The Characterization of a Rodent Model for the Estimation of Folate Bioavailability Using Plasma Homocysteine as an Outcome Measure

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Colleen Patricia O'Connor

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FACULTY OF GRADUATE STUDIES

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Colleen Patricia O'Connor

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirement of the degree

of

MASTER OF SCIENCE

This Thesis

Is dedicated

To the memory of

Marlene Beatrice O'Connor R.N., B.A., M.Ed.

Abstract

The primary role of folate coenzymes is one-carbon metabolism which is important for protein synthesis and cell division. The bioavailability of food folates has been a topic of interest for decades. The sensitivity of current folate bioavailability models is questionable. The recently developed dietary folate equivalents (DFE's) assume that the bioavailability of food folates is only 50% which is likely an underestimation. Therefore, there is a need for the development of a more sensitive model. Plasma homocysteine has been reported as a sensitive indicator of folate status. In the first experiment, the time course for folate depletion and plasma homocysteine elevation was developed using a folate depletion-repletion protocol in a rat model. Sixty male, weanling, Sprague-Dawley rats were fed an amino acid-based diet, with or without folate, for six weeks. The amino acid profiles were determined also. The results showed that four weeks of folate depletion were sufficient to adequately increase plasma homocysteine concentrations and decrease plasma folate concentrations. The results also indicated that folate deficiency causes a significant increase in several plasma amino acid concentrations. The second experiment sought to estimate the folate bioavailability of folate-enriched egg yolks using plasma homocysteine concentrations in a negative slope-ratio assay. Fifty-four rats were depleted of folate for four weeks as outlined in the first experiment. After baseline data were collected, the rats were divided into two groups of 24 rats for the four-week repletion period. The treatment group was fed the basal diet with the addition of folate-enriched egg yolk powder to achieve a folate concentration of 250 μg/kg of diet and the control group was fed the basal diet with the addition of 250 μg/kg of diet of crystalline

folic acid (CFA). The results indicated that, based on this method, the relative bioavailability of folate in folate-enriched egg yolks is 167% compared to crystalline folic acid. It also appeared that plasma homocysteine is a more sensitive outcome measure for the estimation of folate bioavailability in a folate depletion-repletion protocol.

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Abbreviations

[^{3}H]PABA – tritiated *p*-aminobenzoic acid
AI – adequate intake
ATP – adenosine triphosphate
BBFC – brush border folate conjugase
BHMT – betaine:homocysteine transferase
CAD – coronary artery disease
CFA – crystalline folic acid
CVD – cardiovascular disease
DFE – dietary folate equivalent
DNA – deoxyribonucleic acid
DRI - dietary reference intake
dUST – deoxyuridine suppression test
EAR – estimated average requirement
EDTA – ethylenediaminetetracetic acid
HPLC – high pressure liquid chromatography
HRW-bran – hard red wheat bran
ICFC – intracellular folate conjugase
KOH – potassium hydroxide
MC – milk-containing
MCV – mean corpuscular volume
MF – milk-free
NKH – non-ketotic hyperglycinemia
NTD - neural tube defects
PABA - para aminobenzoic acid
PLP – pyridoxal-5'-phosphate
RNA – ribonucleic acid
RDA - recommended daily allowance
RNI – recommended nutrient intake
SAM – S-adenosylmethionine
SAH – S-adenosylhomocysteine
SBDF – 7-flurobenzo-2-oxa-1,3-diazole-4-sulfonic acid
SHMT – serine hydroxymethyltransferase
SWW-bran – soft white wheat bran
TCEP – tris (2-carboxyethyl)-phosphine hydrochloride
THF – tetrahydrofolate
UL – tolerable upper intake level

Chapter 1 - Introduction

Adequate folate status is important throughout the life cycle. Low levels of folate are inversely correlated with increased levels of plasma homocysteine, a sulfur amino-acid and an intermediary in the metabolism of methionine (Wagner, 1995). High plasma homocysteine is considered a risk factor for such conditions as cardiovascular disease and neural tube defects (Brouwer et al., 1999a). Recently, the Dietary Reference Intakes (DRIs) have been established and the Recommended Daily Allowance (RDA) of folate has almost doubled (IOM, 1998). With this comes the need to provide consumers with information concerning sources of dietary folate; however, the bioavailability of the folate consumed must be considered (Bailey, 1988). Unfortunately, the methods established to date for estimating bioavailability of folate have produced variable results (Prinz-Langenohl et al., 1999). House and colleagues have recently developed and produced folate enriched eggs; however, the bioavailability of the folate contained in the eggs has yet to be determined (House et al., 2002). Since, plasma folate and plasma homocysteine are inversely related (Wagner, 1995) and plasma homocysteine levels are a sensitive marker of folate status (Minet et al., 2000), plasma homocysteine levels may offer a sensitive method for estimating the relative bioavailability of dietary folate in rats.

Chapter 2 – Literature Review

2.1 Folic Acid

2.1.1 Chemical Structure and Forms:

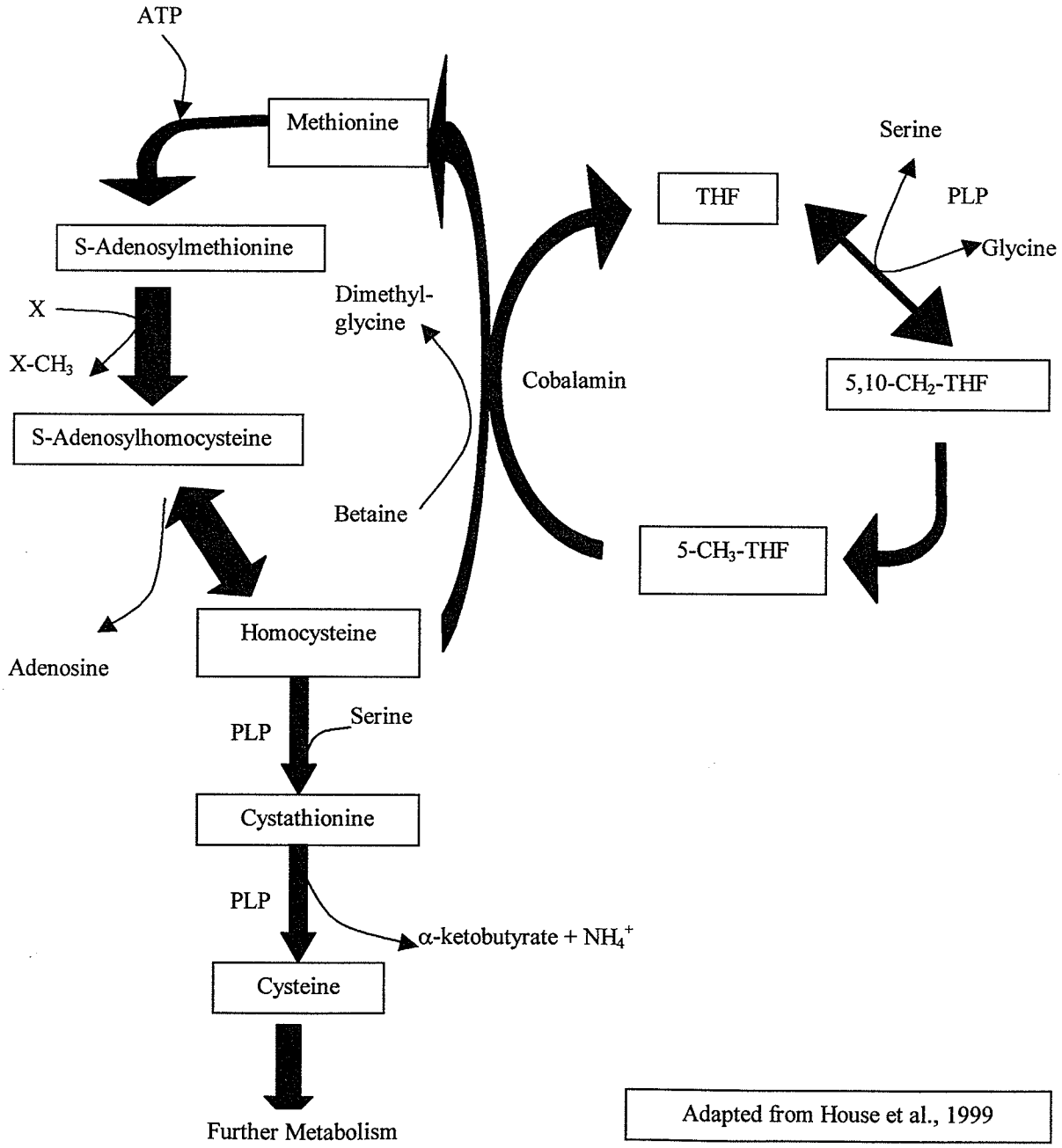
Folic acid, also called pteroylglutamate or pteroylmonoglutamate, consists of three distinct parts which are all needed for the vitamin to be active (Groff and Gropper, 2000). Pterin, also called pteridine, is attached to para aminobenzoic acid (PABA) resulting in pteroic acid, and a glutamic acid residue attached to PABA completes the structure (Bailey, 1992). Pteroylpolyglutamates are formed when one or more glutamic acid residues are attached by way of peptide bonds to the glutamic acid residue of the pterin compound (Rodriguez, 1978).

