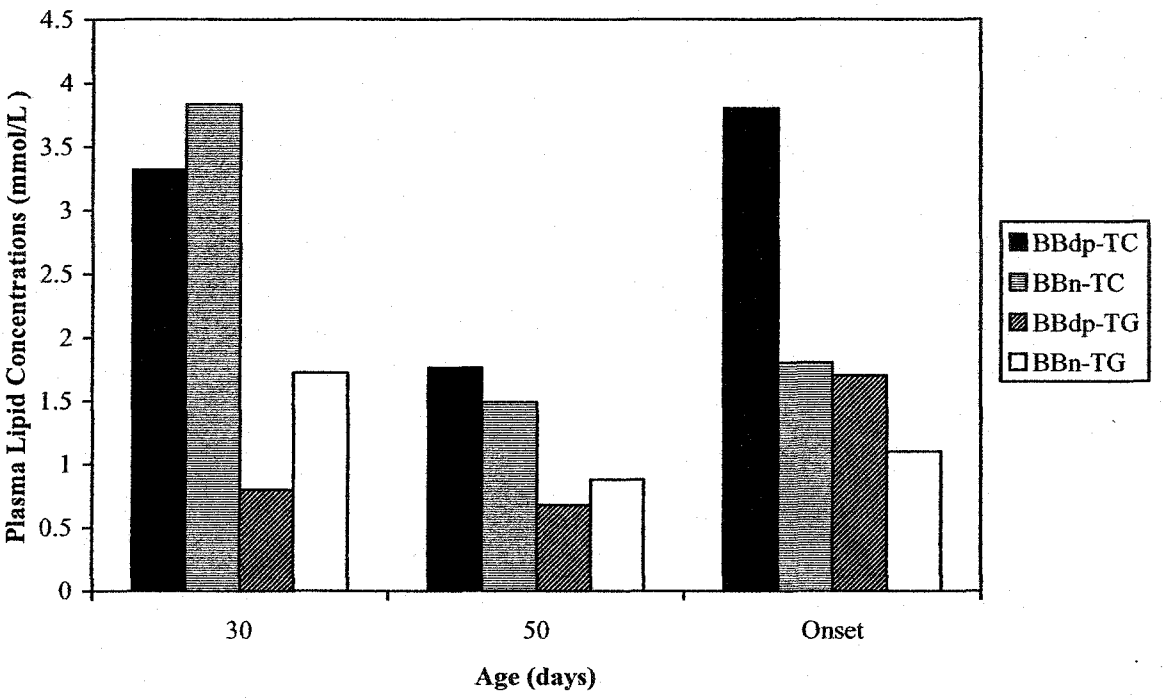


Figure 3-1. Effect of age on plasma total cholesterol (TC) and triglyceride (TG) concentrations in BBdp and BBn rats.

