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Vitamin B-6 status of middle aged women consuming soymilk versus cow's milk

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Vitamin B-6 status of middle aged women consuming soymilk versus cow's milk

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LIST OF ABBREVIATIONS

CV	coefficient of variation
DFE	dietary folate equivalent
DRI	Dietary Reference Intake
FDA	Food and Drug Administration
EAR	estimated average requirement
HPLC	high performance liquid chromatography
4-PA	4-pyridoxic acid
PL	pyridoxal
PLP	pyridoxal 5'-phosphate
PM	pyridoxamine
PMP	pyridoxamine 5'-phosphate
PN	pyridoxine
PNG	pyridoxine glucoside
PNP	pyridoxine 5'-phosphate
RDA	Recommended Dietary Allowance
SA	stimulated activity
SAM	S-adenosylmethionine
SHMT	serine hydroxymethyltransferase
UA	unstimulated activity
USDA	United States Department of Agriculture

ABSTRACT

Vitamin B-6 (B-6), an essential nutrient whose coenzyme form pyridoxal 5′-phosphate (PLP) is required by over 100 enzymes, is involved in various metabolic processes including glycogenolysis, niacin synthesis, lipid metabolism, erythrocyte function, nervous system function, hormone modulation, immune function, and one-carbon metabolism. Pyridoxine glucoside (PNG), a less bioavailable form compared with other forms of B-6, is present in plant foods and absent in animal foods. With the recognition of health effects of soy, such as its cholesterol-lowering effect, there has been an increase in soyfoods consumption. Middle aged women frequently have marginal intakes of B-6, and many drink soymilk for its purported benefit in relieving menopausal symptoms. Although the PNG content of soymilk has not been reported, PNG content of other soyfoods ranges from 57-67% of the total B-6. The effect of substituting soymilk for cow's milk on B-6 status was examined in middle aged women (aged 36-52 y; $n = 16$). The study employed a crossover design: a 14-d adjustment period followed by a 28-d experimental period, during which half the subjects consumed 4 cups of cow's milk and half consumed 4 cups of soymilk per day; a 14-d washout period and a second 28-d experimental period when subjects switched to the other milk. Participants followed a self-selected B-6 restricted diet (~1 mg/d) throughout the study by following a list of prohibited or restricted foods. Three consecutive 24-h urine samples were collected and fasting blood was drawn every two weeks. Methionine loads (0.1 g/kg body weight) were given at the end of each experimental period. Plasma PLP, 4-pyridoxic acid (4-PA), urinary 4-PA, and pre- and postload plasma homocysteine were determined by HPLC analysis. Using crossover ANOVA tests, mean plasma 4-PA ($P = 0.001$), and urinary 4-PA-to-creatinine ratio ($P < 0.001$) were significantly lower when soymilk was consumed. There was no evidence that the mean plasma PLP ($P = 0.107$) and the increase in mean plasma total homocysteine concentrations after a methionine load ($P = 0.316$) were different between the cow's milk and soymilk treatments. We may have reported significant effects during the soymilk versus cow's milk treatment if the study period was longer (>28 d), the diets more rigidly controlled, and our sample size was larger (n >16) regarding these two measures. These results suggest that substituting soymilk for cow's milk has an adverse

effect on B-6 status in middle aged women as reflected by the reduced status indicators after soymilk treatment.

CHAPTER 1. INTRODUCTION

Thesis Organization

The content of this thesis is organized into several chapters. The first chapter includes the organization of the thesis and an introduction to the overall research questions addressed. Chapter 2 is a review of the literature including information that is relevant to the research reported here concerning vitamin B-6 metabolism, bioavailability, and status assessment. Chapters 3 and 4 contain the study regarding the effect of substituting soymilk for cow's milk on B-6 status in middle aged women (aged 36-52 y; $n = 16$) including methods, results, and discussion. Summary and conclusions constitute Chapter 5, followed by an Appendix that provides raw data tables and forms, as well as the complete list of the literature cited throughout this thesis.

Description of the Research Questions

Vitamin B-6 (B-6) via its coenzyme form pyridoxal 5′-phosphate (PLP) plays an important role in various metabolic processes. Pyridoxine glucoside (PNG), the less bioavailable form compared with other B-6 forms, has been found to comprise up to 80% of B-6 in plant foods and is absent in animal foods (Kabir H et al. 1983; Gregory and Ink 1987). It has been shown that PNG is not interconvertible with other B-6 vitamers until its glucosidic bond is cleaved (Gregory 1997). Because approximately half of the dietary vitamin B-6 intake of Americans is from plant sources (Kant and Block 1990), studies addressing PNG bioavailability in vivo have drawn more attention. Human feeding studies comparing B-6 bioavailability in foods with their relative PNG content indicated an inverse relationship between PNG content and bioavailability (Kabir et al. 1983). Hansen et al. (1996b) reported that in a controlled study, the bioavailability of PNG is incomplete and of sufficient magnitude to affect nutritional status. Moreover, apart from its partial bioavailability, PNG was found weakly antagonistic to the utilization of nonglycosylated forms of the vitamin in mammals (Gregory 1998).

With the recognition of the health effects of soy, such as a health claim approved by the Food and Drug Administration (FDA) in 1999 that soy protein reduces the risk of

coronary heart disease by lowering blood cholesterol, there has been an increase in soyfoods consumption (Messina et al. 2002). However, there is limited information about the forms of vitamin B-6 in commercially available soyfoods and soy protein products that have not been fortified with vitamin B-6. The effect of substituting unfortified soy products for animal products on vitamin B-6 status has not been investigated.

It has been found that middle age women (ages 35-55 y) frequently have marginal intakes of vitamin B-6 (Institute of Medicine 1998), and many drink soymilk for its purported beneficial effect in alleviating menopausal symptoms (Kronenberg and Fugh-Berman 2002). There has been limited information regarding the PNG content in soymilk. Moreover, a study in rats showed apart from the effect of PNG content in soy, replacing animal protein with soy protein might have an additional adverse effect on B-6 status (Lu and Huang 1997). Based on the aforementioned evidence, the goal of our study was to determine the effect of substituting soymilk for cow's milk on vitamin B-6 status in middle aged women.

CHAPTER 2. LITERATURE REVIEW AND RATIONALE

Introduction

Vitamin B-6, a water-soluble essential nutrient, is composed of a group of six interconvertable compounds (B-6 vitamers) related chemically, metabolically, and functionally. All six B-6 vitamers are derivatives of 3-hydroxy-5-hydroxymethyl-2-methyl pyridine that can mimic the biological activity of pyridoxine. The three naturally occurring compounds of vitamin B-6 are pyridoxine (PN), pyridoxal (PL), and pyridoxamine (PM), and their respective phosphorylated derivatives are pyridoxine 5'-phosphate (PNP), pyridoxal 5'-phosphate (PLP), and pyridoxamine 5'-phosphate (PMP) (Leklem 1999).

In 1934, Paul Gyorgy first used the term "vitamin B-6" to designate the substance in crude feed supplement that later on proved to be able to heal a florid dermatitis that occurred in rats fed a purified diet supplemented with vitamins B-1 and B-2; subsequently, he named the substance pyridoxine (Esmond 1981; Holman 1995). Several groups in 1938 isolated vitamin B-6 in crystalline form identified as 3-hydroxy-5-hydroxymethyl-2-methyl pyridine (Esmond 1981; Holman 1995; Leklem 1999).

Our understanding of the various roles vitamin B-6 plays has been advanced significantly since its discovery and identification of its structure some 70 years ago. Vitamin B-6 is essential in the metabolism of amino acid metabolism: it is involved in transamination, decarboxylation, and a variety of elimination and replacement reactions (Bender 1992). Vitamin B-6 may have a potential role in the treatment of various chronic and genetic diseases including cancer, asthma, Down's syndrome, sickle cell anemia, and coronary heart disease (Leklem 2001). The major coenzyme form, PLP, is required by over 100 enzymes in diverse metabolic processes including glycogenolysis, niacin synthesis, lipid metabolism, erythrocyte function, nervous system function, hormone modulation, immune function, and one-carbon metabolism (Leklem 1999). The major forms of vitamin B-6 present in animal products are PLP and PMP, which are active coenzyme forms, with PLP being the primary form of biologic interest. In plant-derived foods, the principal forms of vitamin B-6 are PN, PNP, and pyridoxine-ß-D-glucoside (PNG) (Leklem 1999). Moreover, PNG, the conjugated form of vitamin B-6, is virtually absent in animal products (Leklem 1999). The catabolic end product of vitamin B-6 in the liver is 4-pyridoxic acid (4-PA), which excreted in the urine

(Leklem 2001). Urinary 4-PA excretion accounts for 40-60% of daily vitamin B-6 intake in individuals consuming 1-5 mg of the vitamin daily (Leklem 2001). Under normal dietary conditions, the predominant form of vitamin B-6 in human plasma is PLP, which makes up 50-75% of the total, and PL comprises approximately 8-30% of the total, followed by lower concentrations of PN, PMP, and PM (Leklem 2001). Pyridoxine 5'-phosphate is very low or essentially absent in plasma, considering a usual intake of vitamin B-6 (Lekem 2001). Pyridoxine hydrochloride (PN-HCl), a water soluble form, is used in commercial vitamin supplements and fortified foods. Vitamin B-6 is heat-stable in acid but heat-labile in alkaline or neutral solution; it is light-sensitive in solution (Leklem 1999). Muscle serves as a vitamin B-6 reservoir in which a majority of the vitamin is present as PLP bound to glycogen phosphorylase (Coburn et al. 1988).

Absorption, Transport, and Metabolism

The intestinal absorption of nonphosphorylated forms of vitamin B-6 (PL, PM, and PN), primarily occurs in the jejunum to a major extent by a nonsaturable passive process (Leklem 1999). The three phosphorylated B-6 vitamers (PLP, PMP, and PNP) are initially hydrolyzed by alkaline phosphatase in the intestinal mucosa to their respective dephosphorylated forms prior to absorption across the intestine (Bender 1992). When vitamin B-6 intake is high, some intact absorption of the phosphorylated forms does occur, but to a very limited extent (Lekem 2001). Pyridoxine glucoside, however, is absorbed less effectively than are PLP and PMP (Nakano and Gregory 1997). Pyridoxine glucoside needs to be deconjugated by a mucosal glucosidase, releasing metabolically active PN to be passively absorbed by tissues (Gregory et al. 1991; Nakano and Gregory 1995).

The liver is the primary organ responsible for the metabolism of vitamin B-6 and supplies the active form, PLP, to the circulation and other tissue (Lekem 2001). The absorbed forms of vitamin B-6 which are taken up by the liver are primarily the nonphosphorylated forms. Pyridoxine, PL, and PM undergo phosphorylation by attaching a phosphate group at the 5′ position by PN kinase, with zinc and ATP as cofactors (Leklem 1999). Alkaline phosphatase is responsible for the dephosphorylation of PNP, PLP, and PMP by removal of the 5′-phosphate group via hydrolysis (Bender 1992). Primarily the nonphosphorylated forms

are the vitamers that can be transported from one compartment to the other (Institute of Medicine 1998). The two phosphorylated forms, PNP and PMP, are converted to PLP by a flavin mononucleotide (FMN)-dependent PNP/PMP oxidase. The PL, resulting either from dephosphorylation or derived from dietary sources, can be irreversibly oxidized to 4-PA, the primary metabolic end-product, accounting for 40 to 60% of normal daily vitamin B-6 intake, by a flavin adenine dinucleotide (FAD)-requiring aldehyde oxidase or a FAD-dependent dehydrogenase (Bender 1992; Leklem 1991). The elimination of PL to 4-PA is of importance in the overall metabolism of vitamin B-6, serving as the primary route for the catabolism of B-6 vitamers (Leklem 2001). This irreversible reaction, the conversion of PL to 4-PA, plays the important role in metabolizing excess intake of vitamin B-6, which is excreted through the kidneys and thus prevents the accumulation of high concentrations of the reactive PLP form (Leklem 1988a; Leklem 1999). Urinary 4-PA is the main urinary metabolite of vitamin B-6, while there are small amounts of all six B-6 vitamers excreted in the urine (Leklem 1988b).

The metabolically active PLP that formed in the liver is either utilized directly or released into the circulation as a PLP-albumin complex (Bender 1992). In plasma, PLP is the primary form of vitamin B-6, predominantly as a PLP-albumin complex (Fonda et al. 1991). Plasma PLP in the form of a PLP-albumin complex protects it from hydrolysis by phosphatases and allows for the delivery of PLP to other tissues (Lekem 2001).

Functions

Pyridoxal 5'-phosphate, the primary coenzyme form of vitamin B-6, is required by over 100 enzymes including aminotransferases, decarboxylases, racemases and enzymes involved in side-chain elimination and replacement reactions. Of the over 100 enzymatic reactions in which PLP is involved, nearly half are aminotransferase-type reactions in which PLP reacts with ε-amino groups of lysine residues in proteins, forming a Schiff base with amino groups of amino acids so as to stabilize amino acid carbanions and hence to weaken bonds about the α-carbon of the substrate (Bender 1992). The cellular processes in which PLP is involved include amino acid metabolism, immune function, gluconeogenesis, niacin

formation, erythrocyte metabolism and function, lipid metabolism, neurotransmitter synthesis, and hormone modulation (Bender 1992; Leklem 2001).

Gluconeogenesis

Gluconeogenesis plays a key role in maintaining adequate concentrations of glucose during energy deficit (Leklem 2001). Pyridoxal phosphate is involved in gluconeogenesis via its coenzyme role in aminotransferase reactions and in the action of glycogen phosphorylase (Leklem 1999). In a rat study conducted by Angel (1980), results indicated decreased activities of hepatic alanine and aspartate aminotransferase in rats fed vitamin B-6 deficient diets for eight weeks. Glycogen phosphorylase, a key enzyme in the utilization of tissue glycogen reserve, catalyzes the sequential phosphorylysis of glycogen to release glucose-1-phospate, employing PLP as its coenzyme (Bender 1992). The phosphate group of PLP is the reactive moiety in the case of glycogen phosphorylase (Bender 1992). Animal studies have showed that glycogen phosphorylase activity of muscle increases as the intake of vitamin B-6 increases (Black et al. 1977).

Niacin formation, lipid metabolism, and erythrocyte metabolism and function

The direct conversion of tryptophan to niacin requires a PLP-dependent enzyme, kynureninase (Leklem 1999). Kynureninase catalyzes the conversion of 3-hydroxyhynurenine to 3-hydroxyanthranilic acid in niacin formation from tryptophan. Leklem et al. (1975) investigated the effect of vitamin B-6 deficiency on the conversion of trypotophan to niacin. The investigators found that there was evidence that low vitamin B-6 had a moderate negative effect on the conversion of tryptophan to niacin in women consuming a diet containing 0.2 mg of vitamin B-6 daily for 4 weeks. This was indicated by the significantly lower response to a 2 g tryptophan load test of the total urinary excretion of the two major niacin metabolites N-methyl-2-pyridone-5-carboxamide and N′-methylnicotinamide compared with women who consumed 1.8 mg of vitamin B-6 daily.

The role vitamin B-6 plays in lipid metabolism remains controversial. Abe and Kishino (1982) reported that vitamin B-6-deficient rats fed high-protein diets developed fatty livers, whereas others did not (Abe and Kishino 1982; Leklem 1999). The findings from Abe and Kishino suggested that the resultant fatty liver in B-6-deficient rats was due to impaired lysosomal degradation of lipid (Abe and Kishino 1982). Nevertheless, Bender (1992) states

that PLP has a clear role in lipid metabolism as the coenzyme for phosphatidylserine decarboxylation, leading to the formation of phosphatidylethanolamine, an intermediate in the synthesis of choline and phosphatidylcholine. Observations in a rat study conducted by Cunnane and coworkers (1984) suggested that both linoleic desaturation and γ-linoleic acid elongation may be impaired when vitamin B-6 is deficient (Leklem 2001).

Vitamin B-6 is essential in maintaining normal erythrocyte function and metabolism. The synthesis of heme, a component of hemoglobin, requires PLP. Pyridoxal phosphate functions as the coenzyme for δ-aminolevulinic acid synthase involved in the conversion of succinyl CoA and glycine to δ-aminolevulinic acid, the first and rate-limiting step in heme synthesis. Hemoglobin, a protein in erythrocyte, is essential to transport oxygen throughout the body. Apart from PLP as the coenzyme for aminotransferases in erythrocytes, vitamin B-6 via binding of both PLP and PL to hemoglobin plays a key role in maintaining a normal physiological state of the erythrocyte (Leklem 1999). Upon binding of PL to the α chain of hemoglobin, its oxygen binding affinity increased, whereas the binding of PLP to the β chain of the hemoglobin S or A lowers the oxygen binding affinity (Leklem 2001).

Nervous system, immune function, and hormone modulation

Pyridoxal phosphate acts as a coenzyme for decarboxylation reactions that lead to the synthesis of several neurotransmitters, including serotonin, taurine, dopamine, norepinephrine, histamine, and γ-aminobutyric acid (Leklem 1999). Apart from the effect of vitamin B-6 on the direct conversion of tryptophan to niacin, there is another tryptophan pathway that requires a PLP-dependent enzyme, 5-hydroxytryptophan decarboxylase, converting 5-hydroxytryptophan to 5-hydroxytryptamine (Leklem 2001).