Folate and folacin are generic terms used for compounds with similar chemical structure and nutritional properties as folic acid. More than 150 forms of folate have been reported (Sauberlich, 1987). Folates may be present in one of three oxidation/reduction states: the oxidized form, the partially reduced form, and the reduced form (Rodriguez, 1978). Examples for each state include folic acid, 5,6-dihydrofolic acid, and 5,6,7,8-tetrahydrofolic acid, respectively. Folate is found in foods primarily as pteroylpolyglutamates with up to nine glutamic acid residues. The most predominant pteroylpolyglutamates are 5-methyl-tetrahydrofolate (THF) and 10-formyl-THF (Groff and Gropper, 2000). The reduced forms of folate are chemically unstable and easily lose their biological activity (Scott, 1999). Natural folates exist in human tissues as pteroylpolyglutamates (Wagner, 1995). The most predominant circulating form of folate in the plasma is a 5-methyl-THF (Sauberlich, 1987).

2.1.2 Functions

The principal role of folate coenzymes is to accept or donate single-carbon units in key metabolic pathways (Rodriguez, 1978). The synthesis of methyl groups and one-carbon units is tightly controlled (Bailey and Gregory, III, 1999). It is the fully reduced tetrahydro forms of folates which accept or donate single-carbon units, hence these reactions are termed single-carbon metabolism (Wagner, 1995). Metabolism is compartmentalized between the cytosol and the mitochondria since almost all the folate in the cell is distributed more or less equally between the two compartments (Wagner, 1996). Single-carbon metabolism is important in the biosynthesis of such compounds as purines, thymidylate, and methionine. The purine synthesis and thymidylate synthesis pathways are necessary for the *de novo* synthesis of DNA and therefore, for DNA replication and cell division (Steinberg, 1984). It is the methionine synthase reaction that regenerates THF required for the formation of 5,10-methylene-THF and 10-formyl-THF which are used directly in thymidylate and purine synthesis (Bailey and Gregory, III, 1999). The amino acid methionine is essential in the synthesis of proteins and polyamines. Methionine is also the precursor to S-adenosylmethionine (SAM) which is a universal methyl donor in countless enzymatic reactions critical to metabolism (Selhub, 1999). Folate is very important in the remethylation of homocysteine to methionine (**Figure 2.1**) (Brouwer et al., 1999a). Homocysteine, a sulfur amino acid, is an intermediate compound in the metabolism of the essential amino acid, methionine (Green and Jacobsen, 1995). Only trace amounts of homocysteine are obtained in the diet since

Figure 2.1 - Homocysteine Metabolism



there are relatively low amounts found in the tissues of plants and animals (Green and Jacobsen, 1995). Hyperhomocysteinemia, or high levels of total plasma homocysteine, is represented by plasma concentrations exceeding 15 μM (Refsum et al., 1998).

Homocysteine is the result of the transmethylation pathway in which methionine and ATP combine via methionine adenosyltransferase to form S-adenosylmethionine (SAM), a major donor of methyl groups in numerous transmethylation reactions (Green and Jacobsen, 1995). The product of these transmethylation reactions is S-adenosylhomocysteine (SAH), which is then hydrolyzed by SAH hydrolase to form adenosine and homocysteine (Finkelstein, 1990). Although SAH hydrolase is a reversible enzyme, SAH hydrolysis predominates due to the rapid metabolism of homocysteine and adenosine (Finkelstein, 1990). This is an important regulatory measure since SAH is an inhibitor of transmethylases (Finkelstein, 1990). From this point, homocysteine faces one of two fates: a) re-methylation to methionine or b) transsulfuration to form cysteine (Selhub, 1999).

a) Remethylation: The homocysteine remethylation pathway involves the acquisition of a methyl group to form methionine via one of two enzymes: cobalamin-dependent methionine synthase, or betaine:homocysteine transferase (Finkelstein and Martin, 1984). The 5-methyl group of 5-methyl-THF can only be used metabolically in the remethylation of homocysteine, in the presence of methionine synthase, resulting in the regeneration of methionine (Bailey and Gregory, 1999). Although catalyzing a similar reaction, betaine:homocyteine transferase (BHMT) utilizes betaine, an intermediate in choline catabolism, as a methyl donor to synthesize methionine (Finkelstein, 1990). Methionine synthase is found in virtually all mammalian tissues, whereas BHMT is

found almost exclusively in the mammalian liver and kidney and is independent of the folate pool (Green and Jacobsen, 1995, House et al., 1999). This is important in times of folate and cobalamin deficiency, primary or secondary, since homocysteine can be remethylated using the alternate BHMT route; however, this pathway is incapable of handling excessive amounts of homocysteine and homocysteinemia results (Green and Jacobsen, 1995). Folate must be obtained from the diet and if intake is insufficient to meet requirements, there will be a reduction in the methylation cycle as well as the DNA cycle (Scott, 1999). The importance of B12 and folate in re-methylation is well established.

b) Transsulfuration: The transsulfuration pathway is a catabolic route for homocysteine, and its products include cysteine, cysteamine, taurine, and sulfate (Green and Jacobsen, 1995). This pathway, which diverts homocysteine from the methionine cycle, only occurs in cells that contain active cystathione β -synthase (Green and Jacobsen, 1995). The process begins with the condensation of homocysteine with serine using the vitamin pyridoxal-5'-phosphate (PLP)-dependent cystathionine β-synthase to form cystathionine (House et al., 1999). This reaction is irreversible (Finkelstein, 1990). Next, cystathionine is metabolized by another PLP-dependent enzyme, cystathionine-gamma-lyase, to form the non-essential amino acid cysteine, alpha-ketobutyrate and NH_4^+ (Finkelstein, 1990). The resulting cysteine can be used in protein or glutathione synthesis, or it can be further catabolized (Finkelstein, 1990). It is obvious that PLP is needed in this pathway.