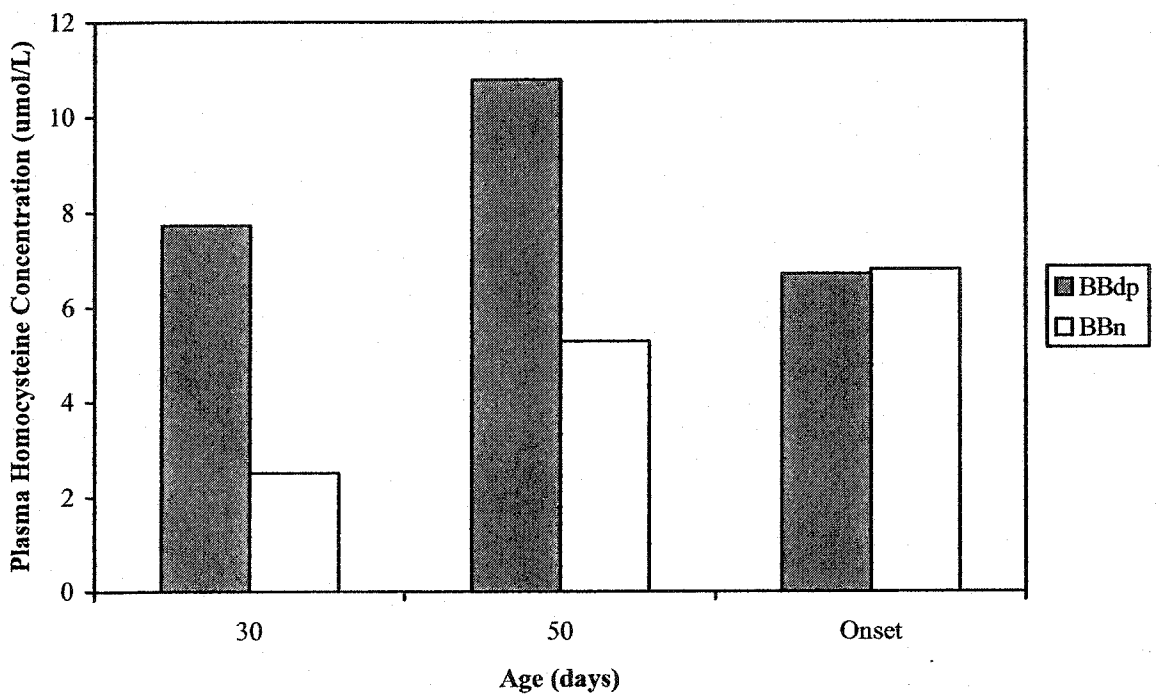


Figure 3-2. Effect of age on plasma homocysteine concentrations in BBdp and BBn rats.

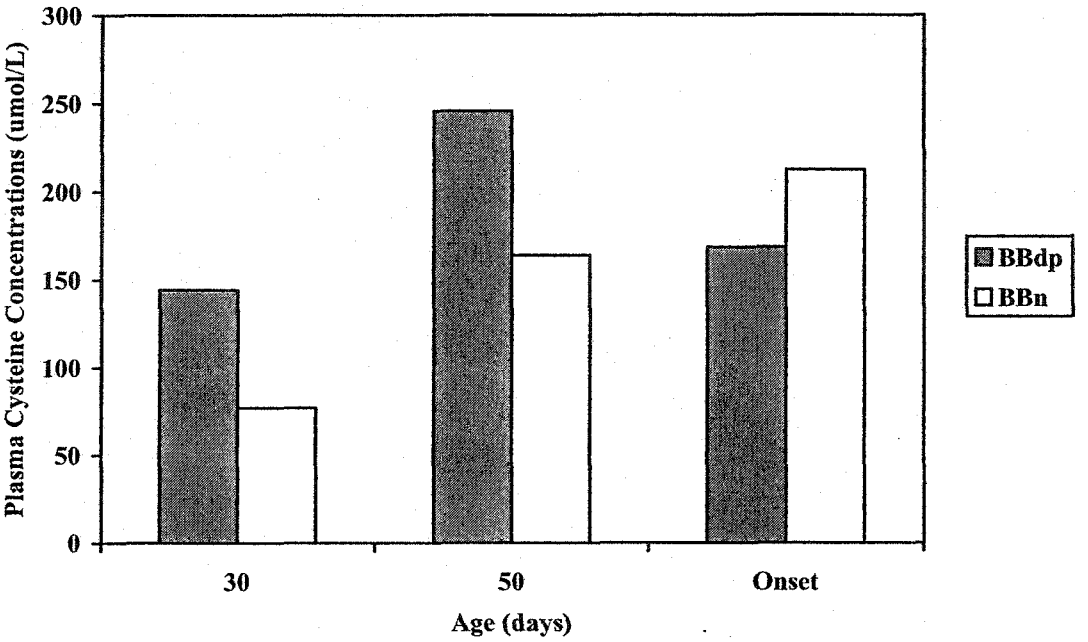


Figure 3-3. Effect of age on plasma cysteine concentrations in BBdp and BBn rats.

CHAPTER 4

DISCUSSION

In recent years, there has been a substantial amount of evidence suggesting that an increased plasma homocysteine concentration can be a risk factor for cardiovascular disease (CVD), independent of other conventional risk factors such as plasma cholesterol and blood pressure (Blum et al, 2001; Bostom et al, 1999; Boushey et al, 1995; Stampfer et al, 1992; van den Brandhof, 2001). Furthermore, Boushey et al (1995) found that the risk of CVD from homocysteine increases significantly with each 5μmol/L increase in plasma homocysteine concentrations. As a comparison, this elevation in plasma homocysteine concentrations is approximately equivalent to an increase of 0.5mmol/L in plasma total cholesterol concentrations, in terms of increased CVD risk (Boushey et al, 1995).