A low intake of vitamin B-6 or decreased status is associated with impaired immune function in both animal (Robson and Schwarz 1975) and human studies (Meydani et al. 1991). In humans, the percentage and total number of lymphocytes, mitogenic responses of peripheral blood lymphocytes to T- and B-cell mitogens, and interleukin-2 production were significantly decreased during vitamin B-6 depeletion in healthy elderly adults (Meydani et al. 1991). This impairment is reversible by vitamin B-6 repletion, suggesting that the indices of cell-mediated immunity may be mediated via one-carbon metabolism, particularly the activity of serine hydroxymethyltransferase (SHMT) and/or hormone modulation

(Trakatellis et al.1992). The PLP-requiring enzyme, SHMT, is one of the key enzymes involved in one-carbon metabolism and the resultant reduced SHMT activity due to B-6 inadequacy or deficiency might adversely alter one-carbon metabolism, leading to impaired changes in nucleic acid synthesis (Leklem 2001).

Pyridoxal phosphate modulates steroid hormone receptors by binding to their receptor, thus inhibiting the binding of the steroid receptor to DNA, resulting in a decreased biological response for a given level of steroid. The Schiff base formed in the reactions of PLP with a lysine residue on the steroid receptor results in the inhibition of the binding of the steroid receptor complex to DNA (Leklem 2001). Pyridoxal phosphate reacts with receptors for estrogen, androgen, progesterone, and glucocorticoids in a reversible manner under conditions of physiological concentrations of PLP. This suggests that the vitamin B-6 status of an individual may have implications for diseases affected by steroid hormones, such as prostate and breast cancers (Leklem 2001).

Amino acid metabolism

Pyridoxal phosphate is involved in amino acid metabolism via reactions with the α-amino group of the substrate. The PLP-requiring reactions include (Leklem 2001): (1) decarboxylation of amino acids to yield amines involving the α carbon of PLP, which are hormones or neurotransmitters, such as serotonin, γ-aminobutyric acid, and noradrenaline (and hence adrenaline); (2) transamination of amino acids to yield their keto-acids (oxo-acids) involving the α carbon of PLP, which are then oxidized as metabolic fuels; (3) reactions involving the side-chains of amino acids, including cystathionine β-synthase and cystathionase in replacement reactions occurring at the β and γ carbons of PLP, respectively, and kynureninase in cleavage reactions occurring at γ carbon of PLP; and (4) decarboxlation of phosphatidylserine to phosphatidyl-ethanolamine in phospholipid synthesis occurring at the α carbon of PLP.

Food Sources and Bioavailability

Vitamin B-6, as an essential nutrient, is not synthesized in the body but obtained from dietary sources. The richest sources of vitamin B-6 include chicken, fish, beef liver and other organ meats, pork, eggs, and yeast, providing more than 0.4 mg/100g serving. Other good

sources include soybeans, oats, peanuts, walnuts, unmilled rice, and whole wheat products; vegetables and cereals (not corn) are intermediate sources. Dairy products, red meat, and fruit (except bananas), are relatively poor sources of vitamin B-6 (NRC 1989). The greatest contribution to vitamin B-6 intake of the U.S. adult population is from fortified, ready-to-eat cereals, followed by mixed foods with meat, fish, or poultry as the main ingredient, white potatoes and other starchy vegetables, and noncitrus fruits (Institute of Medicine 1998).

Nutrient bioavailability from a given food is defined as the amount of a nutrient that is both absorbed and available to cells. When the vitamin is described as "available", it is understood that the vitamin may not be needed by the cell and simply excreted or metabolized to a nonutilizable form, such as urinary 4-PA excretion in the case of vitamin B-6 (Leklem 2001).Vitamin B-6 bioavailability is defined as the proportion of dietary B-6 vitamers that are absorbed and utilized in B-6 metabolism. The evaluation of the bioavailability of vitamin B-6 depends on our understanding of the various forms and the quantities of these B-6 vitamers in foods. As aforementioned, the major forms in animal foods are PLP and PMP, whereas PN and PNP, sometimes in the form of a glucoside or PNG, are primary forms of the vitamin in plant-derived foods. The bioavailability of vitamin B-6 in a mixed diet is considered about 75 percent, with approximately 8 percent of the total contributed by PNG. Pyridoxine glucoside is estimated at 50 to 58 percent of the bioavailability of PN as based on dose studies using deuterium-labeled PNG. The six interconvertable nonglucoside forms of vitamers (PN, PL, PM, PNP, PLP, and PMP) show greater than 75 percent bioavailability (Gregory 1997). Pyridoxine-ß-D-glucoside (PNG), a major naturally occurring form of vitamin B-6 in plant-derived food, is a glycosylated form of vitamin B-6 that is poorly digested and virtually absent in foods of animal origin, but identified to constitute 5-80% of the total vitamin B-6 in plant foods (Gregory and Sartain 1991).

Kabir et al. (1983) conducted controlled-feeding studies in which the relative bioavailability of vitamin B-6 was examined from tuna, whole wheat bread, and peanut butter in eight men with 1.6 mg vitamin B-6 intake daily. Their findings indicate that the percentage of PNG in these foods was inversely correlated with vitamin B-6 bioavailability as based on urinary vitamin B-6 and 4-PA. Leklem et al. (2001) observed an inverse

relationship between vitamin B-6 bioavailability and the glycosylated vitamin B-6 content of six foods, indicating that the glycosylated vitamin B-6 content of foods appears to significantly affect bioavailability. The lower vitamin B-6 bioavailability in plant foods compared with animal products is possibly partially due to the inadequate cleavage of the glucoside moiety of pyridoxine from PNG. Approximately half of the dietary vitamin B-6 intake of Americans is from plant sources (Kant and Block 1990). Thus, studies addressing PNG bioavailability in vivo have drawn more and more attention. Studies with various designs have shown us the diverse aspects of PNG bioavailability.

To determine whether the bioavailability of vitamin B-6 is lower in diets in which vitamin B-6 is mostly from plant sources, comparisons between free-living vegetarian and nonvegetarian populations with self-selected diets showed that diet type (vegetarian or nonvegetarian) did not result in significantly different daily vitamin B-6 intakes. There were no significant differences in plasma or erythrocyte status indicators (Shultz and Leklem 1988; Lowik 1990). To investigate the extent to which PNG bioavailability affects vitamin B-6 status, Hansen et al. (1996b) conducted a controlled study in which young women were fed either low (9%) or moderately high (27%) percentages of glycosylated vitamin B-6 diets. The higher intake of PNG caused a decrease in vitamin B-6 status as indicated by plasma and erythrocyte status indicators. These findings indicate that, in a controlled study, the bioavailability of PNG is incomplete and of sufficient magnitude to affect nutritional status (Hansen et al. 1996b; Gregory 1998). To examine whether the intact glucoside has antagonistic effects on various aspects of vitamin B-6 metabolism, in vivo studies with rats were conducted (Gilbert and Gregory 1992; Nakano and Gregory 1995). Findings indicated that, as reflected by increased urinary excretion and a small accumulation of hepatic PNG, the consumption of the PNG in the presence of PN or alone caused in vivo alteration of vitamin B-6 metabolism (Gregory 1998). The adverse effect of PNG on the utilization of other forms of the vitamin complicates the assessment of the overall bioavailability of total dietary vitamin B-6. Pyridoxine-ß-D-glucosides have great nutritional significance due to their widespread natural occurrence in plants, their role as a partially bioavailable form of vitamin B-6 in human nutrition, and the weak antagonism of the utilization of nonglycosylated forms of the vitamin in mammals (Gregory 1998).

Apart from the effect of PNG content on vitamin B-6 bioavailability, fiber intake appears to be a contributor to bioavailability (Lindberg et al. 1983; Hansen 1996b). Lindberg et al. (1983) have shown that the addition of 6.4 g dietary fiber daily as 15 g of wheat bran to the diet decreased vitamin B-6 bioavailability modestly by up to 17% based on urinary 4-PA and total vitamin B-6. Hansen et al. (1996b) reported that the resultant reduced vitamin B-6 bioavailability of diets that are high in PNG are also high in dietary fiber. This may have an important effect on vitamin B-6 status for people with marginal vitamin B-6 intakes or those consuming diets in which most of the vitamin B-6 intake is from plant foods.

Status Assessment

There are three ways of assessing vitamin B-6 status: direct methods, indirect methods and dietary intake. Direct methods measure the concentration of the B-6 vitamers or the metabolite 4-PA in various biological fluids and erythrocytes. These include measurements of plasma PLP, PL, plasma total vitamin B-6, erythrocyte vitamin B-6 metabolites, urinary 4-PA and urinary total vitamin B-6. The indirect or functional method assesses vitamin B-6 status based on measurement of products in metabolic pathways or enzyme activities that require PLP as a conenzyme. These include measurement of urinary metabolite excretion in response to an oral methionine or typtophan load and measurement of the PLP-dependent enzyme erythrocyte alanine or aspartate aminotransferase activities with and without stimulation by the coenzyme PLP (Leklem 1999; Ink and Henderson 1984). Most investigators suggest using several measures, including both direct and indirect, in addition to an evaluation of dietary intake of both vitamin B-6 and protein (Leklem 1999; Hansen et al. 1997). Protein intake should be taken into consideration because it has been observed that greater intake is associated with reduced plasma PLP concentration and urinary 4-PA excretion (Miller et al. 1985; Hansen et al. 1996a). **Table 2.1** (p.12) lists suggested values for different vitamin B-6 measures indicating adequate status (Leklem 1990).

Direct methods

Plasma vitamin B-6 and its metabolites: Currently, the quantification of fasting plasma PLP concentration is the most frequently used direct method to assess vitamin B-6

Table 2.1. Indices for evaluating vitamin B-6 status and suggested values for adequate status in adults (Leklem 1990).

Index	Suggested value for adequate status
Direct	
Blood	
Plasma pyridoxal 5'-phosphate	> 30 nmol/L
Plasma pyridoxal	[a]Not Available
Plasma total vitamin B-6	> 40 nmol/L
Erythrocyte pyridoxal 5'-phosphate	[a]Not Available
Urine	
4-Pyridoxic acid	> 3.0 μmol/d
Total vitamin B-6	> 0.5 μmol/d
Indirect	
Blood	
Erythrocyte alanine aminotransferase index	< 1.25
Erythrocyte aspartate aminotransferase index	< 1.80
Urine	
2-g tryptophan load; xanthurenic acid excretion	< 65 μmol/d
3-g methionine load; cystathionine	< 350 μmol/d
Dietary Intake	
Vitamin B-6 intake	> 1.2-1.5 mg/d
Vitamin B-6/protein ratio	> 0.020 mg/g
Other	
Electroencephalogram patterns	[a]Not Available

[a]Not Available, adequate value has not been determined

status. Plasma PLP, the primary circulating form of vitamin B-6, comprises approximately 50% to 75% of the total vitamin B-6 in plasma (Leklem 1999). Based on rat studies, plasma PLP concentration correlates well with tissue vitamin B-6, which suggests that it is reflective of tissue stores, thus considered an appropriate index of vitamin B-6 status (Lumeng et al. 1978). Human metabolic studies have shown that when intake is in the range of 0.5 to 1.0 mg/day, plasma PLP concentration changes in response to change in dietary vitamin B-6 intake, taking about 10 days to reach a new steady state (Lui et al. 1985; Leklem 1999). Plasma PLP concentration increases with an increase in dietary vitamin B-6 intake and its concentration falls in response to increased protein intake (Leklem 1999). A cutoff for PLP in plasma of 20 nM was selected by the Dietary Reference Intake (DRI) committee as the basis for the estimated average requirement (EAR) for vitamin B-6, in the absence of evidence linking a particular concentration to favorable or unfavorable health outcomes (Institute of Medicine 1998). However, other investigators suggested a higher concentration of 30 nM as the lower end of normal for plasma PLP based on human metabolic studies (Leklem 1990; Hansen et al. 1996a). The use of plasma PLP concentration, a single measurement, is limited because several factors influence it. Asthma, coronary heart disease, or pregnancy may result in an abnormally low concentration of plasma PLP (Leklem 1999). Thus, the use of several indicators in conjunction with each other has been suggested for evaluating vitamin B-6 status (Leklem and Robert 1981).

Plasma PL, comprising about 8% to 30% of the total plasma vitamin B-6, is the second most abundant form in plasma. Its measurement may be more relevant than that of PLP since it is a form that crosses all membranes and enters the cell (Leklem 1999). However, analysis is technically difficult because its concentration indicating adequate status has not been established. Thus, plasma PL is only recommended as one of the direct vitamin B-6 status measures (Leklem 1999). Additional research is necessary to determine the extent to which plasma PL is a valid vitamin B-6 status indicator.

Erythrocyte PLP: Erythrocyte PLP concentration has been suggested as an additional index to assess vitamin B-6 status. However, it may be unrepresentative of other body tissues due to particular characteristics of the erythrocyte, such as the tight binding ability of hemoglobin to both PLP and PL, together with the relatively long life (~120 d) of the

erythrocyte, complicating the usefulness of erythrocyte PLP concentration as an indicator of vitamin B-6 status (Leklem 1999). Erythrocyte PLP concentration increases to a greater extent than plasma PLP when subjects consume large doses of vitamin B-6 (Bhagavan et al. 1975). The use of blood total vitamin B-6 concentration and the individual concentration of specific B-6 vitamers, such as plasma PL and erythrocyte PLP as status indicators, is limited due to the fact that they tend to fluctuate considerably (Contractor and Shane 1968).

Urinary 4-PA excretion: Urinary 4-PA excretion and urinary total vitamin B-6 (the sum of the nonphosphorylated and phosphorylated forms) have been used extensively to evaluate vitamin B-6 requirements (Institute of Medicine 1998). However, the urinary levels of vitamin B-6 are not useful long-term status indicators because they fluctuate considerably, reflecting recent vitamin B-6 intakes. Urinary 4-PA, the metabolic end-product of vitamin B-6, is considered as a short-term status indicator and represents 40% to 60% of the daily vitamin B-6 intake under normal conditions (Leklem 1999). Its excretion changes rapidly in response to change in vitamin B-6 intake over a 1- to 4-day period and the fluctuation in urinary 4-PA parallel that of plasma PLP in response to change in vitamin B-6 intake. Thus, urinary 4-PA level reflects recent dietary intake (Leklem 1999). A value of greater than 3 μmol/day has been suggested as indicative of adequate status (Leklem 1990). To measure urinary vitamin B-6, several 24-h urine collections during 1 to 3 weeks are required (Leklem 1999). Several dietary and physiologic factors have been shown to affect urinary measurements. For instance, an increase in protein intake results in a decrease in urinary 4-PA excretion. Also, use of vitamin B-6 antagonists, such as the drugs isoniazid, penicillamine, or cycloserine, and injection of oral contraceptives appear to affect this measurement (Leklem 1999). Urinary 4-PA is a valuable vitamin B-6 status indicator if dietary vitamin B-6 intake is controlled.

Indirect methods

Trypotophan load test: A trypotophan load test, based on the requirement for PLP in the major catabolic pathway of trypotophan, has been the most widely used indirect indicator of vitamin B-6 status (Leklem 1999). Greenberg et al. (1949) conducted a vitamin B-6 restrictive diet study in man, which is known as the first investigation to observe an increased excretion of xanthurenic acid, a colored compound, after a load of trypotophan. In the

trypotophan metabolic pathway, under normal conditions, the enzyme tryptophan oxygenase is the rate limiting step. However, when vitamin B-6 deficiency occurs, the activity of the PLP-dependent enzyme kynureninase falls below that of tryptophan oxygenase and results in the accumulation of intermediates and an increased urinary excretion of kynurenic and xanthurenic acid (Holman 1995). For this measure, urinary xanthurenic acid excretion is measured after a 2-g oral test load of L-trypotophan (Leklem 1999). A 24-h urinary excretion of less than 65 μmol post-load xanthurenic acid is considered indicative of normal vitamin B-6 status (Leklem 1999). In subjects with adequate vitamin B-6 status, a urinary excretion of xanthurenic acid usually ranges from 30 to 40 μmol/day in response to the 2-g of tryptophan (Leklem 1999). The reliability of the tryptophan load test has been questioned due to an adverse effect on trypotophan metabolism by several factors other than vitamin B-6 status. These factors include protein intake, exercise, lean body mass, individual variation, loading dose of tryptophan, use of estrogen and oral contraceptives, and pregnancy (Bender 1992; Leklem 1999). Essentially, tryptophan oxygenase activity varies with several factors, such as substrate availability, hormonal induction, feedback by NAD and NADP, and availability of the heme cofactors (Holman 1995). In addition, diseases such as Hodgkin's lymphoma, rheumatoid arthritis, schizophrenia, porphyria, renal tuberculosis, and aplastic anemia have been found to affect the tryptophan load test independently of vitamin B-6 status (Holman 1995). Despite all of these disadvantages of the tryptophan load test, it is regarded as a valid indicator of vitamin B-6 status when conditions that adversely affect test outcome are absent (Leklem 1999).

Methionine load test: The methionine load test, like the tryptophan load test, primarily reflects hepatic vitamin B-6 status and has also been used as an indirect measure of vitamin B-6 status, based on the four PLP-dependent steps in methionine metabolism (Leklem 1999). The step in the methionine pathway that is catalyzed by cystathionase, in which cystathionine is cleaved to form homoserine and cysteine, appears to be especially sensitive to vitamin B-6 deficiency. This is reflected by elevated urine excretions of the metabolites cystathinonine and cysteine sulfonic acid following a methionine load (Leklem 1999). Cystathionase activity falls in vitamin B-6 deficiency, as evidenced by an increase in the tissue content of the inactive apo-enzyme (Bender 1992). Linkswiler (1981) indicated

that there is an increase in the urinary excretion of cystathionine, both after a loading dose of methionine and under basal conditions, and the metabolism does not appear to be subject to the same artifacts as does the tryptophan load test (Bender 1992). Thus, the methionine load test is considered a useful test of vitamin B-6 status and good agreement between impairment of methionine metabolism and other indices of vitamin B-6 status has been established (Bender 1992). However, because the methionine load test has been used in a limited number of studies, the cutoff value for urinary cystathionine of less than 350 μmol/day is based on three studies and no definitive values are available for cysteine sulfonic acid (Leklem 1999).