The methionine cycle cannot function efficiently without adequate folate, cobalamin and PLP. If a deficiency of any of these vitamins arises, the cycle will be affected in some

manner, depending on which vitamin is lacking, and homocysteinemia can result (Selhub, 1999). For example, Selhub and Miller (1994) used a rat model to show that fasting plasma homocysteine concentrations increased 8- to 10-fold in folate-deficient rats, 2.5-fold in cobalamin-deficient rats, but showed no elevation in PLP-deficient rats. Conversely, methionine loading caused a marked increase in plasma homocysteine in the PLP-deficient rats but had no significant effect in the folate-deficient rats (Selhub, 1999). The explanation is that during the fed state, when methionine levels are high, the transsulfuration pathway dominates, whereas in the fasting state, remethylation is of prime importance to replenish methionine (Selhub, 1999).

Other amino acids require folate as a coenzyme as well. For example, folate is required for the interconverison of serine and glycine (Rodwell, 1996). Serine is reversibly degraded in a reaction catalyzed by serine hydroxymethyltransferase which requires THF, and which yields glycine and 5,10-methylene-THF. Folate is again required in the cleavage of glycine via the mitochondrial glycine synthase complex, which also requires PLP (Rodwell, 1996). Glycine and THF are reversibly converted to 5,10-methylene-THF, carbon dioxide (CO_2), and ammonium (NH_4^+). It is clear that folate deficiency would have a severe impact on amino acid metabolism.

2.1.3 Digestion, absorption, transport, and storage

Approximately 80% of dietary folate occurs in the polyglutamate form (Swiatlo et al., 1990). Dietary folates must be hydrolyzed from the polyglutamate forms to the monoglutamate forms in order to be absorbed (Sauberlich, 1987). The enzymes responsible for the hydrolysis of the polyglutamates are termed gamma-

glutamylcarboxypeptidases, or simply conjugases (Sauberlich, 1987). The conjugases found in the human jejunal mucosa are either soluble, or membrane bound in the intestinal brush border (McDowell, 2000). Pancreactic juice, bile, kidney and liver also contain conjugase activity (McDowell, 2000). Conjugase activity can be impaired by zinc deficiency, therefore diminishing folate absorption (Bender, 1992). Some foods, such as legumes, tomatoes and oranges contain conjugase inhibitors, which may prevent the hydrolysis of polyglutamates to monoglutamates (Bhandari and Gregory, 1990). Conversely, endogenous conjugases found in certain fruits and vegetables can cleave glutamic acid residues during preparation producing lower pteroylpolyglutamate content in the cooked product than in the immediately heated product (Konings at al., 2001).

Once hydrolyzed, the monoglutamates are actively transported across the intestinal membrane by a Na^+-coupled, carrier-mediated process, at a maximum pH of approximately 6 (Said et al., 1987). Intraluminal pH has an important role in folate absorption. Russell and colleagues (1979) examined the role of intraluminal pH, and the effect of pancreatic insufficiency, on folate absorption *in vivo*, since pancreatic insufficiency results in abnormal acid-neutralizing function in the upper intestine. They also performed an *in vitro* experiment to examine the effects of pH on intestinal pteroylmonoglutamic acid transport in everted rat jejunal rings using a variety of buffers over a wide pH range (Russell et al., 1979). The results revealed a significant increase in mean fasting pteroylmonoglutamic acid absorption in the patients with pancreatic insufficiency over the controls (Russell et al., 1979). In addition, although mean serum folate levels were not significantly different between the two groups, two patients with pancreatic insufficiency had serum folate levels greater than normal (Russell et al., 1979).

The mean intraluminal pH of two patients with pancreatic insufficiency was 6.1 whereas the mean pH was 7.2 in three control subjects (Russell et al., 1979). The results of the in vitro study indicated the maximum uptake of pteroylmonoglutamic acid occurred at a pH of 6.3, and as the pH increased, uptake decreased to a value of 25% of the uptake observed at pH 6.3 (Russell et al., 1979).

Folate-binding proteins, which appear to form part of a carrier system, are also a part of the transport system responsible for transporting folate across the cell membrane, and are found in the intestinal brush border (Sauberlich, 1987). Absorption is most efficient in the jejunum although it is possible throughout the small intestine (Steinberg, 1984). A small amount of folic acid may also be passively absorbed by diffusion (Russell et al., 1979).

Approximately two-thirds of plasma folate is protein-bound to a variety of proteins, including albumin, while the other third is free folate (Steinberg, 1984). Folate is transported into the tissue cells by a carrier-mediated process, which may or may not require ATP (Groff and Gropper, 2000). Once in the cells, the monoglutamate form is converted to the polyglutamate form, which allows the folate to either be stored in the tissue or used as a coenzyme (IOM, 1998). Polyglutamates are formed, by adding glutamate residues, using the enzyme polyglutamyl synthetase (Steinberg, 1984). The glutamates, added to folate by peptide bonds, allows the production of the various folate coenzyme forms, thus the folates in cells will be a mixture of polyglutamyl THFs and various C1 forms of THF such as 10-formyl- and 5-methylTHF (Scott, 1999). These polyglutamate forms trap the folate inside the cell, since only the monoglutamate forms can be transported across cell membranes (Wagner, 1995).

2.2 Folate Nutrition

2.2.1 Stages of Deficiency

Maintaining folate status is important at every stage of life because of its role in one-carbon metabolism. A folate deficiency will result in a number of metabolic alterations and will firstly affect rapidly proliferating cells since they are especially sensitive to abnormalities in DNA synthesis (Gibson, 1990). The first stage of negative folate balance is represented by a serum folate concentration of 3 ng/mL or less (Bailey, 1990). This value, however, is not reflective of body folate stores since serum folate concentrations fluctuate rapidly with recent changes in folate intakes or temporary changes in folate metabolism, even though body stores are stable (Gibson, 1990). The second stage of negative folate balance is tissue depletion and is assessed by erythrocyte levels that drop below 140 ng/mL (Bailey, 1990). Erythrocyte concentrations are less sensitive to short-term fluctuations in folate status than serum folate levels since they decrease only after several months of inadequate intake (Gibson, 1990). They correlate with liver stores and mirror body folate stores (Gibson, 1990). The youngest bone marrow cells synthesizing DNA take up folate thus erythrocyte folate measures are actually a measure of the folate status at the time the erythrocyte was formed (Bailey, 1990). Once erythrocyte levels reach below 120 ng/mL, biochemical functions are altered and this can be confirmed by an abnormal deoxyuridine suppression test (dUST), which detects erythropoiesis, first in bone marrow cells and then in peripheral blood lymphocytes (Gibson, 1990). The methylation of deoxyuridine (dU) to thymidine requires folate, consequently, in folate deficiency, the incorporation of dU in cells *in vitro* is impaired (Gibson, 1990). In the dUST, normal bone marrow or lymphocyte cells are