CVD is a common secondary complication of diabetes, but little is known of the relationship between homocysteine and CVD, with particular reference to type 1 diabetes. Conflicting results have been found regarding plasma homocysteine concentrations in patients with type 1 diabetes. Reports of elevated (Chiarelli et al, 2000; Drzewoski et al, 2000; Hofman et al, 1997), depressed (Cronin et al, 1998), and unchanged (Cronin et al, 1998; Lanfredini et al, 1998; Pavia et al, 2000) plasma homocysteine concentrations in patients with diabetes compared to healthy controls have all been published. Furthermore, research to date has not examined the metabolism of homocysteine before the onset of diabetes, nor the potential modulative effect of dietary supplementation of vitamins B_6,

B_{12}, and folate on the metabolism of homocysteine at this stage. It has been shown that supplementation with these vitamins can reduce plasma homocysteine concentrations by increasing the metabolism of homocysteine to cysteine and methionine (Beaulieu et al, 1999; Henning et al, 2001; Homocysteine Lowering Trialists' Collaboration, 1998; Naurath et al, 1995), which are further metabolized or used for protein synthesis.

Therefore, the purpose of the present study was to investigate the pattern of change in plasma homocysteine concentrations prior to and at the onset of diabetes in an animal species prone to developing type 1 diabetes. This was accomplished by tracking plasma homocysteine concentrations at various stages of the development of type 1 diabetes in diabetes-prone Bio-breeding (BBdp) rats compared to normal control (BBn) rats. The effect of supplementation with vitamins B_6, B_{12}, and folate versus a control diet on homocysteine status was also compared in each of the rat strains. In addition, plasma homocysteine status was examined in relation to the status of plasma total cholesterol, another major risk factor for CVD. Age-associated increases in plasma homocysteine concentrations were observed prior to the onset of diabetes in BBdp and at the corresponding ages in BBn rats. Elevated homocysteine levels were observed in BBdp rats prior to the onset of diabetes when compared to age-matched control rats; however, these were not seen at the onset of diabetes. No significant effect of vitamin supplementation was observed on plasma homocysteine concentrations before the onset of diabetes in either the BBdp or BBn rats, nor at the onset of diabetes in age-matched BBn rats. On the control diet, plasma total cholesterol concentrations were elevated at the onset of diabetes in the BBd rats compared to the age-matched BBn rats. With supplementation, plasma lipid concentrations were significantly increased at the onset of

diabetes in both BBd rats and their age-matched controls, compared to animals on the control diet.

4.1. *Homocysteine Status*

Prior to the onset of diabetes, age-associated increases in plasma homocysteine concentrations were observed in both BBdp and BBn rats. This finding is supported by numerous studies which have found that plasma homocysteine concentrations are positively correlated with age in adults (Bostom et al, 1999; Jacques et al, 2001; Saw et al, 2001) and in children and adolescents in Britain (Bates et al, 2002), Taiwan (Chang et al, 2003), and Canada (Delvin et al, 2000). In fact, Delvin et al (2000) found that 48% of the variation in plasma homocysteine concentrations was accounted for by differences in the ages of the children studied, while differences in plasma folate concentrations explained 27% of the variation in plasma homocysteine concentrations, and variation in plasma vitamin B_{12} concentrations, only 19%. These findings suggest that age is a stronger predictor of plasma homocysteine concentrations than vitamin status.

While this age-associated increase in plasma homocysteine concentration was seen in BBn, and BBdp rats prior to the onset of diabetes, the increase, however, continued only in the control rats beyond 50 days of age. In contrast, in BBdp rats the plasma homocysteine concentrations were markedly decreased at the onset of diabetes. These results are in parallel with the findings of Cronin et al (1998), who reported lower plasma homocysteine concentrations in male patients with type 1 diabetes compared to age-matched healthy controls. However, these results are not in accordance with the findings of Pavia et al (2000), who found that plasma homocysteine concentrations were

not different between adolescent type 1 diabetes patients and age-matched healthy controls. The present findings are also not in parallel with the findings of Targher et al (2000), who reported elevated plasma homocysteine concentrations in patients with type 1 diabetes, compared to age- and sex-matched healthy controls.