Increase in plasma homocysteine concentration after a methionine load: More recently, an increase in plasma homocysteine after a methionine load has been observed in vitamin B-6 deficiency, providing a potential indirect or functional indicator of vitamin B-6 status (Leklem 1999). There are two PLP-dependent enzymes involved in homocysteine catabolism via transsulfuration to cysteine. Homocysteine can also be remethylated to methionine via folate and vitamin B-12-dependent enzyme (Institute of Medicine 1998). Thus, vitamin B-6, folate, and to a lesser extent vitamin B-12 intakes, are considered to be factors affecting plasma homocysteine concentration (Selhub et al. 1993). With the increased interest in the relationship between plasma homocysteine and risk of coronary heart disease, there have been several studies in which the effect of vitamin B-6 on plasma homcysteine has been examined (van den Berg et al. 1994; Ubbink et al. 1994). van den Berg et al. (1994) found that simple and inexpensive therapy with vitamin B-6 plus folic acid for at least six weeks normalized homocysteine metabolism in 72 patients (aged < 50 y) with cardiovascular disease and mild hyperhomocysteinemia (defined as an increase in the plasma homocysteine concentration after methionine loading to greater than 97.5 percentile of age-matched control subjects but less than 200 μmol/L; van den Berg et al. 1994). A study conducted by Miller et al. (1994) examined responses to methionine loading in vitamin B-6 deficient vs. folate deficient rats. They reported that rats fed vitamin B-6 deficient diets for 4 weeks and given a gastric gavage of methionine (100 mg/kg body wt) exhibited a peak post-methionine load increase (> 300 μmol/L) in plasma homocysteine concentration. In contrast, folate-deficient rats exhibited no significant changes in plasma homocysteine concentration after the load. The investigators explained this phenomenon by the observed increase in hepatic

S-adenosylmethionine (SAM) concentration because of the load. They pointed out that when vitamin B-6 is deficient under conditions of a methionine load, significant increases in the syntheses of both SAM and homocysteine occur. The homocysteinemia was resulted from the following three factors: vitamin B-6 deficiency or enzyme defect led to the reduction of transsulfuration; the increased homocysteine because of the methionine load must be metabolized; and increased SAM inhibits the methylenetetrahydrofolate reductase, thus impairs homocysteine remethylation. The impaired homocysteine remethylation in conjunction with the impaired homocysteine catabolism due to vitamin B-6 deficiency and the increased synthesis of homocysteine due to the methionine load, led to the large elevation of homocysteine in the blood. However, when folate deficiency occurs under conditions of a methionine load, there are again increases in the syntheses of both SAM and homocysteine. Although the increased homocysteine synthesis would be expected to raise plasma homocysteine concentrations, the raise in tissue SAM will activate cystathionine β-synthase, the enzyme that initiates homocysteine catabolism. The increased cystathionine β-synthase induces an acceleration of homocysteine catabolism, which compensates for the increased synthesis of homocysteine due to the load and thus no change in blood homocysteine was observed. The observed increased plasma homocysteine concentration after a methionine load in the face of vitamin B-6 deficiency, therefore, provides a potential indirect indicator of vitamin B-6 status.

Erythrocyte aspartate aminotransferase and alanine aminotransferase: Measurement of erythrocyte aspartate aminotransferase and erythrocyte alanine aminotransferase activity with and without PLP stimulation has been frequently used to evaluate long-term vitamin B-6 status due to the life span of erythrocytes (Institute of Medicine 1998; Leklem 1999). Two measurements are used in the test: the unstimulated, or basal enzyme activity as removed from the subject, and the stimulated enzyme activity after the in vitro addition of excess PLP (Leklem 1999). Two additional values are derived from these data. One is the stimulation index as calculated by the ratio of stimulated activity to unstimulated activity (Leklem 1999). The other is the percent stimulation which is calculated from the following formula

(Leklem 1999): Percent stimulation = $\frac{(SA - UA) \times 100}{UA}$

where SA presents stimulated activity and UA presents unstimulated activity (Leklem 1999).

Human studies provide evidence that activity of these two enzymes is decreased in individuals whose vitamin B-6 reserves have been depleted, whereas activity is increased after the in vitro addition of excess PLP (Leklem 1999). The usefulness of aminotransferase activity measures as an index of a specific vitamin B-6 intake level is limited because the concentration of these enzymes fluctuates in the blood as a result of tissue damage and turnover (Holman 1995). In individuals with apparently adequate vitamin B-6 status, there is considerable interindividual variation in the erythrocyte aminotransferase activity with or without stimulation by added PLP, shown to vary by as much as 25% and 50% for erythrocyte alanine aminotransferase and erythrocyte aspartate aminotransferase, respectively (Leklem 1990). Moreover, the temporal relationship between the measurement of enzyme activity and vitamin B-6 intake is poorly understood (Leklem 1999). No long-term studies (more than six wks) have measured aminotransferase activities after different levels of vitamin B-6 were fed (Leklem 1999). In addition, the existence of three phenotypes of erythrocyte alanine aminotransferase has been documented and their activity differs (Holman 1995; Leklem 1999). Furthermore, the affinity of the albumin to which PLP is bound partially influences the saturation of plasma aminotransferases (Holman 1995). There are additional limitations of this method; for example, abnormalities in erythrocyte aminotransferase arise in liver disease (Holman 1995) or in riboflavin deficiency (Leklem 1999). Also, as influenced by the duration of erythrocyte life span in the circulation (~ 120 d), erythrocyte aminotransferase activation is not as rapidly responsive to acute vitamin B-6 depletion as other indices (Holman 1995). A standardized approach to reporting results from this assay is needed due to a lack of uniform method in reporting data and comparing results among studies.

Serine hydroxymethyltransferase activity: Serine hydroxymethyltransferase (SHMT), a PLP-dependent enzyme, has received attention recently for its importance in homocysteine remethylation and one-carbon metabolism (Davis 2005). Serine hydroxymethyltransferase catalyzes the transfer of the three-carbon of serine to tetrahydrofolate to produce

5,10-methylenetetrahydrofolate, which is reduced by methylenetetrahydrofolate reductase to produce 5-methyltetrahydrofolate (Davis 2005). Five-methyltetrahydrofolate is involved in remethylation of homocysteine by methionine synthase. Thus, if vitamin B-6 deficiency occurs, it reduces SHMT activity that might result in the impairment of homocysteine remethylation .The sensitivity of SHMT activity and homocysteine metabolism to vitamin B-6 status in animal studies (Martinez et al. 2000) suggests that impaired homocysteine remethylation might also occur in vitamin B-6 deficiency in humans (Davis 2005). Human study in healthy, nonsmoking 20-30-y-old men and nonpregnant women (Davis 2005) showed that dietary vitamin B-6 restriction significantly reduced basal and PLP-stimulated lymphocyte SHMT activities in vitro. Further investigation is needed to evaluate the usefulness of SHMT activity as a measure of vitamin B-6 status.

Dietary intake

It is not advisable using dietary intake of vitamin B-6 alone (or the vitamin B-6-to-protein ratio implied) to evaluate status due to the difficulty in obtaining accurate dietary assessment data and lack of complete data for the vitamin B-6 content of foods (Leklem 2001). Several status indicators, including both direct and indirect measures, in addition to an evaluation of both vitamin B-6 and protein intake are necessary to properly assess vitamin B-6 status (Leklem 1990).

Requirement

There are several factors that have been identified that may affect the determination of vitamin B-6 requirement. Dietary factors affecting bioavailability may affect the requirement, such as forms of vitamin B-6 in foods (animal/plant foods). Defects in delivery to tissues and cells, such as impaired gastrointestinal absorption and impaired transport, may play a role affecting vitamin B-6 metabolism and thus requirement. Increased catabolism, growth, gender differences, aging, and physical activity levels are recognized physiological/biochemical factors that influence vitamin B-6 requirement. Nutrient-nutrient interactions such as vitamin B-6 interactions with riboflavin or carbohydrate; genetic factors such as defects in apoenzymes requiring PLP and altered concentrations of apoenzymes requiring PLP are also associated with vitamin B-6 requirement. Additional factors may

affect requirement such as drug interactions, as indicated by people taking isoniazid who have reduced plasma PLP concentrations (Weir et al. 1991), by women using high-dose oral contraceptive agents who have reduced vitamin B-6 status (Shane and Contractor 1975), and by alcoholics consuming alcohol who have a reduced plasma PLP concentration that is distinct from that which is caused by liver disease or poor diet (Institute of Medicine 1998).

Among these factors, the direct relationship between protein intake and the need for vitamin B-6 was employed to set a requirement for vitamin B-6 intake in 1989. A value of 0.016 mg of vitamin B-6 per gram of protein was set as the basis for adults to meet the 1989 RDA value for vitamin B-6 (Leklem 1999). Human feeding studies have provided information for setting vitamin B-6 RDAs for individuals of each gender at different stages of life. Hansen and coworkers (2001) assessed the vitamin B-6 requirements of young women receiving 1.2 g protein/kg body weight for a 7-d period of 1.0 mg B-6/d, followed by three successive 14-d experimental periods of 1.5, 2.1, and 2.7 mg B-6/d. Their findings indicated that dietary vitamin B-6-to-protein ratio was significantly correlated with urinary 4-PA and total vitamin B-6, plasma PLP, total vitamin B-6 and 4-PA, and erythrocyte PLP. They have shown higher vitamin B-6-to-protein ratios of 0.018-0.020 mg/g protein than the recommended ratio in 1989 (0.016) are required to achieve adequacy. This study suggests that the current vitamin B-6 RDA may not be adequate. The most recent RDA for vitamin B-6 is not expressed in terms of protein intake, although the relationship was considered in setting the RDA in 1989. Considering that plasma PLP is reflective of tissue stores of vitamin B-6, the current RDA for vitamin B-6 is based on a plasma PLP concentration of at least 20 nM by the DRI committee (Institute of Medicine 1998).

Methods that have been used to assess the vitamin B-6 requirement include metabolic balance studies, depletion/repletion studies, functional tests such as the tryptophan load test and methionine load test, dietary intake of populations, and metabolic turnover (measurement of pools) (Leklem 2001). Although the latter method above received little attention, it provides the best information upon which to base requirements (Leklem 2001). There are several limitations of methods being used to estimate the vitamin B-6 requirement. First, one of the assumptions for setting the RDA is that the intake data are from a healthy population (Leklem 2001). However, this is not always the case. Often this health information is not

adequately documented in large population-based studies (Leklem 2001). Moreover, the nutrient data bases being used to interpret vitamin B-6 intake are not complete. A significant number of foods do not have vitamin B-6 values, resulting in an underestimation of vitamin B-6 intake (Leklem 2001). In addition, the comparison made from the data across studies is not consistent due to the fact that various levels of protein were utilized. Leklem (2001) suggests that well designed studies with several indices of vitamin B-6 status considered would provide a better understanding of overall metabolism of vitamin B-6. Vitamin B-6 status is best assessed using a direct measure, a short-term indicator, an indirect measure, and appropriate dietary information in a healthy population (Leklem 1990).

The 1989 RDA for vitamin B-6 was 1.6 and 2.0 mg/d for women and men, respectively, ages 19-50 y (Leklem 1999; NRC 1989). However, in 1998 more than 50% of the U.S. population consumed less than the 1989 RDA (Leklem 1999). More recently, the vitamin B-6 EAR and RDA for women and men ages 19-50 y was decreased to 1.1 and 1.3 mg/d, respectively. Considering plasma PLP is reflective of body stores of vitamin B-6, a plasma PLP value of 20 nM was used as the primary criterion to estimate the RDA for vitamin B-6. This was done in the absence of evidence linking a particular concentration to favorable or unfavorable health outcomes (Institute of Medicine 1998). Plasma PLP concentration was chosen as the standard for assessing adequate status because it appears to be reflective of the tissue stores of the vitamin (Liu et al. 1985). A coefficient of variation (CV) of 10% is assumed for vitamin B-6 because information is not available on the standard deviation of the requirement (Institute of Medicine 1998). The RDA for vitamin B-6 is defined as equal to the EAR plus twice the CV to cover the needs of 97 to 98 percent of individuals in the group (Institute of Medicine 1998). That is to say, the RDA for vitamin B-6 has been estimated as being 120 percent of the EAR, which is 1.1 mg B-6/d (NRC 1989). The current vitamin B-6 RDAs are based on metabolic studies, vitamin B-6 intake data, and protein intake of populations (Leklem 1999; Institute of Medicine 1998). A mean PLP concentration close to 20 nM was achieved in past studies (Institute of Medicine 1998) in subjects with an intake of 1.0 mg of vitamin B-6 (1.25 mg of food B-6). In most studies, PN is used and its bioavailability is estimated as 95% compared to 75% for food B-6 (Institute of Medicine 1998). Thus, for the EAR determination, a conversion factor of 1.27 (95÷75) has

been added to dietary B-6 equivalents in the following formula (Institute of Medicine 1998): mg of dietary B-6 equivalents provided = [mg of food B-6 + (1.27×mg of synthetic B-6)]. The RDAs are set to 1.9 mg and 2.0 mg vitamin B-6 per day to meet the additional requirement in pregnancy and lactation, respectively (Institute of Medicine 1998).

Rationale

Our understanding of overall bioavailability of vitamin B-6 in human diets is still incomplete, although several factors affecting vitamin B-6 bioavailability have been identified. Recognition of the health effects of soy, such as a health claim approved by the FDA in 1999 that soy protein reduces the risk of coronary heart disease by lowering blood cholesterol, has led to an increase in soyfoods consumption (Messina et al. 2002). However, there is limited information about the forms of vitamin B-6 in commercially available soyfoods and soy protein products have not been fortified with vitamin B-6. The PNG content in cooked whole soybeans was estimated as 57% of the total vitamin B-6 and the PNG content in soy flour was found to be 67% of the total vitamin B-6 (Gregory and Sartain 1991). Compared with tuna, the bioavailability of vitamin B-6 in soybeans was estimated at 41%. Apart from the effect of PNG content on vitamin B-6 status, there are some other factors that may affect the bioavailability of vitamin B-6 in soy. In an animal study (Lu and Huang 1997), with the same intake of vitamin B-6, rats fed soy protein isolate excreted twice the amount of urinary xanthurenic acid compared to rats fed casein along with a vitamin B-6-deficient diet for 5 weeks and given a tryptophan load. The reduced vitamin B-6 status in the rats fed soy protein was also reflected in decreased erythrocyte aminotransferase activity. These results suggest that, apart from the effect of PNG content on vitamin B-6 status, there might be an additional adverse effect on vitamin B-6 status when replacing milk protein with soy protein.

Middle aged women (ages 35-55 y) were studied because women in this age group frequently have marginal intakes of vitamin B-6 (Institute of Medicine 1998) and many drink soymilk for its purported beneficial health effect on menopausal symptoms, especially alleviating hot flushes and night sweats (Kronenberg and Fugh-Berman 2002). There has

been limited information regarding the PNG content of soymilk. The effect of substituting soy products for animal products on vitamin B-6 status has not been investigated.

Objectives

This study investigated the effect of substituting soymilk for cow's milk on vitamin B-6 status in middle aged women (aged 36-52 y; n=16). The specific aims of this research were to determine the effects of substituting soymilk for cow's milk on: 1) vitamin B-6 metabolite concentrations of plasma PLP, 4-PA and urinary 4-PA; 2) homocysteine concentration in fasting blood and after a methionine load; we also determined the differences in these effects on vitamin B-6 status between soymilk drinkers and milk drinkers. We hypothesized that women substituting soymilk for cow's milk will have decreased concentrations of plasma PLP, 4-PA, and urinary 4-PA, as well as elevated concentrations of plasma homocysteine after a methionine load.

CHAPTER 3. MATERIALS AND METHODS

Methods

Subject selection

Eighteen middle-aged women (aged 36-52 y) were recruited by advertisements in newspapers and announcements posted and circulated throughout the community. Volunteers were interviewed by study personnel, and the study was fully explained, including the risks and benefits to the subject. The volunteers' suitability for the study was initially determined based on the health history, food likes and dislikes, and physical activity level. Potential subjects were additionally screened using a fasting blood draw for evaluation of vitamin B-6 status and clinical chemistry evaluation. Subjects kept a 3-d diet record prior to the study to estimate usual nutrient intake. Nutrient composition of the 3-d diet record was analyzed by Food Processor (Version 8.5.0, ESHA Research, Salem, Oregon). Prior to starting the study, subjects consumed (mean ± SD) 2055 ± 625 kcal/d with a mean (± SD) vitamin B-6 intake of 1.83 ± 0.73 mg/d and a mean (± SD) vitamin B-6-to-protein ratio of 0.0241 ± 0.0094 mg/g.