pre-incubated with non-radioactive dU, which suppresses the marrow cells' ability to incorporate any further added radioactive thymine into DNA. In folate deficiency, the suppression is reduced, resulting in increased incorporation of radioactivity into the DNA. Next, two bone marrow cultures, one with non-radioactive dU and one without, are incubated prior to adding radioactive thymidine. The uptake of radioactivity is counted and elevated dU suppression values >20% are representative of folate deficiency (Gibson, 1990). This is considered stage 2 by some authors (Gibson, 1990) and stage 3 by others (Herbert, 1999) and is characterized by impaired erythropoiesis (Herbert, 1999). DNA synthesis is impaired resulting in a morphological change in the peripheral white blood cells termed neutrophil hypersegmentation (Bailey, 1990). These changes are sometimes referred to as subclinical deficiency (Gibson, 1990). The final stage of negative folate balance is folate-deficiency and it is characterized by macro-ovalocytic erythrocytes and a hemoglobin level less than 13 g/dl (Gibson, 1990). Mean corpuscular volume (MCV) is also increased (Bailey, 1990). Low erythrocyte levels are not specific to folate deficiency since low concentrations also occur in cobalamin deficiency thus to identify folate deficiency, both erythrocyte folate and serum cobalamin levels should be measured (Gibson, 1990). Megaloblastosis results from deranged DNA synthesis of any cause and so in prolonged folate deprivation, megaloblastic anemia will result (Herbert, 1999).

2.2.2 Sources of Folate

Folate is present in a variety of foods. Vegetables such as asparagus, brussels sprouts, leafy greens, and spinach provide an excellent source of folate (Guthrie and

Picciano, 1995). Fruits that are a good source of folate include oranges, avocados, strawberries and melons (Guthrie and Picciano, 1995). Wheat germ, liver, kidney and yeast provide concentrated sources of folate; however, they are a relatively insignificant part of most diets (Guthrie and Picciano, 1995). Since some folate is lost during the process of cooking, due to leaching and chemical degradation, raw foods typically contain more folate (Bailey, 1992). See **Table 2.1** for examples.

2.2.3 Dietary Reference Intakes (DRI's)

In recent years, the Food and Nutrition Board of the Institute of Medicine (IOM), and scientists from Canada, have joined forces to create a comprehensive set of reference values for nutrient intakes of healthy Canadian and United States populations (IOM, 1998). This newly established set of reference values replaces the previously published Recommended Nutrient Intakes (RNIs) for Canada and the Recommended Dietary Allowances (RDAs) for the United States (IOM, 1998). The DRIs consist of a set of at least four nutrient-based reference values with each one having a special use (IOM, 1998). The DRIs include the RDA, the Adequate Intake (AI), the Tolerable Upper Intake Level (UL), and the Estimated Average Requirement (EAR) (IOM, 1998). Current research has suggested that low folate intake is linked to vascular disease and other chronic conditions, along with neural tube defects and other congenital malformations in infants (IOM, 1998). Conversely, high folate intake has been shown to mask the signs of pernicious anemia until after irreversible neurological damage has occurred (IOM, 1998).

Table 2.1 - Food Sources of Folate

Food	Folate (µg per usual serving)	Folate (µg/100 g)
Liver	383	428
Cold cereals (not bran or superfortified)	112	275
Pinto, navy, and other dried beans (cooked)	84	100
Asparagus	82	101
Spinach	70	128
Broccoli	53	65
Avocados	49	62
Brussels Sprouts	47	60
Orange Juice	43	41
Artichokes	43	46
Corn	35	43
Oranges, tangerines	33	26
Cantaloupe	33	17
Eggs	31	43
Cauliflower	28	53
Green Peas	27	46
Beets	27	37
Winter Squash	26	18
Peanuts, peanut butter	25	92
Grapefruit, grapefruit juice	23	10
Papaya, mangos	21	32
Blackberries, raspberries	21	27
Green Beans	21	28
Tomatoes, tomato juice	21	10
2% fat milk	20	5
Green salad	20	48
Bananas	19	19
Strawberries	19	17
Yogurt	18	10
Sweet Potatoes	17	17

Adapted from Subar et al., 1989

Bioavailability and nutrient-nutrient interactions were considered when developing the DRIs for folate and consequently, Dietary Folate Equivalents (DFEs) were established (IOM, 1998).

The poor bioavailability resulting from the poor chemical stability of the natural folates profoundly influences recommendations (Scott, 1999). DFEs are units that account for differences in absorption between food folate and synthetic folic acid from dietary supplements or folic acid-fortified food (Suitor and Bailey, 2000). Therefore, 1 μg DFEs = 1 μg of food folate = 0.5 μg of folic acid taken on an empty stomach = 0.6 μg of folic acid taken with meals (IOM, 1998). The DFEs are based on the assumption that food folates are approximately 50% bioavailable. This translates to folic acid being twice as bioavailable as food folate when taken on an empty stomach, and folic acid taken with food (including folic acid-fortified food) being 1.7 times more bioavailable than food folate (Suitor and Bailey, 2000). The current RDA for folate for both men and women aged 19-50 years is 400 μg/day of DFEs (IOM, 1998). The increase in the recommendation for folate leads to the importance of knowing which foods are a source of folate, and the need to include them in the diet. It is equally important to know which food sources of folate are the most bioavailable. The DFEs facilitate the conversion of micrograms of folic acid to units that are equivalent to folate naturally found in foods (Suitor and Bailey, 2000). The number of micrograms of food folate is equivalent to the number of micrograms of DFEs; therefore, if folate consumption is strictly from food sources, then no adjustments are needed (Suitor and Bailey, 2000). There is also a special consideration for women. It is recommended that women capable of becoming pregnant consume 400 μg of folic acid daily from supplements, fortified foods, or both,

in addition to consuming food folate from a varied diet (IOM, 1998). This consideration is in place to lower the risk of infants being born with neural tube defects (NTDs) (IOM, 1998). Research has shown that folate plays a role in the prevention of NTDs (Brouwer et al., 1999a). Steegers-Theunissen and colleagues (1994) showed that a sub-group of women with a previous NTD affected pregnancy had higher plasma total homocysteine concentrations than healthy control women. It has been suggested that a mildly elevated plasma total homocysteine concentration is a marker for a folate metabolism defect or a folate deficiency and therefore a risk factor for giving birth to a child with a NTD (Steegers-Theunissen et al., 1994). Numerous case-control studies and intervention trials have shown that three-quarters of NTDs could be prevented by the periconceptional ingestion of folic acid; however, it is unclear as to whether the increase in folic acid is treating a folate deficiency or overcoming a metabolic block in folate metabolism (Scott, 1999). In a study performed by Scott et al (1994), the analysis of data revealed that there was a tenfold increase in NTD risk when subjects went from high-normal folate status to low-normal folate status. Their risk of having a NTD-affected birth was strongly reduced even though folate status was initially normal and there were no signs of folate deficiency (Scott, 1999).