Hypoinsulinemia is a common manifestation of type 1 diabetes, as insulin-producing ß-cells are progressively destroyed through auto-immune mechanisms (Committee Report, 2002). It has been found that hypoinsulinemia in Streptozotocin-diabetic rats is associated with decreased plasma homocysteine concentrations (Gursu et al, 2002; Jacobs et al, 1998). Administration of insulin to these animals helped to restore their plasma homocysteine concentrations to the level of their control counterparts (Jacobs et al, 1998). These findings suggest a positive association between plasma insulin and homocysteine concentrations, which supports the results of the present study. While Jacobs et al (1998) have shown a clear association between plasma insulin and homocysteine concentrations, they have also shown that this relationship is not direct. Instead, it has been suggested that the observed relation between plasma homocysteine and insulin concentrations is moderated by the effect of insulin on two of the enzymes involved in homocysteine metabolism, CBS (Dicker-Brown et al, 2001; Jacobs et al, 1998) and MTHFR (Dicker-Brown et al, 2001), since plasma homocysteine concentrations are known to be modified in the presence of altered activity of these enzymes. Dicker-Brown et al (2001) treated human hepatocytes with varying concentrations of insulin and measured the resulting activities of the enzymes CBS and MTHFR. It was found that the activities of these enzymes were decreased when cultured human hepatocytes were presented with increasing doses of insulin (Dicker-Brown et al,

2001). These results suggest an inverse relationship between insulin concentrations and the enzyme activities of CBS and MTHFR. These findings are further supported by Jacobs et al (1998), who showed that the activity of the transsulfuration enzyme CBS was increased in the livers of untreated diabetic (i.e. hypoinsulinemic) rats, and consequently, the plasma homocysteine concentrations were decreased. These findings therefore suggest that increased plasma concentrations of insulin result in decreased enzyme activities; hence plasma homocysteine concentrations would also likely increase due to the limitation of metabolic pathways. Consequently, decreased plasma insulin concentrations would increase the activity of the enzymes CBS and MTHFR, and therefore drive homocysteine metabolism toward the synthesis of cysteine and methionine respectively, thus lowering plasma homocysteine concentrations.

4.2. *Homocysteine Status in Relation to Lipid Status*

Unlike homocysteine, cholesterol status did not differ between strains before the onset of diabetes. At the onset of diabetes, however, elevated cholesterol levels were observed in BBd rats, compared to control BBn rats; plasma triglyceride concentrations followed a similar trend. These results are supported by many clinical studies, as hyperlipidemia is commonly manifested in uncontrolled type 1 diabetes, although it can often be ameliorated with improved blood glucose control (Goldberg, 2000; Goldberg, 2002; Idzior-Walus et al, 2001; Steiner, 2000). There have been a few studies that have examined the relationship between plasma homocysteine and lipid concentrations. These studies have revealed either positive (El-Khairy et al, 1999), negative (Bostom et al, 1999), or non-significant (Pavia et al, 2000) relationships; the results were thus

inconsistent. The present study was first to examine the relationships between homocysteine and lipid studies in BB rats. According to the findings of the present study, significant correlations between plasma homocysteine and total cholesterol concentrations were observed in BBn rats at 50d of age. At the onset of diabetes, no significant correlations were detected between plasma homocysteine concentrations and lipid parameters. The lack of significance in the correlations between plasma homocysteine and lipid (either total cholesterol or triglyceride) concentrations at the onset of diabetes is supported by the findings of Pavia et al (2000), who did not find a correlation between plasma homocysteine and either total cholesterol or triglyceride concentrations in type 1 diabetes patients. On the other hand, El-Khairy et al (1999), found a strong positive association between plasma homocysteine and cholesterol concentrations, while Bostom et al (1999) reported a weak but significant negative correlation between plasma homocysteine and total cholesterol concentrations. However, both of these studies were performed in non-diabetic populations, and therefore the results cannot necessarily be accurately reflected in the presence of diabetes, where the altered status of both homocysteine and lipids is common.