Subject inclusion criteria were as follows: [1] no illness or medical condition requiring a physician's supervision; [2] no history of gastrointestinal disorders which could influence digestion or absorption; [3] no history of heart, liver, or kidney disease, diabetes, alcoholism, cancer, hypertension, epilepsy or related convulsive disease, or other metabolic diseases; [4] for premenopausal women, a regular menstrual cycle length of 26 to 32 d (pregnant and lactating women were excluded from participation); [5] no hormonal therapy (e.g., oral contraceptives, estrogen, androgen, progesterone, or glucocorticoids) or other drugs (e.g., isoniazid, penicillamine, and others), which would influence vitamin B-6 metabolism; [6] no use of vitamin or other nutritional supplements for at least 6 wk prior to the beginning of the study; [7] no food allergies; [8] alcohol consumption less than 56.7 g pure ethanol/wk; [9] no weight change greater than 4 kg within the last year; [10] no participation in strenuous activity such as long-distance running or bicycling; [11] normal blood chemistry evaluation, including liver and thyroid function, glucose, protein, creatinine, electrolytes, triglycerides, and cholesterol; [12] normal vitamin B-6 status.

The characteristics of the subjects are listed in **Table 3.1** (p. 26). The Institutional Review Board of Iowa State University reviewed and approved the screening and experimental procedures, and informed consent was obtained from each subject. Subjects were instructed to maintain their usual physical activity level throughout the study. Eighteen subjects began the study, one subject dropped out during the washout period, and one subject's data were eliminated because of analytical difficulties. Sixteen people were included in our final data analyses. As shown, subjects' initial weight (mean ± SD) was 80.8 ± 20.8 kg and final weight was 80.0 ± 20.7 kg. Their age (mean ± SD) was 41.8 ± 4.9, with 14 Caucasians, 1 African (Zambian), and 1 Asian (Malaysian).

Experimental design

The study employed a crossover design with two 28-d experimental periods preceded by a 14-d adjustment period and separated by a 14-d washout period (**Table 3.2**, p. 26). During the adjustment, experimental, and washout periods, subjects were given a list of foods (**Table 3.3**, p. 27) to eliminate from their diet and foods that were permitted in a given amount per day, to decrease their vitamin B-6 intake to approximately 1.0 mg/day (77% of the RDA). After the 14-d adjustment period, subjects were randomly assigned to dairy milk (A&E, Des Moines, IA) or soymilk (White Wave, Broomfield, Colorado) treatment for the first experimental period. During this 28-d period, subjects consumed 4 cups per day of either dairy milk or soymilk, which provided approximately 0.4 mg vitamin B-6 per day (31% of the RDA) in addition to their usual diet while following the food list protocol. The nutrient composition of soymilk and cow's milk provided is listed in **Table 3.4** (p. 28). Experimental diet was intended to bring the subjects' total daily intake close to the Recommended Dietary Allowance for vitamin B-6 (1.3 mg/d). A 14-d washout period followed, after which subjects crossed over to the other group and consumed either dairy milk or soymilk for the second 28-d experimental period. On day 28 of each experimental period, subjects were given a methionine (L-methionine; Sigma, Saint Louis, MO) load (0.1 g /kg body wt), after fasting blood had been taken.

Diet

The research team instructed the subjects to follow the diet protocol throughout the study. The two types of milk were purchased at a local grocery store and distributed to

Table 3.1. Descriptive characteristics of subjects

Subjects	Age	Height	Weight		BMI[1]	Ethnicity
			Initial	Final		
	y	*m*	*kg*	*kg*	*kg/m²*	
1	37	1.73	81.9	83.3	27.5	Caucasian
2	40	1.68	94.5	95.0	33.6	Caucasian
3	41	1.65	102.6	104.0	37.6	Caucasian
4	47	1.65	67.5	67.5	24.8	African
5	52	1.52	61.7	58.5	26.5	Caucasian
6	51	1.59	56.7	56.7	22.5	Caucasian
7	39	1.77	104.0	96.3	33.4	Caucasian
8	42	1.68	80.1	77.4	28.5	Caucasian
9	39	1.85	73.8	72.5	21.5	Caucasian
10	44	1.65	67.5	64.8	24.8	Caucasian
11	36	1.68	82.8	84.2	29.5	Caucasian
12	43	1.73	139.5	138.6	46.8	Caucasian
13	44	1.63	71.1	71.6	26.9	Asian
14	38	1.73	67.1	66.6	22.5	Caucasian
15	38	1.70	74.7	75.6	25.8	Caucasian
16	37	1.65	67.5	67.5	24.8	Caucasian
Mean ± SD	41.8 ± 4.9	1.68 ± 0.07	80.8 ± 20.8	80.0 ± 20.7	28.5 ± 6.6	

[1]Initial body mass index

Table 3.2. Experimental design

Period	Length (days)	Diet
Adjustment	14	Self-selected, using food list – no dairy milk or soymilk
Experimental period 1	28	Self-selected, using food list plus 4 cups dairy milk or soymilk
Washout	14	Self-selected, using food list – no dairy milk or soymilk
Experimental period 2	28	Self-selected, using food list plus 4 cups dairy milk or soymilk

Table 3.3. List of prohibited foods and foods permitted only in designated amounts

Prohibited foods:	
Fruits: banana, watermelon, avocado	**Fish**: salmon
Vegetables: potato, squash, sweet potato	**Cereals**: fortified cereals not listed below; instant oatmeal, cream of wheat, or cream of rice
Legumes: soybeans	**Dairy milk (yogurt, pudding, ice cream) or soy milk other than that provided; other dairy not listed below**
Nuts: chestnuts, hazelnuts, pistachios, walnuts	
Seeds: sunflower seeds	
Foods permitted in designated amounts:	
Juices: Orange or tomato,1cup(8 fl oz) or less; Grape, pineapple or vegetable juice cocktail (V-8®), ½ cup or less; Prune or carrot, ¼ cup or less	**Vegetables:** Mushrooms, kohlrabi, onions, cooked carrots, cooked broccoli, bamboo shoots, cooked cauliflower, artichokes, parsnips, rutabagas, sauerkraut, ½ cup or less
Legumes (other than soybeans): ½ cup or less	Brussels sprouts or peppers, red or green, ¼ cup or less; Raw carrots, 1 carrot; Cucumber, 1 cucumber
Fruits: Prunes, ¼ cup or less; Dates or raisins, pineapple, apricots, melon, grapes, ½ cup or less; Mango, ½ mango	**Miso:** ½ cup or less
Greens: Spinach, ¼ cup or less; Turnip greens, collards, Chinese cabbage, beet greens, kale, mustard greens, cooked cabbage, dandelion greens, ½ cup or less	**Dairy:** Cottage cheese or ricotta cheese, ½ cup or less; American pasteurized process cheese food, 1 slice; Cheese sauce, ¼ cup or less; Cheddar, colby, mozzarella, muenster, provolone or swiss cheese, 1 slice or 1 oz. or less; Parmesan cheese, ¼ cup; Cream cheese, 4 TBS or less
Nuts, seeds (other than those prohibited): ¼ cup or less	
Meat, poultry, fish (other than salmon), **shellfish**: 3 oz. or less	
Cereals permitted (* in designated amounts):	
Cold cereal:	**Hot cereal**:
Flavorite® Bite-size shredded wheat *(1¼ cups or less); Post® Original shredded wheat *(2 biscuits or less); Spoon size shredded wheat *(1¼ cups or less); Weetabix® Whole grain cereal *(2 biscuits or less); Arrowhead Mills® Amaranth flakes; Health Valley® Original oat flakes *(¾ cup or less); Barbara's Bakery® Multigrain shredded spoonfuls; Shredded wheat *(2 biscuits or less); Sunbelt Snacks and Cereals® Berry basic, Fruit & nut, Granola;Kashi®; Seven whole grain & sesame; Organic promise; Sweethome® Low-fat crunchy muesli; Low-fat granola with raisins; Honey oat clusters with almonds; Fresh vanilla almond crisp; Quaker® Oats & honey (& raisin) granola; Sweet puffs *(1 cup or less); Puffed wheat; Puffed rice	Nabisco® Cream of rice, Cream of wheat Stone-buhr® Bran flakes; Kretschmer® Oat bran cereal; Quaker® Oat bran cereal; Quick or old-fashioned oatmeal; Albers® Hominy grits; Krusteaz® Zoom hot wheat cereal Wheatena® **Cereal and snack bars:** Sunbelt® Chewy granola bar; M&M® Kudos bar; Quaker® Granola bar; Nature Valley® Granola bar

Table 3.4. Average daily nutrient composition of 4 cups (C) milk or soymilk consumed by 16 subjects during each experimental period [1].

Nutrient	unit	4 C Plain Soymilk	4 C Vanilla Soymilk	4 C Chocolate Soymilk	4 C A&E Fat Free Skim Milk
Total Energy	*kcal*	400	400	560	320
Total Fat	*g*	16	14	14	0
Cholesterol	*mg*	0	0	0	<20
Total Carbohydrate	*g*	32	40	92	92
Fiber	*g*	4	4	8	0
Protein	*g*	28	24	20	32
Vitamin A	*IU*	2000	2000	2000	2000
Vitamin C	*mg*	0	0	0	4.8
Calcium	*mg*	1200	1200	1200	1200
Iron	*mg*	4.32	4.32	5.76	0
Vitamin D	*IU*	480	480	480	400
Riboflavin	*mg*	2.04	2.04	2.04	*1.784
Folate (DFE[2])	*μg*	96	96	96	*49
Vitamin B12	*μg*	12	12	12	*5.194
Phosphorus	*mg*	240	*470.4	240	*725.2
Iodine	*μg*	180	NA[3]	NA[3]	NA[3]
Magnesium	*mg*	160	160	*182.4	*88.2
Zinc	*mg*	2.4	2.4	2.4	*8.33
* Vitamin B6	*mg*	0.392	0.392	0.392	0.363

*Computer analysis using First DataBank Nutritionist Pro™

[1] From manufacturers' food labels or nutritional information

[2] DFE represents dietary folate equivalent

[3] NA represents not available

subjects prior to each experimental week. Preference for flavors was taken into consideration for the soymilk distribution, with plain, vanilla, and chocolate flavor soymilk (White Wave, Broomfield, Colorado) provided as options. Cow's milk purchased for the study was fat-free skim milk with vitamin A and D added (A & E, Des Moines, IA).

Sample collection

Weights of the subjects were recorded at the beginning and the end of the study. Subjects completed 3-d diet records once during the adjustment and washout periods and twice during each experimental period in order to assess their total daily intake of vitamin B-6 and other nutrients. Nutrient analysis was performed by Food Processor (Version 8.5.0, ESHA Research, Salem, Oregon). Missing values in the Food Processor program were filled in using the online information from the USDA (United States Department of Agriculture) Nutrient Data Laboratory.

To assess creatinine and 4-PA concentrations, subjects collected three consecutive 24-h urine collections on days 12-14 of the adjustment, experimental, and washout period, and days 26-28 of the experimental periods using toluene as a preservative. Total daily urine volume was measured and aliquots of urine were frozen (-20°C) until analysis. Blood was drawn from fasting subjects on the morning of day 1 of the adjustment and experimental periods, and days 15 and 28 of the experimental periods. A second blood sample was drawn six hours after the methionine load on day 28 of the experimental periods. Blood samples were collected into heparinized Vacutainer tubes (BD Vacutainer® CPT™) after an overnight fast. Heparinized tubes were immediately placed on ice. After the removal of aliquots of whole blood for hematocrit measurements, heparinized tubes were centrifuged (at 4°C) and plasma separated, aliquoted, and frozen at -30°C until analysis.

Sample analyses

Determination of creatinine and urinary 4-PA quantification: Urine was analyzed for creatinine concentration (Pino and Gordnya, 1965) by using a microplate reader procedure. This commercial kit (Teco Diagnostics, Anaheim, CA) contained 10 mM picric acid; a solution containing 10 mM sodium borate, 240 mM sodium hydroxide, and surfactant; and creatinine standard (5.0 mg/dL). Creatinine working reagent contained a mixture of equal volumes of picric acid and borate buffer. A 1:10 dilution of urine was centrifuged for 5 min and 10 μL supernatant was pipetted into each well of a 12 × 8 geometric microplate with 210 μL creatinine working reagent added to each well. The microplates then were placed in 37°C heating water bath for 15 minutes. Absorbance was read at a wavelength of 490 nm by microplate reader (Bio-Tek instruments.INC, Winooski, VT).

Urinary 4-PA was determined by HPLC with fluorometric detection (Gregory and Kirk 1979). The HPLC system (Varian Inc, Palo Alto, CA) consisted of a Model 210 liquid chromatograph, Model 410 auto injector, Model 363 fluorescence detector, and HPLC column (Alltech Econosil C18 10μ 250 X 4.6 mm). Reagents for this assay included: mobile phase consisting of 0.34 M K_3PO_4, pH 2.2, 1.25% acetonitrile, and 5% methanol (V:V:V); internal standard prepared using 0.6 μM pyridoxamine dihydrochloride (PM); 4-PA stock standard (0.11 μmol/mL) frozen at -30°C; and synthetic urine using a solution of 0.5% urea and 0.225% NaCl (W:V).

Subjects' samples were prepared as follows: 0.05% of a subject's total urine volume was mixed with 0.2 mL PM (internal standard) and the total volume brought to 5 mL with deionized water. For the standard (0.44 nmol/mL) preparation, 0.1 mL 4-PA stock standard was combined with 1.0 mL PM and brought to 25 mL total volume in a volumetric flask with synthetic urine. Recovery samples were made as follows: 0.625 mL of 1:10 diluted 4-PA stock standard was added to 0.05% total volume of well mixed control urine sample and brought to 5 mL with deionized water. The amount of diluted 4-PA was equivalent to an added 13.65 μmol 4-PA/d. All samples were assayed in duplicate. The subjects' samples, standards, and recovery samples were centrifuged for 10 min, and the supernatants were filtered into HPLC sample vials using 0.45 μm nylon syringe filters. All 4-PA samples then were injected into HPLC column (Column: Alltech Econosil C18 10μ 250 X 4.6 mm; flow rate: 1.9 mL/min; injection volume: 50 μL; run time: 10 min), and quantified by fluorescence detection at 320 μm excitation and 425 μm emission wavelengths. Mean recovery of urinary 4-PA was 101 ± 5% with a control interassay coefficient of variation of 4.8%.

Plasma PLP and 4-PA quantification: All regents were analytical grade unless otherwise specified. PLP, 4-PA, PMP, PL, PN, PM, and DPN were obtained from Sigma Chemical Company (Saint Louis, MO). Three-mL aliquots of 1 mM stock solutions of PLP, PM, PN, PMP, PL, 4-PA, and 4-deoxypyridoxine (DPN, internal standard) were stored at -20°C. The working standard solutions were prepared daily and further diluted with deionized water, DPN, and trichloroacetic acid (TCA) to obtain standards with 12.5, 25, 50, 100, and 200 nM of each standard. Standards were kept on ice and in the dark. Standards were prepared in parallel with the blood samples prior to injection into the HPLC system.

Prior to analysis proteins were precipitated with 100% TCA. Plasma samples and standards were centrifuged for 3 min, and the supernatants were filtered into HPLC autosampler vials through 0.2 μm syringe filter (Fisher, Dubuque, IA).

Plasma PLP and 4-PA were determined by HPLC with fluorometric detection (Sharma and Dakshinamurti 1992). The analytical HPLC column, Microsorb-MV 100-3 C18, 100X4.6 mm (Varian, Lake Forest, CA), was protected by use of a guard column, Ultrasphere C18 5μ, 7.5X 4.6 mm (Varian). Mobile phase A consisted of 0.033 M H_3PO_4, 8.0 mM octane sulfonic acid at pH 2.2, while mobile phase B consisted of 0.033 M H_3PO_4, 18% isopropanol at pH 2.2. The pre-column derivitization solution consisted of 1 M NaK_2PO_4 and 2 g/L potassium bisulfite at pH 7.5. The fluorescence detector excitation and emission wavelengths were set at 295 nm and 405 nm, respectively. The pump flow was 1.0 mL/min with a premix of 125 μL sample and 15 μL of 2g/L bisulfite solution and an injection volume of 50 μL. The solvent gradients were 0% B to 10% B in 7.5 min; 10% B to 40% B in 7.5 min; 40% B for 7 min; 40% B to 0% B in 10 min; and 0% B for 10 min for a total analysis time of 42 min. To identify and quantify vitamin B-6 metabolite concentrations, retention times and peak areas were compared with standards. Plasma PLP and 4-PA concentration analyses were performed in duplicate.

Homocysteine analysis: Chemicals and reagents included 4-fluoro-7-sulfobenzofurazan (ammonium salt, 0.1%)(SBD-F), N-acetyl-L-cysteine (2.5 mM), and tri-n-butylphosphine purchased from Sigma. Other reagents were of analytical grade. Total homocysteine concentration in fasting and post-methionine load plasma samples from day 28 of each experimental period was determined using HPLC and fluorescence detection by the method described by Ubbink et al. (Durand et al. 1996; Ubbink et al. 1991). The analytical HPLC column was Alltech Econosil ODS-2 C18 5μ 4.6 X 150 mm. The pump flow was 1.0 mL/min with an injection volume of 50 μL. The fluorescence detector excitation and emission wavelengths were set at 385 nm and 515 nm, respectively. The total analysis time was 10 min. For derivatization, plasma (120 μL) mixed with 30 μL of N-acetyl-L-cysteine (2.5 mM in 0.9% NaCl and 4 mM EDTA) as internal standard (the internal standard allows the rapid separation of plasma total SBD-F-derivatized thiols with accurate quantification; Durand et al. 1996) was incubated with 15 μL 10% tri-n-butylphosphine in

dimethylformamide for 30 min at 4°C. The reaction was terminated with 0.6 M ice-cold perchloric acid containing 1 mM EDTA (150 μL). Following centrifugation for 10 min, supernatants (50 μL) were added to a 185 μL of incubation solution containing borate buffer (0.125 M, pH 9.5), sodium hydroxide (1.55 M), and 0.05 mg SBD-F. Samples were injected onto the HPLC column equilibrated in a mobile phase consisting of 2% methanol in 0.1 M sodium acetate/acetic acid solution (pH 4.0).