An elevated plasma total homocysteine concentration is also a risk factor for cardiovascular disease (CVD) (Brouwer, 1999b). More than 80 clinical and epidemiological studies support this claim (Refsum et al., 1998). Many studies have shown that elevated homocysteine levels are often found in patients with arteriosclerosis affecting coronary, cerebral, and peripheral arteries (Boushey et al, 1995). Clinical and experimental evidence has indicated that increased homocysteine levels cause vascular

lesions (Refsum and Ueland, 1990). Unfortunately, the exact mechanism by which homocysteine may cause vascular lesions is currently unknown (House et al, 1999). A possible explanation is that homocysteine exerts its negative effects by interacting with platelets and/or endothelial cells (Green and Jacobsen, 1995). Folic acid supplementation, as well as the intake of folate-dense foods, improve folate status and decrease elevated plasma homocysteine concentrations in both men and women (Brouwer et al., 1999). This also implies that folate could play a role in lowering the risk of CVD related to hyperhomocysteinemia (Boushey et al, 1995).

Although the DFEs have been developed to address the issue of folate bioavailability, they are not without criticism. The arbitrary assignment to folate bioavailability of all foods of 50%, based on research by Sauberlich and colleagues (1987), was used in establishing the DRIs for folate (IOM, 1998). The DRIs also assume that folic acid consumed with food is 85% based on research executed by Pfeiffer and coworkers (1997) and it is also stated that this value is probably an underestimate of the true bioavailability which may lead to an underestimation of folate requirements (IOM, 1998). An in-depth knowledge of folate bioavailability and the numerous factors involved is needed to understand the complexity of developing an appropriate method for establishing reliable estimates of folate bioavailability.

2.2.4 Bioavailability

Bioavailability is defined as the degree to which an ingested nutrient from a particular source can be absorbed in a form that can be utilized in metabolism (Baker, 1995). Folate is of great interest due to the uncertainty surrounding the bioavailability

(Gregory et al., 1990). Factors that affect folate bioavailability include polyglutamate absorption, intraluminal pH, conjugase inhibitors, milk folate-binding proteins, thermal processing, form of folate, drugs and alcohol, and aging (Bailey, 1988). Generally, the bioavailability of folate is quite variable compared to folic acid. Folate from plant derived foods is typically less bioavailable than from animal products (Combs, 1998). Other factors affect folate availability as well. The reduced forms of pteroylpolyglutamates in foods are labile and easily oxidized resulting in an estimated loss of one-half to three-quarters of initial folate activity due to food preparation and processing techniques (Scott, 1999).

A variety of experimental designs have been developed to estimate the bioavailability of food folates in both humans and animals (Clifford et al, 1990). Bioassays with measurement of increases of plasma, red blood cell or urinary folate in response to single or multiple test doses, isotope techniques based on the recovery of labeled folate or metabolites in the urine after a test dose, and area-under-the plasma-curve method have all been employed (Prinz-Langenohl et al, 1999). The variety of methods used has contributed to the variability of current estimates of food folate bioavailability (Clifford et al, 1990). For example, beef folates were noted to be 50%, 70%, or 100% bioavailable, and orange juice was noted to be 31% or 100% bioavailable, based on different studies (Clifford et al, 1990). The variation in results is due in part to the number of different protocols used to estimate bioavailability, as well as the argument over the appropriate response criterion (Clifford et al, 1990). As a result of the inconsistencies in folate bioavailabilities that now exist, clearly new and more sensitive

protocols are needed. Firstly, a review of previously developed methods for estimating folate bioavailability will be examined.

2.3 Bioavailability Studies

Countless studies have been performed attempting to determine the bioavailability of folic acid and its derivatives. Here they are divided into human models, rat models, and *in vitro* studies.

2.3.1 Human Models

Tamura and Stokstad (1973) were among the first and are consequently often cited in folate bioavailability research. They introduced the concept of maintaining subjects in a 'saturated condition' while estimating folate bioavailability of certain foods by measuring urinary excretion of folate. This was achieved by loading the subjects with large oral doses of folic acid at the beginning of the experiment and continuing regular loading throughout the experiment on alternate days, so that fasting serum folate levels were maintained between 20-30 ng/mL. The authors noted that an oral dose greater than 0.4 mg was needed to cause a significant increase in urinary folate excretion. The results showed the bioavailability of the tested foods to be quite variable. For example, the relative bioavailability of egg yolk folate to folic acid was reported as 39% with a range of 0 to 129%. This is an important study since it explores the use of urinary folate excretion as a method for estimating folate bioavailability. It demonstrates that a substantial dose of folate is needed to reveal an increase of urinary folate excretion, and it considers the saturation protocol as a way to increase urinary response to folate intake.

However, the physiological relevance of the high doses of test foods administered to the subjects must be questioned, as well as the precision of the saturation protocol.

Babu and Srikantia (1976) incorporated the procedure described by Tamura and Stokstad (1973) in a study that sought to determine the bioavailability of some commonly consumed Indian foods and yeast. Ten healthy male subjects between the ages of 25 and 35 years were presaturated with 5 mg of synthetic folic acid orally every day for 6 days, before the bioavailability studies began and continued taking 2 mg on alternate days to maintain saturation (Babu and Srikantia, 1976). The tests were performed on every second day when the subjects did not receive the 2 mg dose of folic acid. The foods tested included: Bengal gram, green gram, spinach, banana, tomato, hen's egg, goat liver, and brewer's yeast. The tests involved the consumption of a known amount of the test food at 9:00 am, followed by measurement of urinary excretion of folate for 24 hours. The bioavailability was calculated from the individual dose-response curves. The authors noted a linear increase in urinary excretion of folic acid with increasing intake of folic acid, along with a high degree of correlation ($r= 0.9683$) between the dose and the urinary excretion of folic acid. The responses were similar within an individual when tested on more than one occasion; however, there were wide variations between individuals in the urinary excretion of folic acid at all levels of intake of the vitamin. Intakes of folic acid below 400 μg resulted in inconsistent urinary excretion of folic acid; consequently, a basal dose of 400 μg was given in addition to the test food, since consuming high enough amounts of the test food was not possible (Babu and Srikantia, 1976). The results revealed a considerable variation in bioavailability of folate among the different foods. The average availability of folate from egg, liver, Bengal gram, green gram, spinach,

banana, and brewer's yeast was 72, 70, 70, 70, 63, 50, and 10%. Also worth mentioning is that five of the seven test foods had upper range values in excess of 100%, which begs the question of whether or not certain food folates are more bioavailable than folic acid, or whether the administration of certain foods can promote the excretion of stored folate (Babu and Srikantia, 1976). Although this method may provide useful information in the area of folate bioavailability research, the small number of subjects and the inter-subject variability is too great to deduce reliable estimates. Clearly an improvement on this method is needed.