4.3. *Effects of Vitamin Supplementation*

Common nutritional factors that can influence plasma homocysteine concentrations include folate, vitamin B_6, and vitamin B_{12} status. Plasma concentrations of these B-vitamins have been reported to be inversely associated with plasma homocysteine concentrations (Saw et al, 2001). Depletion studies of folate (O'Leary et al, 2001), vitamin B_{12} (Stangl et al, 2000) and vitamin B_6 (Martinez et al, 2000) in animals

have also been found to results in significant increases in plasma homocysteine concentrations.

The Dietary Reference Intake (DRI) of folate is 400 μg/d, of vitamin B_{12} is 2.4 mg/d, and of vitamin B_6 is a mean of 1.5 mg/d (American Dietetic Association & Dietitians of Canada, 2000). Supplementation studies in humans have used levels of vitamins up to 12.5 times the DRI of folate, 30 times the DRI of vitamin B_6, and up to 400 times the DRI of vitamin B_{12} (Homocysteine Lowering Trialists' Collaboration, 1998). Supplementation levels as low as 1.3 times the DRI of folate have successfully reduced plasma homocysteine concentrations (Malinow et al, 1998), as well as levels as low as 1.3 times the DRI of vitamin B_6, and 8.3 times the DRI of vitamin B_{12} (Homocysteine Lowering Trialists' Collaboration, 1998). Furthermore, Jacques et al (1999) reported significant reductions in plasma homocysteine concentrations since the inception of the folate fortification program in the United States, which provides 140μg of folate per 100g of flour product. This program supplies an additional 0.1-0.2 mg of folate per day to the average North American diet (Ray et al, 2002), which corresponds to approximately 25 to 50% of the DRI.

In the present study supplementation of 10 times the dietary levels of vitamin B_6, vitamin B_{12}, and folate did not have a significant effect on plasma homocysteine concentrations in either the pre-onset or onset stages of diabetes in BBdp rats. However, plasma homocysteine concentrations were significantly decreased with vitamin supplementation in BBn rats age-matched to the onset of diabetes compared to BBn rats at the same stage on the control diet. According to the Institute for Laboratory Animal Research (1995), the estimated requirements of pertinent vitamins for growing rats are:

1.0 mg folic acid/kg diet; 6.0 mg pyridoxine/kg diet; and 50.0 μg vitamin B_{12}/kg diet. Vitamin levels of the control diet were therefore 3 times higher than the requirements for folic acid, 1.6 times the requirements of vitamin B_6, and 0.9 times the required level of vitamin B_{12}. Supplementation in the present study therefore provided 30 times the requirements of folic acid, 17 times the requirements of pyridoxine, and 10 times the requirements of vitamin B_{12}. These vitamin levels were similar to, or higher than, the mean supplementation levels, which resulted in a reduction of plasma homocysteine concentrations in humans studies, of vitamin B_6 (11 times the DRI) and folate (6.9 times the DRI), but were lower than the mean levels of vitamin B_{12} (208 times the DRI) (Homocysteine Lowering Trialists' Collaboration, 1998). However, the above stated requirement levels of rats are only estimations as it is known that vitamin requirements of rats vary with strain, sex, age, developmental state, and whether the colony is inbred or outbred (Institute for Laboratory Animal Research, 1995).

The decrease in plasma homocysteine concentrations in supplemented BBn rats, age-matched to the onset of diabetes, is supported by many supplementation studies in healthy populations, which were able to reduce plasma homocysteine concentrations by supplementing combinations of vitamin B_6, vitamin B_{12}, and/or folate (Homocysteine Lowering Trialists' Collaboration, 1998; Malinow et al, 1998; Mansoor et al, 1999; Vermeulen et al, 2000). The lack of response of plasma homocysteine concentrations in BBd rats to vitamin supplementation at the onset of diabetes cannot be explained. As described above, insulin appears to play a role in determining plasma homocysteine concentrations, but it was expected that vitamin supplementation would further reduce the plasma concentrations of this amino acid. This finding is interesting, but cannot be

explained with the current results. Furthermore, as homocysteine-lowering supplementation studies have not been performed in diabetic populations, it is not known whether this finding is paralleled in the human species.