Statistical analyses

Data were analyzed using SAS statistical analysis computer software programs (version 9.1; SAS Institute Inc., Cary, NC). Results were expressed as group means and standard deviations. Urinary 4-PA was averaged over the last three days of each period before performing statistical analyses, to minimize the effect of day-to-day variation. Crossover ANOVA test was conducted to test significant effects of dairy milk and soymilk treatments. Statistical comparisons were considered significant at $P < 0.05$.

CHAPTER 4. RESULTS AND DISCUSSION

Results

Eighteen volunteers were initially selected to participate in this study. Fifteen subjects were Caucasians, one African (Zambian), one Hispanic (Brazilian), and one Asian (Malaysian). One subject dropped out during the washout period (Brazilian), and one subject's data were eliminated because of analytical difficulties. Consequently, data from these two subjects were eliminated from statistical analyses.

Subject characteristics

The means for body weight and hematocrit did not change significantly during the study. Their mean body weight was 80.8 ± 20.8 kg before the study and 80.0 ± 20.7 kg at the end of the study. Their mean hematocrit was 41 ± 3 % before the study and 40 ± 3 % at the end of the study.

Diet

Dietary intakes (mean ± SD) during each period of study are listed in **Table 4.1** (p. 34) and **Table 4.2** (p. 35).There were no significant differences in the mean total energy, carbohydrate, protein, and total vitamin B-6 intake when cow's milk or soymilk was consumed. The mean total fat intake was 13% higher (9 g/d more) when soymilk was consumed ($P < 0.01$). Twelve of the 16 subjects (75%) had a higher total fat intake during the soymilk than during the cow's milk consumption period. Moreover, the mean total vitamin B-6-to-protein ratio was 14% higher (0.0025 mg/g greater) when soymilk was consumed ($P < 0.01$). Twelve of the 16 subjects (75%) had a higher ratio during the soymilk than during the cow's milk consumption period.

In addition, there were significant differences in the mean total dietary fiber, vitamin B-12, and folate (DFE) intakes between the cow's milk and soymilk consumption periods. The mean fiber intake was 19 ± 7 g/d during the cow's milk treatment and 24 ± 4 g/d during the soymilk treatment. The mean fiber intake was 21% higher (5 g/d more) when soymilk was consumed ($P = 0.017$). All subjects except one had a higher fiber intake during the soymilk treatment. The mean vitamin B-12 intake was 6.5 ± 0.8 µg/d during the cow's milk treatment and 13.4 ± 0.9 µg/d during the soymilk treatment. The mean vitamin B-12 intake was 52% (6.9 µg/d more) higher during the soymilk treatment ($P < 0.0001$). All subjects

Table 4.1. Dietary intakes of 16 subjects during combined adjustment and washout periods (14-d each), and 28-d experimental periods during which 4 cups cow's milk or 4 cups soymilk were consumed daily[1]

	Baseline	Adjustment / washout	Cow's milk treatment	Soymilk treatment
Energy intake (kcal)	2055 ± 625	1777 ± 412	1966 ± 390	2057 ± 424
Protein (g)	78 ± 17	63 ± 18	91 ± 14	84 ± 19
(% energy)	*16 ± 3*	*14 ± 3*	*19 ± 2*	*16 ± 3*
(g/kg body weight)	*1.0 ± 0.2*	*0.8 ± 0.3*	*1.2 ± 0.2*	*1.1 ± 0.3*
Carbohydrate (g)	251 ± 72	239 ± 73	270 ± 63	274 ± 66
(% energy)	*49 ± 8*	*53 ± 8*	*55 ± 5*	*53 ± 4*
Fat (g)	86 ± 42	65 ± 18	61 ± 18 [a]	70 ± 15 [b]
(% energy)	*37 ± 10*	*33 ± 6*	*28 ± 5*	*31 ± 4*
Vitamin B-6 intake (mg)	1.83 ± 0.73	1.07 ± 0.35	1.33 ± 0.21	1.42 ± 0.26
B-6 to protein ratio (mg/g)	0.0241 ± 0.0094	0.0173 ± 0.0046	0.0149 ± 0.002 [a]	0.0174 ± 0.0033 [b]

[1]Mean ± SD, n=16. Values for treatments within a row with different superscript letters are significantly different, $P < 0.05$.

Table 4.2. Dietary intakes during each period of study in which 16 subjects consumed 4 cups cow's milk or 4 cups soymilk daily during each 28-d experimental period

Period	Total energy intake	Total protein intake	Vitamin B-6 intake from milk/soymilk	Vitamin B-6 intake from diet	Total vitamin B-6 intake	Vitamin B-6 to protein ratio
	(kcal/d)	*(g/d)*	*(mg/d)*	*(mg/d)*	*(mg/d)*	*(mg/g)*
Adjustment period *(14 d)*	1771 ± 412	62.2 ± 16.7	0	1.12 ± 0.31	1.12 ± 0.31	0.0184 ± 0.0046
Experimental period 1 *(28 d)*						
Subjects on cow's milk (n=8)	2053 ± 459	91.5 ± 16.2	0.36	0.93 ± 0.21	1.30 ± 0.21	0.0144 ± 0.0020
Subjects on soymilk (n=8)	1970 ± 378	78.7 ± 13.1	0.39	1.05 ± 0.25	1.44 ± 0.25	0.0185 ± 0.0028
Washout period *(14 d)*	1784 ± 426	63.6 ± 19.5	0	1.02 ± 0.39	1.02 ± 0.39	0.0161 ± 0.0044
Experimental period 2 *(28 d)*						
Subjects on cow's milk (n=8)	1879 ± 313	90.2 ± 11.9	0.36	1.00 ± 0.21	1.37 ± 0.21	0.0154 ± 0.0021
Subjects on soymilk (n=8)	2144 ± 474	89.1 ± 23.6	0.39	1.01 ± 0.28	1.40 ± 0.28	0.0163 ± 0.0037
Mean of cow's milk drinkers (n=16)	1966 ± 390	90.9 ± 13.7	0.36	0.97 ± 0.21	1.33 ± 0.21	0.0149 ± 0.0020 [a]
Mean of soymilk drinkers (n=16)	2057 ± 424	83.9 ± 19.2	0.39	1.03 ± 0.26	1.42 ± 0.26	0.0174 ± 0.0033 [b]

[1]Mean ± SD, n=16. Values for means of treatments within a column with different superscript letters are significantly different, $P < 0.05$.

had a higher vitamin B-12 intake during the soymilk treatment. In our study, during both treatments, the mean vitamin B-12 intakes were adequate based on its RDA (2.4 μg/d). The mean folate intake was 290 ± 112 μg/d during the cow's milk treatment and 345 ± 128 μg/d during the soymilk treatment. The mean folate intake was 16% (55 μg/d more) higher under soymilk treatment ($P < 0.01$). Fourteen of the 16 subjects (75%) had a higher folate intake during the soymilk treatment. In this study, during both treatments, neither of the mean folate intakes was above the RDA for folate (400 μg/d).

Urinary 4-PA status measure

Most studies to date have expressed urinary 4-PA excretion using 24-h urine collections (μmol/d). In our study, the urinary creatinine excretion was measured to determine whether the urine sample was a complete 24-h sample. Most of the sample collections were determined to be incomplete compared to the expected 24-h urinary creatinine values according to height for adult females (Blackburn et al. 1977). Thus, instead of using daily urinary 4-PA excretion, a urinary 4-PA-to-creatinine ratio was employed as a measurement of vitamin B-6 status in this study. The daily estimated urinary 4-PA excretion was calculated using expected 24-h urinary creatinine values based on height for adult females (Blackburn et al. 1977). The mean urinary 4-PA-to-creatinine ratio was 4.56 ± 1.04 μmol/g when the subjects consumed cow's milk and 3.69 ± 0.74 μmol/g when they consumed soymilk. The mean ratio during the soymilk treatment was 19% (0.87 μmol/g lower) lower than during cow's milk treatment ($P < 0.001$, **Figure 4.1;** p. 37). The estimated mean urinary 4-PA excretion was 4.76 ± 1.13 μmol/d, representing 61 ± 16% of the total B-6 intake (7.88 ± 1.24 μmol/d) that was excreted as urinary 4-PA when subjects consumed the cow's milk and 3.87 ± 0.84 μmol/d, representing 47 ± 14% of the total B-6 intake (8.41 ± 1.52 μmol/d) excreted as urinary 4-PA when subjects consumed soymilk. The estimated mean urinary 4-PA excretion was 19% lower (0.89 μmol/d less) when the soymilk than when the cow's milk was consumed ($P < 0.001$). Fourteen of the 16 subjects (87.5%) excreted less 4-PA at the end of the soymilk than at the end of the cow's milk consumption period. Urinary 4-PA excretion ≤ 3.0 μmol/d is considered indicative of inadequate vitamin B-6 status (Leklem 1990). By the end of soymilk treatment, only one subject excreted ≤ 3.0 μmol/d, and none were by the end of the milk treatment.

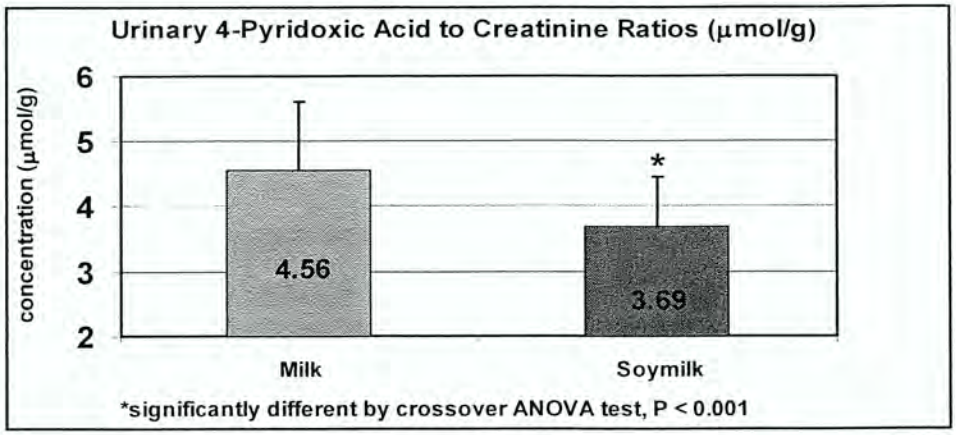

Figure 4.1 Urinary 4-pyridoxic acid (4-PA) to creatinine ratio in 16 middle aged women who consumed 4 cups soymilk or cow's milk daily for 28 days. Asterisk (*) denotes means that are significantly different between soymilk and cow's milk treatments ($P < 0.001$).

Plasma PLP and 4-PA status measures

The mean plasma PLP concentration was 36.2 ± 11.9 nM when the subjects consumed cow's milk and 32.2 ± 11.3 nM when they consumed soymilk. Thirteen of the16 subjects (81%) had a lower plasma PLP concentration at the end of the soymilk than at the end of the cow's milk consumption period. By the end of cow's milk treatment, six subjects (37.5%) had plasma PLP concentrations ≤ 30 nM. By the end of the soymilk treatment, five subjects (31%) had PLP concentrations ≤ 30 nM and three of these subjects were ≤ 20 nM.

The mean plasma 4-PA concentration was 13.2 ± 4.5 nM when the subjects consumed cow's milk and 10.1 ± 3.7 nM when they consumed soymilk. The data for this measure were from 15 subjects because the plasma 4-PA for one subject was not detectable using our methods. The mean plasma 4-PA concentration was 23.5% lower (3.1 nM less) when the soymilk than when the cow's milk was consumed ($P = 0.001$, **Figure 4.2**; p. 38). Thirteen of the 15 subjects (86.7%) had a lower plasma 4-PA concentration at the end of the soymilk than at the end of the cow's milk consumption period.

Plasma homocysteine measures

The mean pre-methionine load plasma total homocysteine concentration was 7.2 ± 1.8 µM when the subjects consumed cow's milk and 7.0 ± 1.8 µM when they consumed soymilk. Nine of the 16 subjects (56%) had a lower pre-methionine load concentration at the

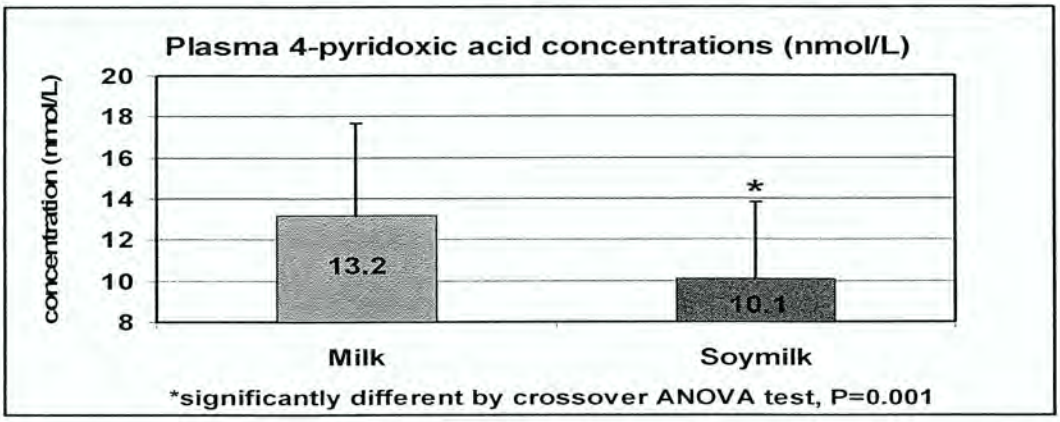

Figure 4.2 Plasma 4-pyridoxic acid (4-PA) concentration in 16 middle aged women who consumed 4 cups soymilk or cow's milk daily for 28 days. Asterisk (*) denotes means that are significantly different between soymilk and cow's milk treatments (P = 0.001).

end of the soymilk than at the end of the cow's milk consumption period.

The subjects' mean post-methionine load plasma total homocysteine concentration was 24.1 ± 7.8 μM when they consumed cow's milk and 26.1 ± 5.9 μM when they consumed soymilk. Eleven of the 16 subjects (69%) had a higher post-methionine load concentration at the end of the soymilk than at the end of the cow's milk consumption period.

The increase in the mean plasma total homocysteine concentration after a methionine load was 17.8 ± 6.3 μM when the subjects consumed cow's milk and 19.1 ± 5.2 μM when they consumed soymilk. Twelve of the 16 subjects (75%) had a greater increase in plasma total homocysteine concentration at the end of the soymilk than at the end of the cow's milk consumption period.

Discussion

This study evaluated the effects of substituting soymilk for cow's milk on vitamin B-6 status in middle aged women (36-52 y; n=16). The unique aspects of this study are: first, it was a crossover design with two 28-d experimental periods preceded by a 14-d adjustment period and separated by a 14-d washout period. Fourteen days were included to separate the experimental periods because previous research has shown it takes about 10-14 days for all B-6 vitamers to plateau after a change in vitamin B-6 intake (Hansen et al. 1997). Secondly, the subjects were following a food list designed to limit their daily vitamin B-6

intake to less than 1.0 mg; their daily milk or soymilk intake was intended to bring their daily total vitamin B-6 intake to the B-6 RDA (1.3 mg/d) for this age group. No studies have been published in healthy middle-aged women who were not B-6 depleted, assessing the effect of substituting soy products for animal products on vitamin B-6 status. This study provides data on vitamin B-6 status indicator values in middle-aged women consuming self-selected diets following a vitamin B-6 restricted food list. Thirdly, the methionine load test was chosen as an indirect method to assess status to provide further information of its usefulness as a potential indirect indicator of vitamin B-6 status.

Dietary factors affecting status

Among several factors that affect vitamin B-6 status, dietary protein has been the most frequently considered factor to date. Studies have found that the percentage of dietary vitamin B-6 excreted as urinary 4-PA and vitamin B-6 decreases as protein intake increases (Miller et al. 1985; Hansen et al. 1996a). A vitamin B-6-to-protein ratio of 0.016 mg/g was employed for setting the B-6 RDAs in 1989 (RDA 10th ed.; NRC 1989), although currently the RDAs for B-6 are not based on protein intake. The current RDA is based on a plasma PLP concentration of at least 20 nM because plasma PLP is reflective of tissue stores of vitamin B-6 (Institute of Medicine 1998). Data under controlled metabolic conditions (depletion/repletion studies) show strong interrelationships between vitamin B-6 status indicators and B-6 to protein ratios (Donald et al. 1971; Kretsch et al. 1995). In this study, when cow's milk was consumed, the mean (± SD) ratio was 0.0149 ± 0.002, which was lower than the ratio recommended in 1989; when the soymilk was consumed, the mean (± SD) ratio was 0.0174 ± 0.0033, which was higher than the ratio recommended in 1989. Thus, according to the ratios, we would have expected that during soymilk treatment, subjects had better B-6 status. However, the mean values of the status indicators measured indicated reduced vitamin B-6 status when soymilk was consumed.