Gregory and colleagues (1990) aimed to determine the suitability of using deuterium-labeled folates in humans, and to compare the bioavailability of d2 and d4 forms of folic acid. Stable-isotopic methods had been used *in vivo* for the study of many nutrients, since such methods were reported to provide the specificity attained with radioisotopic procedures, but without the associated concerns (Gregory et al., 1990). They had recently developed methods for synthesizing stable-isotope-labeled folates which produced d4-glutamate-labeled tetradeuterofolic acid (d4-folic acid) and benzene-ring-labeled 3',5'-bideuterofolic acid (d2-folic acid) (Gregory et al., 1990). In addition, a mass spectrometric method was developed which permitted the quantification of isotopic ratios of urinary folates (Gregory et al., 1992). The advantage of concurrent administration of the two isotopes was that the bioavailability of two chemical forms of folate could be compared at the same time; however, it must be ascertained that the labeling has no effect on the absorption or metabolism of the vitamin (Gregory et al., 1990). The study involved 11 male subjects, between 20 and 35 years of age, with normal blood chemistry. They were each given a 1mg tablet of folic acid every morning

and evening, for 7 days prior to the administration of the isotopically labeled folates, to enhance urinary excretion of folates (Gregory et al., 1990). Twenty-four hour urine collections began the morning before the test day and, after an overnight fast, the subjects were given the equimolar mixture of d2- and d4-folic acid in aqueous solution added to 120 mL of apple juice. Urine collections continued for the following 48 hours. The results revealed that there were no differences between d2- and d4-labeled folates in absorption, transport, metabolism, or excretion (Gregory et al., 1990). In addition, d2- and d4-labeled folates appear to behave *in vivo* similarly to unlabeled folates. The authors concluded that this protocol was suitable for *in vivo* human studies of folate bioavailability and kinetics (Gregory et al., 1990). A disadvantage was that the amount of folate ingested (5700 nmol or 2.5 mg) per day is much higher than the daily requirement of 0.4 mg, and thus a physiological irrelevant dose. It would be difficult to consume such high amounts of folate from dietary sources. As well, this method would not be viable for estimation of bioavailability of specific food sources of folate since it is not possible to label folates in intact foods.

Gregory and colleagues (1991, 1992) expanded their stable-isotope protocol in two consecutive studies. The objectives of the first study were to determine the relative bioavailability of d2-labeled mono- and polyglutamyl folates administered orally with a simultaneous dose of d4-folic acid administered intravenously, and to further examine the suitability of single-dose protocols utilizing stable-isotopes for folate bioavailability research in humans (Gregory et al., 1991). The subjects were seven men aged 20-30 years. To enhance folate excretion, the subjects underwent a seven-day saturation period during which time they received a 1 mg folic acid tablet taken twice daily. During the

three-day experimental period all subjects received similar diets which provided approximately 400-500 μg folate each day. Urine collections began on day one, 24 hours before administration of the labeled dose. The first trial involved an overnight fast, followed by omission of the morning folic acid tablet, and an oral 677 nmol dose of d2-folic acid in 120 mL apple juice including a rinse with 100 mL of apple juice. The oral dose was instantly followed by an intravenous dose of 502 nmol d4-folic acid. Urine collections continued for 48 hours. The second trial occurred three weeks later and employed generally the same protocol except that d2-pteroylhexaglutamic acid was administered orally instead of d2-folic acid. The results revealed a significant difference between the trials in excretion of d2-folates but not d4-folates. In the first trial, the 48-hour urinary excretion of d2 folates from the oral dose was greater than the excretion of d4-folates from the intravenous dose, implying *in vivo* differences in activity between d2- and d4-folates administered orally and intravenously, respectively. These differences may occur in folate distribution, enterohepatic recirculation, metabolism or excretion. In addition, the excretion of d2-pteroylhexaglutamic acid was significantly less than the excretion of d2-folic acid in the first trial, and was also significantly less than the excretion of d4-folates in each trial. The authors stated that under the conditions of this study, the bioavailability of the pteroylhexaglutamic acid was approximately half that of the folic acid. These results were consistent with many other studies in that incomplete availability of the polyglutamate folate form was shown; however, there is much variability in results among the various studies. Possible limitations of this design included the high amount of test doses administered to the subjects and the assumption that the excretion of the labeled folates is directly related to its intestinal absorption.

The protocol for the sequel to this study was identical except that different form of folate were tested (Gregory et al., 1992). Instead of pteroylhexagultamate, Gregory and colleagues (1992) examined the bioavailability of various d2-tetrahydrofolates (THFs): 10-formyl-THF, 5-methyl-THF, 5-formyl-THF, and THF. The different forms of folate were tested in the same subjects at three-week intervals. The results showed a progressive increase in 24-hour pre-dose urinary excretion of folate. The estimation of relative bioavailability among the various d2-H4folates based on d2-folate excretion was not possible due to variations in excretion of total and d4-folates over the course of the study, likely as a result of the saturation protocol. The authors utilized d2/d4 ratios to draw conclusions on apparent bioavailability under these conditions. Based on 48-hour urinary d2/d4 ratios for the d2-H4folates, all were significantly lower than the d2-folic acid from the previous study. Noteworthy is that no significant differences were observed among the d2/d4 ratios of the d2-H4folates. The results suggest differences in the *in vivo* processing of monoglutamyl forms of folate. The authors acknowledged that the differences observed in this study may have been the result of differences in mucosal metabolism of the d2-folates, selectivity of folate transport into tissues, or differences in polyglutamylation of the tested H4folates in various tissues. The authors postulated that the high tissue folate concentrations caused by the saturation protocol may have altered *in vivo* tissue uptake and retention. Although differences in apparent bioavailability of the various H4folates were observed and the mechanisms for this phenomenon were not understood, the authors were certain that all were extensively absorbed. The same limitations raised in the previous study hold true for the present study. A disadvantage to this study was the repeated use of the same subjects which, as a result of the saturation

protocol, caused variations in folate excretion. Different subjects for each trial, or a recovery period between trials, in which folate intake was normalized, may have allowed for more conclusive bioavailability results. These studies provided important information regarding the bioavailability of the various forms of folate; however, they cannot predict the availability of endogenous food folates. A protocol is needed in which physiologically relevant doses of endogenous folates are administered under physiological conditions. Radiolabeled folate raises safety issues, and although stable isotopes are better suited for humans, they require sophisticated and expensive equipment for synthesis and measurement of the labeled substances (Prinz-Langenohl et al., 1999).

2.3.2 Rat Models

In 1982, Keagy and Oace questioned the possibility of using the relationship between dietary folic acid and liver folate concentrations as a quantitative standard response curve for a folate bioassay in rats. Previous research had provided convincing evidence that liver folate concentrations were influenced by folate intake, even though folate synthesis by intestinal bacteria reportedly met a considerable share of the rat's requirement (Keagy and Oace, 1982). They were interested in liver folate concentration as the response variable since many early animal assays concerning folate research had focused on non-specific responses such as growth or hematological values in deficient animals (Keagy and Oace, 1982). Antibiotics were not used in this study since the authors wanted the determination of the effect of foods on folate status to be performed under normal physiologic conditions. The objectives of this study were to examine the time required for liver folate depletion as well as the effect of the supplementation period

on the range of the linear response and the precision of the standard curve. Weanling, male, Fisher rats were divided into control and treatment groups. The treatment rats were fed a basal low folate (0.24 mg/kilogram of diet) diet in which 'vitamin free' casein was substituted for soy protein. The control rats received the basal diet with the addition of 4 mg of folic acid per kilogram of diet (Keagy and Oace, 1982). Food was provided *ad libitum*. On day 31, the folate depleted rats were further divided into six groups and given diets containing 0, 0.25, 0.5, 1.0, 2.0, and 4.0 mg of folic acid per kilogram of diet for a period of 28 days (Keagy and Oace, 1982). Livers were analyzed for total folate concentration. The results showed that liver folate concentrations decreased rapidly until day 14, increased slightly at days 28 and 33, and then continued to decrease. The authors concluded that since there was an unexplained increase in liver folate concentrations at 28 days, a depletion period of 35 days is recommended (Keagy and Oace, 1982). They also reported that the 7, 14, or 28 day repletion curves all provided sufficient precision for use as a standard curve for a rat bioassay of relative folate bioavailability. They suggested that 1 week of repletion is adequate when using 1-4 mg folate per kilogram of diet, and 4 weeks when the diet provides less than 1 mg (Keagy and Oace, 1982). This study provided a good basis for continued research in folate bioavailability.