The lack of response to vitamin supplementation of plasma homocysteine concentrations of rats prior to the onset of diabetes is likely due to a combination of factors. The control diet alone provided the vitamins at close to, or greater than, the requirements of the rats, and it is possible that higher levels of these B-vitamins added to the diet were simply excreted as they were not needed. Plasma concentrations of each vitamin were significantly increased after supplementation, indicating that the supplementary vitamins were being absorbed. It would be advantageous to collect urinary samples for analysis of concentrations of the vitamins' metabolites in a future study. Plasma concentrations of these vitamins tend to reflect only short-term changes in intake, and do not necessarily represent the body pool of the vitamin (Basu & Dickerson, 1996). It is possible that supplementation was not done over a sufficient period of time, and that supplementation for a longer period might have resulted in lowered plasma homocysteine concentrations, as the mean length of supplementation in human studies is 6 weeks (Homocysteine Lowering Trialists' Collaboration, 1998), whereas the present study supplemented rats for approximately 1 week and 3.5 weeks in the 30 and 50 d old groups, respectively.

At 50 days of age, a decrease was seen in plasma cysteine concentrations in BBdp rats on the supplemented diet, despite no significant difference in plasma homocysteine concentrations. Plasma cysteine concentrations are strongly associated with other risk factors for CVD such as body mass index, plasma total cholesterol concentrations, and

blood pressure (El-Khairy et al, 1999), therefore a decrease in plasma cysteine concentrations may be a positive indicator of decreased CVD risk. Furthermore, cys, like homocysteine, is a sulfur amino acid, and is present in significantly higher concentrations in the plasma than homocysteine, suggesting that the increased CVD risk often attributed to homocysteine could be due to a general action common to sulfur amino acids, or that elevated plasma homocysteine concentrations may be a result of an increased plasma cysteineconcentration (El-Khairy et al, 1999). Although CVD patients have been found to have significantly higher plasma cysteine concentrations than controls (van den Brandhof et al, 2001), the strong association of plasma cysteine concentrations with other risk factors of CVD (and therefore the presence of multiple confounding factors) precludes the definition of plasma cysteine as an independent risk factor for CVD. Therefore, the implication of the observed decrease in plasma cysteine concentrations in BBdp rats at 50 days of age in the present study is unclear. Since the supplemented vitamins are not known to be involved in the metabolism of cysteine beyond the transsulfuration pathway of cysteine synthesis (El-Khairy et al, 1999), it cannot be stated that the observed decrease in plasma cysteine concentrations is due to the supplementary vitamins.

An interesting finding of the present study is the simultaneous significant elevations of plasma triglyceride and total cholesterol concentrations in supplemented diabetic and control rats at the onset of diabetes. In addition, an elevation in plasma total cholesterol concentrations was also found in supplemented 50-d old BBdp rats. The mechanisms behind these elevations are unknown. Vitamins B_6, B_{12}, and folate are not thought to be involved in the metabolism or synthesis of lipids in humans (Basu &

Dickerson, 1996), although this cannot be ruled out as a possibility in BB rats, as a supplementation study of this nature has not previously been performed in this type of animal. Homocysteine studies in humans have not reported alterations in lipid status after the supplementation of folate, vitamin B_6, or vitamin B_{12}; consequently this could be a novel finding limited to the BB strain of rats. This finding also indicates that the BB strain of rat may not be an appropriate model for vitamin or lipid research, as the control strain of the BB rat (BBn) did not respond similarly to the control (or healthy) groups in human studies, in terms of elevated lipid levels in the presence of vitamin supplementation.

4.4. *Limitations*

An obvious limitation of this study is that it was performed in rats and therefore the results cannot necessarily be extrapolated to the humans. However, due to the difficulty in predicting the onset of diabetes in humans, and in consideration of the objectives of the study, it seemed appropriate to utilize an animal model of diabetes. Furthermore, the incidence of diabetes in man is allegedly mitigated by several environmental factors (Knip & Akerblom, 1999), which can be controlled for with the use of an animal model of the disease.