Pyridoxine glucoside, a unique form of vitamin B-6 presented in plant foods, has been found to comprise up to 80% of vitamin B-6 in plant foods. More importantly, PNG has been considered a less bioavailable form compared with the other six vitamin B-6 forms that are present in both animal and plant foods (Gregory 1991). Hansen et al. (1996b) conducted a PNG controlled feeding study in women, investigating the extent to which vitamin B-6

bioavailability affects vitamin B-6 status, and thus requirement. These researchers showed that women consuming a diet containing a higher percentage of the total vitamin B-6 intake as PNG exhibited a decrease in vitamin B-6 status indicators, equal to a loss of 15%-18% of the total vitamin B-6 intake. This suggested that the reduced bioavailability of PNG and its presence in higher amounts in some diets should be considered during the determination of the RDA for vitamin B-6. Some soyfoods that have been analyzed for PNG content have been found to contain PNG that contributes 57-67% of the total vitamin B-6 (Leklem 2001). There has been no information published regarding the PNG content of soymilk. This study provides information regarding bioavailability of vitamin B-6 in soymilk on vitamin B-6 status when substituting soymilk for cow's milk. While subjects were during the soymilk treatment, following a vitamin B-6 restricted food list, their daily soymilk consumption contributed a higher percentage of the less bioavailable PNG than while during cow's milk treatment.

An additional issue regarding the effect of substituting soy products for animal products on vitamin B-6 status is soy protein versus animal protein. In a rat study, after being fed the same vitamin B-6 intake in a B-6 deficient diet for 5 wk and given a tryptophan load, rats fed soy protein isolate excreted twice the amount of urinary xanthurenic acid in 24 h compared with rats fed casein. Moreover, lower erythrocyte aminotransferase activity also indicated reduced vitamin B-6 status in the rats fed soy protein (Lu and Huang 1997). The results suggest that the source of dietary protein significantly affected the B-6 status in these rats. Therefore, it further suggests that apart from the effect of PNG content in soy, replacing milk protein with soy protein may have an additional adverse effect on vitamin B-6 status. We expected that a similar diet using cow's milk protein would have produced status indicators better than soy protein. In this study, subjects had a mean (±SD) soy protein daily intake of 24 ± 3 g/d, representing 30 ± 8% of daily total protein intake. We cannot determine whether this contributed to the reduced vitamin B-6 status. The amino acid composition differences between milk protein (primarily casein) and soy protein might affect vitamin B-6 status measurement. Ni et al. (1998) indicated that soy protein isolate differs from casein in the proportion of methionine (14 vs. 31 g/kg protein). Matthias et al. (1996) showed that oral administration of high doses of methionine to normotensive and hypertensive rats resulted in

a marked elevation of serum homocysteine (34.2 and 61.0 μM for normotensive and hypertensive rats, respectively). Thus, it is expected that the high proportion of methionine in casein (milk protein) would result in more homocysteine. In our case, the amino acid composition differences between soy protein and casein would affect the increase in plasma homocysteine after a methionine load, the indirect vitamin B-6 status indicator chosen.

Some of the confounding factors that may have affected vitamin B-6 status indicator measurements include dietary fiber, vitamin B-12, and folate intakes. There were significant differences between the diets during cow's milk and soymilk treatments in dietary fiber, vitamin B-12, and folate intakes. The diets when soymilk was consumed had 20.8% higher ($P = 0.0167$; 5 g/d more) dietary fiber intake than when cow's milk was consumed. This was partially due to the fiber content differences of the commercial cow's milk and soymilk chosen. There is no fiber content in cow's milk, whereas there is 4 g in 4 cups plain or vanilla flavor soymilk and 8 g in 4 cups chocolate flavor soymilk. Lindberg et al. (1983) found that the addition of 15 g of wheat bran (6.4 g dietary fiber) to the diet decreased the vitamin B-6 bioavailability modestly (by up to 17%) in 10 men aged 20 to 35 years consuming bran and nonbran diets, providing 1.69 and 1.66 mg/d vitamin B-6, respectively. Vorster et al. (1987) reported that 7.5 to 19 g of supplemental dietary fiber decreased the plasma PLP and pyridoxal concentrations in their subjects, although not significantly. Thus, the higher fiber intake during the soymilk consumption period may have contributed to the reduced B-6 bioavailability.

The vitamin B-12 and folate intakes were significantly higher ($P < 0.01$) when soymilk was consumed. This was the result of the fortification of vitamin B-12 and folate in the commercial soymilk chosen. Four cups of cow's milk contain 5.2 μg vitamin B-12 whereas this is 12 μg for 4 cups of soymilk (56.7% higher in soymilk). As far as folate content (daily folate equivalent; μg/d) in the commercial milk, it is 96 μg in 4 cups soymilk, whereas is only 49 μg in 4 cups cow's milk (49% higher in soymilk).Vitamin B-12 and folate intakes could affect the vitamin B-6 indirect status indicator chosen, altering plasma homocysteine concentration after a methionine load. Homocysteine, an intermediate in the metabolism of methionine, is eliminated by two metabolic pathways: remethylation and transsulfuration. In remethylation pathway, by acquiring a methyl group either from

N-5-methyltetrahydrofolate or from betaine, homocysteine is remethylated to methionine. The reaction with N-5-methyltetrahydrofolate is vitamin B-12 dependent, whereas the reaction with betaine is vitamin B-12 independent. In transsulfuration pathway, homocysteine reacts with serine to form cystathionine in an irreversible manner catalyzed by the cystathionine β-synthase, a PLP- dependent enzyme. Cystathionine is then hydrolyzed by a second PLP-dependent enzyme, γ-cystathionase, to form cysteine. Excess homocysteine that is not required for methyl transfer is effectively catabolized to cysteine via this transsulfuration pathway. Both of these aforementioned metabolic pathways contribute to the metabolism of homocysteine. Thus, if either vitamin B-12 or folate inadequacy occurs, homocysteine will accumulate, despite vitamin B-6 intake being adequate. According to the current RDAs for adults, the vitamin B-12 RDA is 2.4 μg/day and the folate RDA is 400 μg/day (Institute of Medicine 1998). In our study, during both treatments, the mean vitamin B-12 intakes were adequate, whereas neither of the mean folate intakes were above the RDA for folate. This may have contributed to the lack of significant difference between the mean increases in plasma homocysteine concentration after a methionine load at the end of the cow's milk and soymilk treatments. If folate intakes were adequate, the mean increase in plasma homocysteine concentration after a methionine load with soymilk treatment might have become significantly greater than with cow's milk treatment.

Direct measures of vitamin B-6 status

The major end-product of vitamin B-6 metabolism is 4-pyridoxic acid. Urinary 4-PA excretion has been considered a short term indicator of vitamin B-6 status that is directly reflective of recent B-6 intake (Leklem 1990). A previous human study conducted by Schuster et al. (1984) indicated that expressing urinary 4-PA excretion as the ratio 4-PA/creatinine is representative of the total 4-PA excretion in 24-h samples. Moreover, their results suggest that the 4-PA/creatinine ratio in random urine samples reflects the total 4-PA excretion in a 24-h period. The mean 4-PA-to-creatinine ratio during soymilk treatment was significantly lower than during cow's milk treatment ($P < 0.001$), indicating reduced vitamin B-6 status at the end of soymilk treatment. To date, no inadequacy cutoff for 4-PA-to-creatinine ratio has been established. Hence, we further expressed the ratio as the estimated daily urinary 4-PA excretion using the expected 24-h urinary creatinine values

based on height for adult females (Blackburn et al. 1977). Thus, we can compare our results with the other studies which expressed their results as daily 4-PA excretion, as well as compare the individual values with the inadequacy cutoff (< 3 μmol/d; Leklem 1990). The individual data for the estimated daily urinary 4-PA concentrations showed urinary 4-PA excretion reached values indicative of adequate status in all subjects during both treatments, except for one subject who was excreting less than 3 μmol/d (2.6 μmol/d) with a total vitamin B-6 intake of 5.92 μmol/d (1.00 mg/d) during the soymilk treatment. The calculated mean estimated daily urinary 4-PA excretion values were in agreement with the mean urinary 4-PA-to-creatinine ratio that indicated reduced vitamin B-6 status when soymilk was consumed ($P < 0.001$). Because the urinary 4-PA excretion is considered a short term status indicator, it best describes how recent dietary intake affects the vitamin B-6 status. Thus, the significantly lower mean values at the end of the soymilk treatment strongly support our hypothesis that it was the soymilk treatment, the recent dietary intake, that resulted in reduced vitamin B-6 status.

Fasting plasma PLP concentration, the most frequently used direct status indicator, is reflective of tissue stores of vitamin B-6 (Leklem 1990). There was no evidence that the mean fasting plasma PLP concentration was different when soymilk or cow's milk was consumed. The mean values during both treatments were above the recommended cutoff of 30 nM. However, the cutoff values are less useful for comparison of mean values than for the comparison of individual data (Hansen et al. 1997). Notably, when soymilk was consumed, three subjects (19%) had a plasma PLP concentration less than 20 nM; their fasting PLP concentrations were 12.9, 12.4, and 19.8 nM, with total vitamin B-6 intakes of 1.00 (5.92 μmol/d), 1.35 (8 μmol/d), and 0.92 (5.42 μmol/d) mg/d, respectively, whereas during cow's milk treatment, no subjects had a value below 20 nM. Moreover, no evidence of significance was shown for the mean plasma PLP between the treatments could be due that, compared with the urinary 4-PA excretion, the plasma PLP is relatively more reflective of long-term vitamin B-6 status. Thus, in a short-term study (28 d), it might not change as rapidly as the urinary 4-PA excretion in response to the treatments.

There has been limited research regarding the usefulness of fasting plasma 4-PA concentration as an index of vitamin B-6 status. The measurement of fasting plasma 4-PA

concentration in our study provides further information of its usefulness as a potential direct indicator of vitamin B-6 status. The mean fasting plasma 4-PA concentration was significantly lower during the soymilk than during the cow's milk treatment ($P = 0.001$). The lower plasma 4-PA concentration when soymilk was consumed may be reflective of the fact that less vitamin B-6 was being metabolized to 4-PA because less was available in soymilk. These results suggest reduced vitamin B-6 status at the end of the soymilk treatment, reflected by the urinary 4-PA and plasma 4-PA measurements. The results further suggest that plasma 4-PA may be a valid vitamin B-6 status indicator. Further research investigating the usefulness of plasma 4-PA as a direct status indicator of vitamin B-6 is necessary.

Indirect measures of vitamin B-6 status

Methionine load test was chosen as the indirect measure to assess vitamin B-6 status in our study. Because vitamin B-6 (PLP) plays a role in the catabolism of homocysteine to cysteine, an increase in plasma homocysteine concentration after a methionine load has been considered useful in assessing vitamin B-6 status. Limited information about the usefulness of this measure as a potential indirect indicator of vitamin B-6 status is available, whereas our study provides information convincing evidence.

Some dietary factors may have affected the results, including: folate intake which is an important potential contributor to the insignificance in the means of increase in the plasma homocysteine concentration after a methionine load. During both treatments the mean folate intakes were slightly below the current RDA for folate (400 μg/d), although they were both above the RDA for folate in 1989 (200 μg/d). The mean folate intake was 16% higher (55 μg/d more) during soymilk treatment. Due to the role that folate plays in homocysteine remethylation, inadequate intakes of folate might have resulted in the decreased elimination of homocysteine via the remethylation pathway involved in both folate and vitamin B-12, despite adequate vitamin B-6 intake that ensured the catabolism of homocysteine to cysteine via transsulfuration. If these participants had experienced an improved folate status (~ 400 μg/d) during the soymilk treatment, the change in plasma homocysteine after the methionine load might have become significant. Additionally, we may have reported a significant increase in plasma homocysteine concentration after a methionine load during the soymilk

versus cow's milk treatment if the study period was longer (>28 d), the diets more rigidly controlled, and our sample size was larger (n >16).

CHAPTER 5. SUMMARY AND CONCLUSIONS

This study was conducted to determine the effect of substituting soymilk for cow's milk on vitamin B-6 status in middle aged women. Our hypotheses were that women who substituted soymilk for cow's milk would have decreased concentrations of plasma PLP and 4-PA, and urinary 4-PA, and increases in plasma homocysteine concentration after a methionine load.

Throughout the study, our subjects followed a vitamin B-6 restricted diet designed to limit their daily vitamin B-6 to less than 1.0 mg, apart from the approximately 0.4 mg of daily vitamin B-6 intake either from soymilk or cow's milk provided by the investigators. During either the cow's milk or soymilk treatment, the mean vitamin B-6 intake was above the RDA for vitamin B-6. Compared with the recommended vitamin B-6-to-protein ratio by the DRI committee in 1989 (0.016 mg/g protein); however, the mean ratio was lower during the cow's milk treatment, whereas it was higher during the soymilk treatment. After 28 d of soymilk consumption, our subjects had significantly lower mean urinary 4-PA-to-creatinine ratio, estimated daily urinary 4-PA excretion, and plasma 4-PA concentrations compared to the end of 28 d of cow's milk consumption. These results indicate reduced vitamin B-6 status at the end of soymilk treatment. There was no evidence of differences in either the mean plasma PLP ($P = 0.107$) or the mean increase in plasma homocysteine concentration after a methionine load ($P = 0.316$) between the treatments. With longer study periods, more rigidly controlled diets, and larger sample size, it may be possible that the difference would become significant. The reduced vitamin status during soymilk treatment may be due to the PNG content in soymilk. Pyridoxine glucoside, a form of B-6 with a reported 58% bioavailability compared with the other six commonly consumed forms of B-6 (Gregory et al. 1991), is only present in plant foods. Thus, the relatively higher percentage of vitamin B-6 as PNG in the diet when soymilk was consumed may account partially for the reduced status indicator measurements we have reported. The amount of four cups of soymilk that has contributed to 0.4 mg of vitamin B-6 daily intake was chosen in our study because the FDA-approved health claim states that 25 g of soy protein daily has a significant cholesterol lowering effect

and the amount of soy protein in the commercial soymilk chosen provided approximately that amount daily.

For determining adequacy of vitamin B-6 status, a functional measure related to a specific health outcome ideally would be the best status measure. Plasma PLP concentration was chosen as the standard for adequate status because this measure appears to be reflective of tissue stores (Lui et al. 1985). Without evidence linking a particular concentration to favorable or unfavorable health outcomes, a fasting plasma PLP concentration of 20 nM was chosen as the standard for adequacy by the DRI committee in 1998. However, other investigators consider a higher concentration of >30 nM for adequacy based on results from human feeding studies (Leklem 1990; Hansen et al. 2001). The vitamin B-6 RDA was calculated based on an EAR of 1.1 mg/d, the intake of vitamin B-6 required for a plasma PLP concentration of 20 nM (Institute of Medicine 1998). Findings from our study showed that based on the 20 nM cutoff, all subjects exhibited adequate status when cow's milk was consumed whereas during soymilk treatment, 19% (3 of 16) had an inadequate value. However, considering 30 nM cutoff, 37.5% (6 of 16) had inadequate status when they consumed cow's milk and 31.3 % (5 of 16) exhibited inadequate status during soymilk treatment.

Urinary 4-PA excretion, as a short-term vitamin B-6 status indicator, is reflective of recent vitamin B-6 intake, with a value of less than 3 μmol/d considered inadequate (Leklem 1999). In this study, when soymilk was consumed, there was one subject who had a urinary 4-PA excretion value that fell below 3 μmol/d, whereas no subjects fell below this cutoff when cow's milk was consumed. Moreover, as urinary 4-PA excretion reflects the recent vitamin B-6 intake, the significantly lower mean urinary 4-PA-to-creatinine ratio and estimated urinary 4-PA excretion at the end of the soymilk treatment, strongly support our hypothesis that it was the soymilk treatment, the recent diets, that resulted in the reduced status of this vitamin B-6 indicator compared to the end of cow's milk treatment.

There are several vitamin B-6 metabolites that do not have an established, defined status indicator concentration, such as plasma PL and 4-PA. Thus, it is difficult to conduct status assessment when considering them as biomarkers. The mean plasma 4-PA concentration was significantly lower during the soymilk treatment than that during the cow's

milk treatment (P = 0.001). A lower plasma 4-PA concentration when soymilk was consumed maybe reflects that less vitamin B-6 was available for metabolism.

The methionine load test was chosen as the indirect status measurement. The methionine load given in this study was 0.1 g/kg body weight, which was approximately 3 to 5 times higher than the amount of average methionine intake from a typical diet. In this study, this measure showed our expected greater increase (albeit not significantly) in mean plasma total homocysteine concentration after a methionine load for the soymilk compared to cow's milk treatment. The relatively short study period (28 d), small sample size (n = 16), uncontrolled diets (self-selected diets) might have contributed to the insignificant increase for this measure. Based on our results, study design employing a longer period, larger sample size, and a more rigidly controlled diet would be warranted to assess the usefulness of this measure as a potential indirect or functional vitamin B-6 status indicator.

In summary, a diet composed of natural foods with a relatively high amount of PNG content contributed by 4 cups daily of soymilk reduced the urinary 4-PA-to-creatinine ratio, estimated urinary 4-PA excretion, and plasma 4-PA concentrations in middle aged women after 28 days. When soymilk was consumed, subjects had a significantly higher mean dietary vitamin B-6-to-protein ratio. We would expect the vitamin B-6 status measurements to improve during soymilk treatment compared to cow's milk treatment. However, the results showed reduced vitamin B-6 status at the end of soymilk treatment. The decrease in vitamin B-6 status indicators when soymilk was consumed suggested that substituting soymilk for cow's milk had an adverse effect on vitamin B-6 status in middle aged women as reflected by the reduced status indicators at the end of soymilk consumption. All of the findings indicate that vitamin B-6 in soymilk is less bioavailable than from cow's milk. The results of our study further suggest that the RDA for vitamin B-6 should take into consideration the reduced vitamin B-6 bioavailability of a high PNG content diet. The long-term effect of consuming a high PNG content diet on vitamin B-6 status needs to be investigated. The long-term consumption of high PNG content diets on vitamin B-6 status may greatly affect people with marginal intakes or consuming diets in which most of the B-6 intake is from plant foods. Thus, future research determining the quantitative importance of the effect of vitamin B-6 intake from PNG is needed.