Following several studies indicating that dietary fiber affected folate bioavailability, Keagy and Oace (1984) employed their previously developed rat bioassay to examine the possible interaction between dietary fiber and folic acid. Male, weanling Fisher rats, individually housed in wire-bottomed cages, were used in this trial. During the three week depletion period, the rats were permitted *ad libitum* access to the basal diet which consisted of 'vitamin free' casein as the protein source. For the repletion period,

the rats were assigned to either the fiber-free diet with graded levels of added folic acid, or one of several test diets which contained two levels of fiber or fibrous foods and either 0, 1, or 2 mg of folic acid per kilogram of diet. The test substances were cellulose, xylan, pectin, soft white wheat bran (SWW-bran), hard red wheat bran (HRW-bran), cooked dried beans, and extracted cooked dried beans. The rats receiving the fiber-free diet received 0, 1, 2, 2.5, or 3 mg of folic acid per kilogram of diet. The repletion period lasted seven days. Body weights and food intake were recorded frequently, and beginning on day 5, fecal samples were collected from the rats receiving no added folic acid. The results showed the bran-fed rats had a significantly higher intake during the repletion period than all other diets; however, once corrected for intake, it appeared that food efficiency ratios were similar in all other groups except for that of the higher level of pectin. With the exception of pectin, the dilution of a diet with fiber does not affect growth performance. The results from the liver folate concentrations suggested that many of the fiber sources either supplied folate to the diet or encouraged intestinal folate synthesis. Of the purified fibers, the only effects of cellulose were to increase fecal weight and dilute fecal folate concentrations. The higher level of xylan significantly increased liver folate concentrations likely as a result of fermentation by intestinal bacteria capable of synthesizing folate. The lower level of pectin had no significant effect on liver or fecal folate concentrations; however, it did increase fecal weight. Conversely, the higher level of pectin showed higher fecal folate concentrations with a negligible increase in fecal weight suggesting microbial fermentation resulting in folate synthesis, though liver folate concentrations were not significantly different from the basal diet. A positive significant correlation was noted between fecal and liver folate

concentrations. The authors stated that unabsorbed dietary folate, intestinal folate synthesis, and folate stores evading enterohepatic circulation, may all give rise to fecal folate concentrations. Since the rats were not receiving folic acid, and folate stores were low, it is likely that the increase in fecal folate concentrations was a result of intestinal synthesis. Thus, fermentable fiber may alter folate status in the rat. The authors suggest that rat studies ought to include cellulose as the source of fiber since it neither affects the availability of added folic acid, nor does it stimulate fermentation and thus, intestinal synthesis of folate. Of the natural fiber sources, wheat bran sources had endogenous folate in significant amounts which resulted in an increase in liver folate concentrations. The increase in fecal excretion may have been explained by lack of absorption or by fermentation. Beans also increased liver and fecal folate concentrations and, similar to wheat bran, the increases may have been a result of dietary folate, intestinal folate synthesis, or both.

Abad and Gregory (1987) chose to expand further on the rat bioassay procedure developed by Keagy and Oace (1982). Their objectives were to examine variations for measurement of folate bioavailability, to determine the folate bioavailability for rats of certain foods, and to determine the influence of the tested foods on the bioavailability of exogenous polyglutamyl folate in the diet. The modifications made to the procedure of Keagy and Oace (1982) included the addition of pair feeding, the assessment of plasma folate as a response variable, and the evaluation of the effects of coprophagy. The first experiment began with a 28-day depletion period in which male, weanling Sprague-Dawley rats were fed the basal diet *ad libitum*, which included 'vitamin-free' casein and no added folate. The rats were housed individually in wire-bottomed stainless-steel

cages. For the 12-day repletion period, the rats were randomly divided into groups of ten, and pair-fed one of five diets containing either graded amounts of added folic acid or 15% (wt/wt) orange juice solids. The pair-fed rats received the amount of food consumed by the rats receiving the unfortified diet. The pair-feeding was introduced to reduce any effects caused by the possible differences in palatability of the different diets, although folate deficiency reportedly has little effect on intake. Half of the rats were fitted with tail cup devices for the entire repletion period to prevent coprophagy. The rats were then terminated by decapitation after an overnight fast. The second experiment examined the bioavailability of folate found in orange juice solids and dried cabbage, as well as the effect of these foods on the bioavailability of exogenous folic acid pentaglutamate, using slope-ratio methods but without controlling for coprophagy. The rats endured the same depletion protocol as described in the first experiment. Prior to the 14-day repletion period, the rats were randomly divided into 13 groups of 8 rats each. The diets consisted of the basal diet with the addition of either a graded amount of folic acid, folic acid pentaglutamate, or dried test foods (orange juice solids and dried cabbage). The rats were terminated as described in the first experiment. The results from the first experiment revealed that when coprophagy was prevented, plasma folate under fasting conditions was linearly related to the level of dietary folic acid. Conversely, there was no significant relationship between liver folate levels and dietary folic acid concentration. The use of tail cups was successful in preventing coprophagy resulting in a consistent and significant decrease in plasma folate levels between all groups but with no significant difference in liver folate concentrations. The results suggest that fecal folate recycling contributes to folate status in rats thus demonstrating that dietary

components that stimulate folate synthesis by intestinal microorganisms may cause overestimation of folate bioavailability. The second experiment confirmed a relationship between amount of added dietary folate and fasting plasma folate concentrations. Fasting plasma folate concentrations appeared to be a sensitive indicator of folate bioavailability in short term studies. The apparent bioavailability of folate from orange juice solids and dried cabbage was 146 and 68%, respectively. The apparent available folate in the orange juice solids was significantly greater than the total folate, as it was in experiment one. The authors concluded that the bioavailability of folate in the orange juice solids could not be accurately estimated in this study, although effective utilization by the rats is believed. In addition, it appeared that for all three diets, the bioavailability of folic acid pentaglutamate was not significantly different from 100%, relative to folic acid, indicating that neither orange juice solids, nor cabbage, had an effect on the deconjugation and utilization of folic acid pentaglutamate. The authors suggested that test materials be analyzed in the bioassay for at least three levels of inclusion to the basal diet to facilitate evaluation of the dose-response curves; however, this may prove to be lengthy, costly and labor-intensive. Other factors that may affect the results obtained by a rat bioassay include large differences in rate of folate absorption and intestinal transit time, and dietary interference with enterohepatic circulation. Consideration to possible influences of the components of the basal diet on folate utilization must also be given.

The development of folate deficiency in rats fed either amino-acid based diets or diets containing casein was evaluated by Walzem and Clifford (1988). The main objective of their research was to develop a standardized protocol for the production of severe, yet uncomplicated, folate deficiency in rats during maximum growth stage, within

a period of 4 weeks, while maintaining a nutritionally balanced diet. The results revealed that that an amino acid-based, folate-free diet was more effective than a casein-based, folate-free diet at producing folate deficiency since casein has an endogenous folate content of 27 μg/kg. Also, the addition of 1% succinylsulfathiozole to the diets was necessary for maximum reduction of liver and whole blood folate levels. The findings of this study succeeded in elucidating key components necessary to produce folate deficiency in rats.