Within the experimental design, limitations include the use of a single dose level of vitamin supplementation, as well as an inability to distinguish the effects of each individual vitamin. However, since a homocysteine-lowering effect of vitamin supplementation was not observed in the present study in diabetic rats, a future study examining the effects of various preparations of folate, vitamin B_{12}, and/or vitamin B_6 on

plasma homocysteine concentrations in BBd rats would be redundant. Also, the present study included only male rats; therefore the results cannot be extrapolated to female rats. However, due to the reported inverse association between homocysteine status and plasma estrogen concentrations (Dimitrova et al, 2002), female rats were excluded from the present study to eliminate any potentially confounding variables.

Furthermore, urinary concentrations of vitamins, as well as plasma methionine concentrations, would have been valuable in meeting the objectives of the study and in further characterizing homocysteine metabolism before and at the onset of diabetes in BB rats.

4.5. *Conclusions*

The present study established the presence of elevated homocysteine levels in diabetes-prone BB rats prior to the onset of diabetes, although the supplementation of vitamins B_6, B_{12}, and folate did not have a significant homocysteine-lowering effect at this stage. The presence of elevated homocysteine levels before the onset of diabetes is a novel finding, as homocysteine status has not previously been elucidated prior to the onset of diabetes in BB rats. At the onset of diabetes, elevated homocysteine levels were not found, refuting our hypothesis. However, this finding does support the research of others (Gursu et al, 2002; Jacobs et al, 1998), where positive associations between plasma homocysteine and insulin concentrations have been reported in Streptozotocin-diabetic rats. The supplementation of vitamins B_6, B_{12}, and folate had no effect on plasma homocysteine concentrations in diabetic rats; however vitamin supplementation did result

in increased plasma lipid concentrations at the onset of diabetes in both BBd and age-matched BBn, which was another novel finding of the study.

Due to the lack of significant effect of vitamin supplementation on plasma homocysteine concentrations prior to or at the onset of diabetes in BBdp rats, in combination with the observed elevations in plasma lipid concentrations in supplemented diabetic and control animals at the onset of diabetes, along with the possibility that the BB rat is an inappropriate model for vitamin or lipid research, a recommendation cannot be made at this time regarding the use of supplements of folate, vitamin B6, and vitamin B12 in the time frame leading up to the onset of diabetes. Further research is required to elucidate the mechanisms behind the increased plasma lipid concentrations observed with supplementation in the present study.

4.6. *Future Research Directions*

Future research in this area could follow many directions. An in-depth investigation into the mechanisms underlying the effect of insulin on homocysteine status in the diabetic state is warranted, with particular reference to the effect of insulin on the enzymes involved in the metabolism of homocysteine, such as CBS or MTHFR. By the same token, expansion of the current experimental design with BB rats to include a time frame post-onset of diabetes with the administration of insulin, would further address the questions regarding the association between plasma insulin and homocysteine concentrations, and would also help to more completely elucidate the status of homocysteine in BB rats. Furthermore, expanding the current study to include rats at

different ages, perhaps at shortly before or after weaning, as well as later in life, would help to define homocysteine status over the life span of the BB rat.

Another area that could be expanded in relation to the current study is the amount of time for which the animals are supplemented. Starting the animals on the supplemented diet earlier, perhaps before weaning, or even in utero (by feeding the supplemented diet to the dams), could have an impact on plasma homocysteine concentrations in BBdp rats before the onset of diabetes, as elevated homocysteine levels were present in this strain in the pre-onset of diabetes stages, although no effect of vitamin supplementation was seen on plasma homocysteine concentrations at these ages. Finally, further research is warranted to investigate the incidence of elevated lipid levels observed in BB rats with the supplementation of vitamins B_6, B_{12}, and folate. Current research has shown no association between the status of any of these vitamins and the lipid parameters (Wasilewska et al, 2003), and supplementation studies in humans have not reported hyperlipidemic side effects. Investigations in this area could focus on the dose-responses of plasma total cholesterol and triglyceride concentrations to varying levels of vitamin supplementation in BB rats, or on the incidence of this phenomenon in other animal models of type 1 diabetes, such as the non-obese diabetic (NOD) mouse or the Streptozotocin-diabetic rat.

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