APPENDIX A – RAW DATA TABLES

Table A1. Cow's milk and soymilk distribution during each experimental period.

	Experimental Period 1				Experimental Period 2			
Subject	Week 1	Week 2	Week 3	Week 4	Week 1	Week 2	Week 3	Week 4
1	4V,3C	4V,3C	3V,4C	4V,3C	7M	7M	7M	6M
2	4C,3P	4C,3P	4C,3P	3C,4P	7M	7M	7M	6M
3	4V,3C	7C	7C	7C	7M	7M	7M	6M
4	7V	7V	7V	7V	7M	7M	7M	6M
5	7P	7P	7P	7P	7M	7M	7M	6M
6	1C,6P	1C,6P	1C,6P	1C,6P	7M	7M	7M	6M
7	7V	7V	7V	7V	7M	7M	7M	6M
8	4V,3P	7V	7V	7V	7M	7M	7M	6M
9	7M	7M	7M	7M	4V,3C	4V,3C	4V,3C	3V,3C
10	7M	7M	7M	7M	7P	7P	7P	6P
11	7M	7M	7M	7M	4V,3C	4V,3C	7C	6C
12	7M	7M	7M	7M	7P	7P	7P	6P
13	7M	7M	7M	7M	7C	7C	7C	6C
14	7M	7M	7M	7M	7P	7P	7P	6P
15	7M	7M	7M	7M	7C	4V,3C	4V,3C	6C
16	7M	7M	7M	7M	4V,3C	4V,3C	4V,3C	3V,3C

Volume of Milk/soymilk container is 1 quart (946mL)
V stands vanilla soymilk, P stands for plain soymilk, C stands for chocolate soymilk, and M represents fat free skim cow's milk

Table A2. Subject age, height (m), weight (kg), and initial and final BMI (kg/m^2)

	Age	Height	Weight				BMI		Ethnicity
	y	*m*	*kg*				*kg/m²*		
Subject			*5/14/2004*	*6/10/2004*	*6/25/2004*	*7/22/2004*	*Initial*	*Final*	
1	37	1.73	81.9	81.0	81.5	83.3	27.5	27.9	Caucasian
2	40	1.68	94.5	95.4	94.5	95.0	33.6	33.8	Caucasian
3	41	1.65	102.6	102.6	104.0	104.0	37.6	38.1	Caucasian
4	47	1.65	67.5	67.1	67.1	67.5	24.8	24.8	African
5	52	1.52	61.7	59.4	59.9	58.5	26.5	25.2	Caucasian
6	51	1.59	56.7	56.3	57.2	56.7	22.5	22.5	Caucasian
7	39	1.77	104.0	98.6	97.2	96.3	33.4	30.9	Caucasian
8	42	1.68	80.1	79.2	77.4	77.4	28.5	27.5	Caucasian
9	39	1.85	73.8	72.9	73.4	72.5	21.5	21.1	Caucasian
10	44	1.65	67.5	66.2	66.2	64.8	24.8	23.8	Caucasian
11	36	1.68	82.8	83.7	84.6	84.2	29.5	29.9	Caucasian
12	43	1.73	139.5	140.0	140.9	138.6	46.8	46.5	Caucasian
13	44	1.63	71.1	71.6	72.9	71.6	26.9	27.1	Asian
14	38	1.73	67.1	65.7	65.7	66.6	22.5	22.3	Caucasian
15	38	1.70	74.7	76.1	75.6	75.6	25.8	26.1	Caucasian
16	37	1.65	67.5	67.1	67.5	67.5	24.8	24.8	Caucasian

Table A3. Methionine load of 0.1 g/kg body weight given to each subject consuming 4cups cow's milk or soymilk for 4 weeks on day 28 of each experimental period

Subject #	Weight on 5/14/2004 *kg*	Methionine Load on 6/10/2004 *g*	Weight on 6/25/2004 *kg*	Methionine Load on 7/22/2004 *g*
1	81.9	8.19	82.3	8.23
2	94.5	9.45	95.5	9.55
3	102.6	10.26	105.0	10.50
4	67.5	6.75	67.7	6.77
5	61.7	6.17	60.5	6.05
6	56.7	5.67	57.7	5.77
7	104.0	10.40	98.2	9.82
8	80.1	8.01	78.2	7.82
9	73.8	7.38	74.1	7.41
10	67.5	6.75	66.8	6.68
11	82.8	8.28	85.5	8.55
12	139.5	13.95	142.3	14.23
13	71.1	7.11	73.6	7.36
14	67.1	6.71	66.4	6.64
15	74.7	7.47	76.4	7.64
16	67.5	6.75	68.2	6.82

Table A4. Hematocrit measurements (%) at 7 time points during the study period of subjects consuming 4 cups cow's milk or soymilk for 4 weeks

Subject	Baseline	Adjustment	Experimental Period 1		Washout	Experimental Period 2	
#	4/30/2004	5/14/2004	5/28/2004	6/10/2004	6/25/2004	7/9/2004	7/22/2004
1	36	39	39	38	40	40	38
2	47	43	45	43	45	47	42
3	39	39	41	45	40	43	40
4	45	46	41	47	43	41	43
5	43	39	38	37	40	42	34
6	43	43	46	40	42	43	44
7	43	43	43	42	Missing	41	42
8	44	44	44	45	44	46	42
9	35	43	44	42	43	43	38
10	42	36	35	35	36	36	39
11	40	44	43	44	43	44	40
12	39	40	40	41	39	41	40
13	38	34	42	39	42	39	38
14	43	36	38	36	43	39	39
15	42	42	40	42	42	39	42
16	39	38	37	37	39	36	36

Table A5. Plasma pyridoxal 5'-phosphate (PLP) measurements (nmol/L) of subjects consuming 4 cups cow's milk or soymilk for 4 weeks, taken at 7 time points during the study period

Subject	Baseline	Adjustment	Experimental Period 1		Washout	Experimental Period 2	
#	4/30/2004	5/14/2004	5/28/2004	6/10/2004	6/25/2004	7/9/2004	7/22/2004
1	76.3	25.5	28.6	31.7	29.4	36.8	34.6
2	38.2	30.3	37.1	40.2	39.7	36.8	42.2
3	43.8	38.3	36.4	34.9	63.5	51.1	51.5
4	42.0	37.7	40.8	30.7	27.1	26.3	27.7
5	28.4	36.8	40.8	49.2	30.4	32.4	43.5
6	60.4	35.2	37.4	36.5	32.9	43.2	40.2
7	64.7	43.0	46.1	52.2	39.7	50.4	58.6
8	26.7	18.5	19.9	12.9	12.8	20.9	21.3
9	319.9	60.9	47.7	38.3	42.7	44.3	36.4
10	56.0	45.7	37.1	38.1	30.4	29.8	31.1
11	54.5	62.0	84.9	35.4	57.1	36.6	27.5
12	28.2	24.8	27.5	23.6	23.4	23.9	23.0
13	40.5	39.4	45.7	54.0	32.0	41.6	34.9
14	31.1	12.4	20.4	22.7	15.1	14.8	41.7
15	37.7	24.1	14.6	23.5	24.6	19.2	12.4
16	47.5	31.6	32.3	23.2	29.5	24.9	19.8

Table A6. Plasma 4-pyridoxic acid (4-PA) measurements (nmol/L) of subjects consuming 4 cups cow's milk or soymilk for 4 weeks, taken at 7 time points during the study period

Subject	Baseline	Adjustment	Experimental Period 1		Washout	Experimental Period 2	
#	4/30/2004	5/14/2004	5/28/2004	6/10/2004	6/25/2004	7/9/2004	7/22/2004
1	20.5	10.9	8.5	6.5	6.2	8.1	9.8
2	14.5	11.6	12.0	14.4	10.9	12.8	16.1
3	10.2	7.5	7.6	8.6	23.7	11.9	8.8
4	13.6	9.8	11.9	7.6	7.3	7.5	8.3
5	12.7	16.4	18.9	16.5	10.6	13.2	19.1
6	25.0	6.8	6.1	5.4	7.8	9.7	8.3
7	13.0	10.0	7.7	11.0	10.2	11.7	9.6
8	18.8	9.7	13.4	8.2	8.3	9.8	13.6
9	139.4	22.6	19.6	20.5	11.5	14.3	11.9
10	19.2	12.3	19.0	16.6	8.9	11.6	13.5
11	14.3	14.1	21.3	12.6	7.5	12.5	ND[1]
12	29.6	15.2	13.3	14.5	9.1	19.1	15.6
13	18.9	13.3	13.5	19.3	10.2	12.4	11.3
14	27.5	9.6	6.1	13.0	8.0	6.7	6.3
15	21.0	12.3	15.2	13.4	13.7	11.5	9.6
16	9.0	8.7	7.2	6.6	9.9	7.3	5.2

ND[1] not detectable using our methods

Table A7. Urinary 4-pyridoxic acid measurements (nmol/24h) in subjects consuming 4 cups cow's milk or soymilk for 4 weeks, measured in three 24-h urine collections taken 6 times during the study

Subject	Adjustment			Experimental Period 1						Washout			Experimental Period 2					
#	5/11	5/12	5/13	5/25	5/26	5/27	6/7	6/8	6/9	6/22	6/23	6/24	7/6	7/7	7/8	7/19	7/20	7/21
1	3.99	1.91	5.19	3.69	2.75	2.78	2.67	3.33	4.58	3.22	2.49	1.34	9.88	3.62	2.31	3.46	3.58	5.99
2	2.85	3.66	5.03	5.84	5.74	5.33	4.68	5.82	5.19	4.06	4.86	4.33	5.33	5.51	5.95	5.95	5.55	6.81
3	2.21	2.41	2.99	2.20	2.04	3.29	3.02	2.31	4.23	5.95	5.39	14.38	7.90	7.92	6.90	6.54	2.57	4.16
4	5.34	5.48	5.72	5.33	6.03	4.66	4.17	4.62	3.18	4.32	3.97	4.11	7.00	5.55	6.81	7.09	6.58	6.97
5	2.66	2.55	3.31	3.98	4.65	4.74	3.84	3.97	3.95	2.88	4.30	1.38	3.86	4.49	4.28	3.25	4.86	5.62
6	3.91	3.23	3.13	2.51	4.13	3.56	2.22	2.80	3.33	2.96	3.43	3.21	4.14	2.83	3.54	5.54	4.73	5.02
7	4.72	4.24	4.24	5.06	3.30	6.10	5.00	4.28	5.36	4.83	4.08	4.14	6.52	6.47	6.55	5.61	5.76	5.67
8	2.60	3.05	3.21	3.82	3.09	3.31	2.16	2.75	1.64	2.58	2.45	2.11	3.23	4.29	4.35	4.26	4.69	4.72
9	NV[1]	NV[1]	4.19	5.33	6.19	4.47	4.68	3.93	3.75	2.47	2.87	2.72	4.98	3.36	3.21	4.96	4.95	3.33
10	5.58	5.21	4.55	5.28	5.49	4.26	5.07	5.36	4.79	4.69	4.39	4.14	4.89	4.17	4.97	4.08	4.47	4.08
11	3.73	5.34	1.90	13.27	8.36	8.70	3.23	4.28	5.68	2.65	2.37	4.33	2.55	7.19	4.23	1.39	2.54	2.61
12	4.88	4.12	4.50	5.58	4.56	5.14	4.68	4.30	4.38	2.44	3.74	2.81	4.20	3.68	4.96	5.30	5.23	4.81
13	1.41	2.62	2.42	2.47	3.64	3.13	1.74	3.07	3.61	1.65	2.01	1.96	1.61	1.72	1.36	1.51	1.83	2.75
14	2.88	2.20	3.69	3.69	4.37	3.26	2.72	3.05	3.58	2.30	2.52	2.30	2.31	2.64	2.33	1.25	3.29	2.45
15	5.45	4.52	4.13	4.19	3.71	5.39	4.19	7.84	4.67	5.39	11.19	6.40	4.03	3.50	3.33	3.72	4.66	5.44
16	4.58	3.93	3.95	3.25	2.99	3.81	1.22	1.52	4.42	2.01	2.40	4.48	2.88	4.10	1.70	1.94	1.45	3.09

NV[1] urine sample on the day is not available for analysis

Table A8. Urinary creatinine measurements (nmol/24h) in subjects consuming 4 cups cow's milk or soymilk for 4 weeks, measured in three 24-h urine collections taken 6 times during the study

Subject	Adjustment			Experimental Period 1						Washout			Experimental Period 2					
#	5/11	5/12	5/13	5/25	5/26	5/27	6/7	6/8	6/9	6/22	6/23	6/24	7/6	7/7	7/8	7/19	7/20	7/21
1	383	812	652	532	906	807	447	531	768	436	495	527	398	507	444	414	519	833
2	1016	1263	1427	1502	1341	1528	1431	1422	1098	1029	1371	1274	1482	1366	1616	1353	1416	1534
3	901	1069	1556	760	719	1284	611	762	1155	993	1048	1097	1021	1238	1040	1003	564	1082
4	1469	1725	1684	1332	1567	1534	1688	1720	1119	1736	1547	1784	1592	1601	1769	1687	1608	1783
5	619	601	780	773	1022	1022	851	899	946	931	1441	442	803	1047	911	664	865	834
6	745	640	706	492	771	844	590	783	870	635	760	749	691	557	786	826	730	829
7	1519	1458	1504	1438	1031	1953	1604	1269	1573	1434	1325	1130	1520	1582	1635	1503	1612	1558
8	1042	1271	1200	1190	1263	1227	975	1217	822	1260	1289	1059	1109	1272	1264	1218	1317	1159
9	NV[1]	NV1	911	1100	1199	855	1123	882	798	938	1049	1092	1135	791	877	1094	1192	915
10	1161	1430	1226	1177	1316	1060	1230	1308	1202	1191	1192	1136	1267	1163	1202	1224	1242	1229
11	874	1276	476	834	596	918	712	922	1178	1029	886	1393	647	1877	1174	513	869	971
12	1862	1633	1584	1649	1564	1655	1692	1549	1565	1325	1569	1503	1401	1206	1797	1677	1569	1475
13	383	812	652	532	906	807	447	531	768	436	495	527	398	507	444	414	519	833
14	891	734	1181	748	890	821	740	789	858	802	868	800	735	793	815	597	1251	872
15	1021	1008	1051	921	1024	1108	943	1109	910	785	930	957	732	900	810	939	881	1035
16	970	961	1041	748	829	1079	408	475	1503	609	692	955	593	1111	533	552	422	994

NV[1] urine sample on the day is not available for analysis

Table A9. Urinary 4-pyridoxic acid (4-PA) (nmol/sample),creatinine (mg/sample), urinary 4-PA to creatinine ratio (nmol/mg) and estimated urinary 4-PA excretion (μmol/d) in subjects consuming 4 cups cow's milk or soymilk for 4 weeks. Averages of three 24-h urine collections taken twice during each experimental period

	Milk Treatment				Soymilk Treatment			
Subject #	Measured Urinary 4PA *nmol/sample*	Measured Creatinine *mg/sample*	U4PA/ creatinine *nmol/mg*	*Estimated U4PA *μmol/d*	Measured Urinary 4PA *nmol/sample*	Measured Creatinine *mg/sample*	U4PA/ creatinine *nmol/mg*	*Estimated U4PA *μmol/d*
1	4.81	1111	9.93	11.01	3.30	969	5.27	5.85
2	5.85	1461	4.01	4.19	5.43	1387	3.96	4.13
3	6.00	991	5.95	5.98	2.85	882	3.32	3.34
4	6.67	1673	3.99	4.01	4.67	1493	3.15	3.17
5	4.39	854	5.17	4.53	4.19	919	4.57	4.00
6	4.30	737	5.80	5.51	3.09	725	4.31	4.09
7	6.10	1568	3.89	4.57	4.85	1478	3.29	3.86
8	4.26	1223	3.48	3.63	2.80	1116	2.47	2.58
9	4.73	993	4.76	5.90	4.13	1001	4.11	5.09
10	5.04	1215	4.15	4.17	4.44	1221	3.64	3.66
11	7.25	860	8.90	9.29	3.42	1009	3.28	3.43
12	4.77	1612	2.96	3.28	4.70	1521	3.10	3.43
13	2.94	665	4.49	4.38	1.80	519	3.49	3.41
14	3.44	808	4.26	4.72	2.38	844	2.81	3.12
15	5.00	1002	4.95	5.32	4.11	883	4.67	5.03
16	2.87	841	3.44	3.46	2.53	701	3.64	3.66

*Calculated using expected 24 h urinary creatinine values based on height for adult females In: Blackburn et al. (1977) Nutritional and metabolic assessment of the hospitalized patient. *JPEN* 1: 11-12

APPENDIX B - FORMS

INFORMED CONSENT DOCUMENT

Title of Study: **"Bioavailability of Vitamin B-6: Determination of Pyridoxine Glucoside Content in Unfortified Soy Foods, and the Effect of Substitution of Soy Milk for Cow's Milk on Vitamin B-6 Status"**

Investigators: **Christine M. Hansen, Ph.D.**
Lester A. Wilson, Ph.D.
Michele Beattie, M.S., R.D.
Yana Chen, M.S. graduate student

This is a research study. Please take your time in deciding if you would like to participate. Please feel free to ask questions at any time.

INTRODUCTION

Vitamin B-6 is an essential nutrient that is involved in fundamental metabolic processes. In recent years, American consumption of soy foods has increased due to numerous health claims. The U.S. Food and Drug Administration has approved a food label health claim that soy protein reduces the risk of coronary heart disease by lowering blood cholesterol. The purpose of this study is to determine if substituting soy milk for cow's milk in the diet has a detrimental effect on vitamin B-6 status. Women in your age group frequently have marginal intakes of vitamin B-6. They may also increase sources of soy (soy foods) in their diet because of the reported benefits from soy on perimenopausal symptoms and heart disease risk. You are being invited to participate in this study because you are a healthy woman between the ages of 35 and 55 years. Women who are pregnant or lactating are not eligible for participation in this study.

DESCRIPTION OF PROCEDURES

If you agree to participate in this study, your participation will last twelve weeks and will include bi-weekly or weekly visits to the Human Metabolic Unit (HMU) in the Center for Designing Foods to Improve Nutrition. During the study, you may expect the following study procedures to be followed. During subject screening, you will be asked to complete a health history questionnaire, give a blood sample (two 10 mL tubes, less than 2 tablespoons of blood) for clinical chemistry and vitamin B-6 analysis, and record all food and beverages consumed over a three-day period. For the first two weeks of the study period (adjustment period), you will be asked to follow a restricted vitamin B-6 diet by eliminating or limiting servings of foods on a list we will provide. A fasting blood sample (two 10 mL tubes, less than 2 tablespoons of blood) will be taken at the end of this two-week adjustment period. In addition, for the last three days of the adjustment period you will collect all of your urine as instructed (three consecutive 24-hour collections), and keep a record of food eaten during those three days. During the next four weeks of the study (first experimental period), you will continue following the vitamin B-6 restricted diet with the addition of four cups of cow's milk or soy milk daily. You will come to the HMU weekly to pick up your milk or soymilk. Fasting blood samples (two 10 mL tubes, less than 2 tablespoons of blood) will be taken after two weeks and at the end of the four-week

experimental period. In addition, you will again collect three consecutive 24-hour urine collections and keep a three-day diet record at the end of this period. On the final day of the first experimental period, after fasting blood is taken, you will be given a methionine load (0.1 g methionine per kilogram body weight, approximately 0.2 to 0.4 ounces, in a glass of juice) with breakfast. Methionine is a component of protein that is found in the diet. The amount of methionine you will be given is approximately three times the average daily intake of methionine and poses no health risks. Another sample of blood (one 10 mL tube, less than a tablespoon) will be taken 5 to 6 hours after the methionine load. The next two weeks (washout period), you will follow the vitamin B-6 restricted diet, without cow's milk or soy milk. A fasting blood sample (two 10 mL tubes, less than 2 tablespoons of blood), three-day urine collection and three-day diet record will be collected at the end of this period. For the final four-week period (second experimental period), you will continue to follow the restricted vitamin B-6 diet with the addition of either cow's milk or soy milk (whichever you did not consume during the first experimental period). You will come to the HMU weekly to pick up your milk or soy milk. Fasting blood samples (two 10 mL tubes, less than 2 tablespoons of blood) will be taken after two weeks and at the end of the four-week experimental period. In addition, you will again collect three consecutive 24-hour urine collections and keep a three-day diet record at the end of this period. On the final day of the period, after fasting blood is taken, you will again be given a methionine load (0.1 g/kg body weight) with breakfast, and another sample of blood (one 10 mL tube, less than a tablespoon) will be taken 5 to 6 hours after the methionine load. You will be giving a total of less than three-quarters of a cup of blood during the twelve weeks of the study. You will be providing a total of four three-day urine collections and four three-day diet records.

RISKS

While participating in this study you may experience the following risks: Drawing blood sometimes causes bruising. Blood will be taken by a professional phlebotomist to minimize any risk. Collecting urine can be cumbersome; we will provide containers and supplies to make it as convenient as possible. Your diet will be restricted in vitamin B-6 by eliminating or limiting servings of high vitamin B-6 foods, but your vitamin B-6 intake will be marginally adequate during the adjustment and washout periods, and at the level of the Recommended Dietary Allowance (RDA) during the experimental periods. Vitamin B-6 intake will not be low enough to cause deficiency at any time during the study.

BENEFITS

If you decide to participate in this study there may be no direct benefit to you. You will have access to your screening blood chemistry report and vitamin B-6 status assessment if requested. It is hoped that the information gained in this study will benefit society by providing valuable information about the effect on vitamin B-6 status of substituting soy milk for cow's milk. If soy milk is found to reduce vitamin B-6 status, soy foods manufacturers may be encouraged to fortify soy milk with vitamin B-6 to enhance the health benefits of soy milk consumption.

COSTS AND COMPENSATION

You will not have any costs from participating in this study. You will be compensated for participating in this study. At the end of the study, participants will be paid $5.00 for each day of participation, for a total of $420 if the subject completes the study. If a subject drops out or is terminated from the study, she will be paid $5.00 for each day of participation.

PARTICIPANT RIGHTS

Your participation in this study is completely voluntary and you may refuse to participate or leave the study at any time. If you decide to not participate in the study or leave the study early, it will not result in any penalty or loss of benefits to which you are otherwise entitled. You may be terminated from the study if you do not comply with the dietary restrictions and requirements, if you do not complete the urine collection or diet records, or if you miss a blood draw appointment.

RESEARCH INJURY

Emergency treatment of any injuries that may occur as a direct result of participation in this research is available at the Iowa State University Thomas B. Thielen Student Health Center, and/or referred to Mary Greeley Medical Center or another physician or medical facility at the location of the research activity. Compensation for any injuries will be paid if it is determined under the Iowa Tort Claims Act, Chapter 669 Iowa Code. Claims for compensation should be submitted on approved forms to the State Appeals Board and are available from the Iowa State University Office of Risk Management and Insurance.

CONFIDENTIALITY

Records identifying participants will be kept confidential to the extent permitted by applicable laws and regulations and will not be made publicly available. However, federal government regulatory agencies and the Institutional Review Board (a committee that reviews and approves human subject research studies) may inspect and/or copy your records for quality assurance and data analysis. These records may contain private information.

To ensure confidentiality to the extent permitted by law, the following measures will be taken. Subjects will be assigned a unique code number, which will be used on forms instead of their name. Any identifiable data will be kept in a locked filing cabinet and destroyed after results of the study have been published. Only the key personnel will have access to study records. If the results are published, your identity will remain confidential.

QUESTIONS OR PROBLEMS

You are encouraged to ask questions at any time during this study. For further information about the study contact Dr. Christine M. Hansen, (515) 294-9567. If you have any questions about the rights of research subjects or research-related injury, please contact the Human Subjects Research Office, 2810 Beardshear Hall, (515) 294-4566; austingr@iastate.edu or the Research

Compliance Officer, Office of Research Compliance, 2810 Beardshear Hall, (515) 294-3115; dament@iastate.edu

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SUBJECT SIGNATURE

Your signature indicates that you voluntarily agree to participate in this study, that the study has been explained to you, that you have been given the time to read the document and that your questions have been satisfactorily answered. You will receive a copy of the written informed consent prior to your participation in the study.

Subject's Name (printed) _____

_____ _____

(Subject's Signature) (Date)

INVESTIGATOR STATEMENT

I certify that the participant has been given adequate time to read and learn about the study and all of their questions have been answered. It is my opinion that the participant understands the purpose, risks, benefits and the procedures that will be followed in this study and has voluntarily agreed to participate.

_____ _____

(Signature of Person Obtaining Informed Consent) (Date)

Telephone Pre-Screening
Soymilk/Vitamin B-6 Status Spring 2004

Name_____ Phone_____

Address_____ Age_____ Height_____ Weight_____

1. Are you pregnant, nursing an infant, or taking hormones (contraceptives or hormone replacement therapy)? Yes___No___

2. Do you participate in strenuous physical exercise more than 3 hours a week? Yes___No___

   If yes, what type of exercise, how often do you exercise and for how long each time?

   _____

3. Are there any medications that you take on a regular basis? Yes__No___

   If yes, what do you take and for what medical condition?

   _____

4. Are you lactose intolerant or have any allergies to milk or soy? Yes___No___

5. Are you currently a smoker? Yes___No___

6. Do you take any dietary supplements, vitamins, minerals or herbal supplements? Yes___No___

7. Can we schedule a time for you to come in to learn more about the study and what will be expected of the subjects? Date_____Time_____

SOYMILK/VITAMIN B-6 STATUS STUDY
DEPARTMENT OF FOOD SCIENCE AND HUMAN NUTRITION
IOWA STATE UNIVERSITY

General Questionnaire

Today's Date _____ / _____ / _____
month day year

Name _____
LAST First Initial Maiden

I like to be called _____

Address _____
Street

_____ _____ _____
City State Zip Code

Phone (____) _____ Daytime
(____) _____ Home
(area code)

Birthdate _____ / _____ / _____ **Age** _____
Month Day Year Years

State (or Country) of Birth _____

Current HEIGHT _____ / _____ Current WEIGHT _____
feet inches pounds

Have you lost or gained weight in the last 6 months? _____Yes___No How Much?_____ gain loss
lbs (please circle)

Ethnic Group (please circle one)
(a) African American
(b) Caucasian
(c) Native American
(d) Chinese
(e) Other Asian (specify)_____
(f) Hispanic_____
(g) Other (specify)_____

Some questions about your health. Please indicate for each disease on the left if you are currently being treated or in the right column if you are considered cured, the last year you were treated.

Medical History	Currently have or being treated for	Had in the past Year of last treatment
Diabetes	_____	_____
Hypothyroidism	_____	_____
Hyperthyroidism	_____	_____
Goiter	_____	_____
Hypoadrenalism (Addison's Disease)	_____	_____
Hepatitis	_____	_____
Cirrhosis	_____	_____
Gall Bladder Disease	_____	_____
Kidney Stones	_____	_____
Other kidney disease	_____	_____
Cystitis	_____	_____
Cancer: Skin	_____	_____
Cervical	_____	_____
Uterus	_____	_____
Prostate	_____	_____
Breast	_____	_____
Bowel/Colon	_____	_____
Fibroids of the uterus	_____	_____
Angina	_____	_____
Heart Disease	_____	_____
Mental Depression requiring medication	_____	_____
Insomnia requiring frequent medication	_____	_____
Ulcers (stomach or small intestine)	_____	_____
Epilepsy	_____	_____
Osteoporosis	_____	_____
Hypertension (high blood pressure)	_____	_____
Allergies to toiletries/cosmetics	_____	_____
Food allergies	_____	_____
Alcoholism	_____	_____
Arthritis	_____	_____
Eating Disorders (e.g. anorexia, bulimia, not eating, binging, purging)	_____	_____

Medication History. Please circle the medications that you take or have taken on a regular basis in the last year.

a. Sleeping pills
b. Tranquilizers
c. Barbiturates
d. Blood pressure pills
e. Antibiotics
f. Androgens (male hormones)
g. Estrogens (female hormones)
h. Aspirin
i. Oral contraceptives
j. Thyroid (Thyroxin)
k. Insulin
l. Cortisone
m. Isoniazid (for Tuberculosis)
n. Other drugs (specify)
_____
_____
_____
_____

What over-the-counter, non-prescription medicines are you presently taking (e.g., cough medicine, Tylenol, Nytol, No-doz)?

Are you presently taking vitamin/mineral, herbal or other food supplements?

None___ Yes, daily _____ Yes, in the winter only ____ Yes, frequently ____ Yes, occasionally____

What type _____

How long have you been taking them? _____(years/months) How often?_____

Was this on the advice of a physician? Yes _____ No _____

Smoking History.

1. Are you currently a smoker? Yes ___ No ___

Dietary History

1. Are you vegetarian? Yes____ No ____

If yes, circle one:

a. lacto-ovo (consumes dairy and eggs)
b. ovo (consumes eggs)
c. lacto (consumes dairy)
d. vegan (consumes no animal products)
e. no red meat

If no, circle one:

a. I could try a vegetarian diet
b. I could not get along without at least chicken or fish

How long have you been vegetarian? _____ years

2. Are there any foods you cannot tolerate (allergy, upset stomach, etc)? Tell us which foods:

3. Are there particular foods that you dislike so much you cannot eat them? If so, tell us which ones:

4. Do you include soy foods in your diet? Yes_____ No_____

   What foods and how often do you eat them?

Exercise and Hygiene

1. Do you, one or more times per week, engage in any regular activity like brisk walking, jogging, bicycling, aerobic exercises long enough to work up a sweat? Yes____ No ____

   If yes, how many times per week? _____
   Specify activity: _____

2. Do you frequently, on the weekend, do outdoor activities (e.g., backpack, rafting, bird watching, softball)? If so, what and how often/month?

Recreation	Frequency/month

3. Are you in competitive sports, such as swimming, basketball, crew, cross-country? If so, what and how often in the next 6 months?

Competitive Sport	Frequency next 6 months

4. On the average, how long is your regular menstrual period?
   a) Not applicable, I have none
   b) Not applicable, I am past menopause
   c) _____ days between flows

   Is your menstrual flow Average ____ Heavy all the time____ Usually light ____?

   Dates of last menstruation period: _____

5. If applicable, during your menstrual period can you use tampons? (circle one)
   a) No
   b) Never tried
   c) Yes
   d) Yes, with pad

Thank you for answering this questionnaire.

Now we need a 3-day record of your food intake. You will be given a form to record your intake. Please choose 1 weekend day to include in your 3-day diet intake record.

DIRECTIONS FOR RECORDING DIET RECORD

Attached is a three day food intake record to be competed prior to your appointment. The record should include two week days and one day of the weekend.

Following the guidelines below, record all food, fluid, medication, and vitamin/mineral preparations consumed over the three day period. Include brand names where applicable and indicate whether food are fresh, frozen, or canned. Also, please indicate the source of meals/food items which are restaurant, fast food, or take out. (Ex. McDonalds Grilled Chicken Sandwich)

MILK	The amount of milk consumed should be recorded in ounces or by carton size. The kind of milk should also be recorded as whole milk, evaporated milk (diluted or undiluted), nonfat milk, buttermilk. Also note whether or not the milk is fortified.
FRUIT	Fruit juice or other beverages consumed should be recorded in ounces or by size. List the kind of juice as orange, grape, etc. Canned Fruits should be reordered by name and cup portions Whole Raw Fruits should be recorded by name, number and size (small, medium or large)
VEGETABLES	List name and how prepared. Cooked vegetables should be recorded in cup portions or number and length of spears. (Asparagus, broccoli). Raw vegetables should be recorded in number and size of pieces such as two carrot sticks, 4 inches long.
CEREALS	List specific name such as Cheerios or Oatmeal. Cooked cereal should be recorded in level tablespoon or cup portions after cooking. Dry cereal should be recorded in level tablespoons or cup portions. Biscuit cereals should be recorded as number of biscuits eaten.
BREADS	Record as wheat, white, rye, tortilla. List whether corn or flour, enriched or restored.
MEATS, POULTRY, FISH	List kind and how prepared as baked, broiled, boiled or fried. The amount should be listed in raw weight before cooking.
FATS	Record level teaspoon or tablespoon. Include amounts used in cooking. If butter is used, list as butter, if margarine, list brand, if oil, list kind as corn, vegetable, olive, canola, etc.
DESSERTS	Describe size of portion and provide a description/name of dessert.
CANDIES	List with exact amount and number, such as four large gumdrops.
MIXED DISHES	For stews, casseroles, other mixed dishes, list amount of ingredients in each serving. Space is provided on the reverse of each day's record for the recipes of mixed dishes.
SUGAR	List sugar added to cereals, tea or used on other foods. Record in level teaspoons or tablespoons.
VITAMINS	If vitamins and/or minerals are used, please list brand name and ingredients as listed on the bottles.

INSTRUCTIONS FOR 24-HR URINE COLLECTIONS

1. Collect **all** urine in containers provided. Pour the urine into the liter bottles labeled with your initials and the date. (Make sure the date is correct.) Rinse the collection container with the squirt bottle and add the rinse water to the liter bottle with the urine. The liter bottles contain a preservative which will evaporate, so please keep the lids tightly closed. The squirt bottle can be refilled with tap water when needed.

2. Each liter bottle should be labeled with your initials and subject number and the date you began the collection.

3. You will be collecting three 24-h specimens. Urine collections are on a 24-hr basis and run from, for example, 7:00 AM one day until 7:00 AM the next day.

4. The day you begin collecting urine, note the time of your first urination but ***do not collect this sample***. You have emptied your bladder and will begin collecting urine from this point on.

5. The last sample you save will be at the same time the next day. For example, you get up at 7 AM the day you begin collecting urine and urinate, noting the time but not saving that specimen. You will save **all** of your urine from that point on until 7 AM the next day, when you will urinate and ***save that specimen as the last of your 24-hr collection*** (the date on the container will be the previous day).

6. All urine collected after that on the second day goes into the bottles labeled with the second day's date.

7. Store urine in a cool place and protected from light as much as possible.

8. The day you finish collecting your 24-hour sample, bring your urine collection in the ***labeled*** (subject number and date) containers provided to the refrigerator in Room 2124 HNSB and pick up your bottles for that day's collection. If you can't return your bottles each day, return all three day's collections when you come in for the blood draw.

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