Swiatlo and colleagues (1990) examined the bioavailability of folate in human, bovine, and goat milk, relative to a milk-free control diet. They employed slope-ratio statistics to estimate the bioavailability, and also compared the sensitivity of plasma, kidney, liver, and red blood cell folate concentrations as indicators of folate bioavailability (Swiatlo et al., 1990). A rat model was used. The study design consisted of a 12 week depletion period during which the rats were fed a folate deficient (66 μg/kg diet), semipurified diet, formulated according to the AIN-76A diet, which utilized 'vitamin-free' casein as a protein source (Swiatlo et al., 1990). The control rats were fed the semipurified diet with the inclusion of folic acid (1400 μg/kg diet). Food and demineralized water were provided *ad libitum*. Following the depletion period, the depleted rats were randomly assigned to 1 of 16 test diets. Four groups were given milk-free (MF) diets with graded amounts of folate, and the remaining 12 groups were given one of three milk-containing (MC) diets (human, bovine, or goat) with graded amounts of folate (Swiatlo et al., 1990). All test diets were formulated to contain 0, 200, 400, or 600 μg of folic acid/kg diet. The repletion period lasted four weeks. The results suggested that human milk incorporated into the diet increased folate bioavailability so that the

human MC diet had a 40% and 133% greater bioavailability than bovine and goat MC diets, respectively (Swiatlo et al., 1990). Regarding the indicators of folate bioavailability, plasma folate concentrations appeared to be the most significantly correlated to total folate intake while kidney concentrations showed a less sensitive but linear relationship. There was no significant relationship between either liver nor red blood cell folate concentrations and total folate intake (Swiatlo et al., 1990).

In 1991, Rong and colleagues confirmed that folate synthesized by the microflora in the intestine is indeed incorporated into host tissue folate pools. Tritiated *p*-aminobenzoic acid ([^{3}H]PABA) was injected directly into the rat cecum to label the folates that are synthesized by the intestinal microflora. Coprophagy was prevented. The liver and kidneys were analyzed for folate content. Approximately 7% of the total [^{3}H]PABA administered was recovered as [^{3}H]folate synthesized by the intestinal bacteria. The findings of this research provide continuous support for the use of antibiotics in establishing folate deficiency.

2.3.3 *In Vitro* Studies

An important consideration when studying the bioavailability of folate is the deconjugation of the polyglutamate form to the monoglutamate form via folate conjugases. Wang and colleagues (1985) cited the differences in conjugase activity between rats and humans. Research had shown that the rat intestinal folate conjugase is a soluble enzyme with an optimum pH of 4.5. On the contrary, humans exhibit two separate conjugase activities. The human brush border folate conjugase (BBFC), located on the surface of the cell, exhibits exopeptidaselike activity and functions best at a pH of

6.5. The human intracellular folate conjugase (ICFC) is soluble with a pH optimum of 4.5. Wang and colleagues argued that the differences in conjugase activity between humans and rats presented a limitation in using rat models for folate bioavailability research. Previous research had shown similarities in folate conjugase activity between pig and human intestine. Wang and colleagues (1985) compared folate conjugase activity of the brush border and supernatant fractions from human, pig, rat, and monkey intestinal mucosa. Folate conjugase was measured both in the brush border and the prepared supernatant fraction. In the human and the pig brush border fractions, folate conjugase was active from pH 4.5 to 8.0, while very little activity was present in the rat or the monkey brush border fractions. In the supernatant fractions, folate conjugase was active over a pH range of 4.5 to 5 in the human, the pig, and the monkey mucosa, and active over a pH range of 4.0 to 7.5 in the rat. The addition of zinc caused a significant increase in folate conjugase activity in both the human and the pig brush border fractions. Except for the pig, there was no effect of zinc on the folate conjugase activity in the supernatant fractions. The folate conjugase activity in the supernatant fractions was similar in the pig, the rat, and the monkey; however, it was twofold higher in the human. The results of this *in vitro* study demonstrated the similarities in folate conjugase activity between the human and the pig, and suggested that the pig is more suitable than the rat or the monkey as a model for research pertaining to folate conjugases and folate absorption in humans. Noteworthy is that neither the rat nor the monkey was exposed to polyglutamyl folates in their diets and previous research had shown that the activity of brush border folate conjugase may be increased by exposure to this form. A further step would be to examine the brush border folate conjugase activity in the rat and the monkey

after being exposed to polyglutamyl folates in their diets. Although the pig may prove to be a more precise model for folate bioavailability, the rat model has provided researchers with much valuable knowledge. The rat model provides a rapid turnover of folate with high sensitivity when employing a depletion-repletion protocol, and the ability to quantify folate bioavailability using plasma and various tissues (O'Leary and Sheehy, 2001). The cost to perform similar studies on the pig must also be considered.

In 1990, Bhandari and Gregory decided to further examine brush border folate conjugase in the human and the pig. Wang and colleagues (1985) had previously shown that the folate conjugase activities of the human and the pig were similar in their action. Bhandari and Gregory (1990) thought it important to determine whether components of certain foods inhibited folate conjugase activity and thus, deconjugation of polyglutamyl folates *in vitro*. Human and porcine jejunal segments were used. The test foods included: red kidney beans, green beans, black-eyed peas, yellow-corn flour, whole-wheat flour, medium rye flour, wheat bran, cauliflower, cabbage, lettuce, spinach, banana, tomato, whole egg, evaporated milk, and orange juice solids. The prepared food extracts were added to the assay mixture for determination of their *in vitro* effects on enzyme activity. For evaluation of food components that could possibly bind or trap triglutamyl folates, the food extracts were incubated with triglutamyl folates in a manner similar to the enzyme assay but excluding the enzyme. There was a 25-35% inhibition of conjugase activity in the pig by red kidney beans, pinto beans, green beans, and black-eyed peas, while the inhibition by banana and spinach was 20-25%. Tomato and orange juice solids showed a much greater inhibition at 46 and 80%, respectively. The extracts from whole-wheat flour, medium rye flour, yellow-corn flower, wheat bran, whole egg,

milk, cabbage, cauliflower, and lettuce did not significantly affect enzyme activity. The same foods that caused inhibition in the pig brush border also caused inhibition in the human brush border. The range for pinto beans, lima beans, and black-eyed peas was 29-35%, while the range for red kidney and green beans was lower at 16-19%. There was also a 25% inhibition by banana. The range for spinach, cauliflower, and wheat bran was lower but still significant at 14-16%. Finally, tomato and orange juice solids again showed a strong inhibitory effect of 46 and 73%, which was similar to the results seen in the pig. In general there was an inhibition of folate conjugase activity in the jejunal brush border by legumes, orange juice solids, and tomatoes, which increased with increasing concentrations. The incubation of the food extracts (pinto beans, lima beans, tomato, and orange juice solids) with the triglutamyl folate revealed no indication of binding of the triglutamyl folate by components in the foods. In regards to the high inhibition caused by tomato and orange juice solids, the authors reasoned that the high concentration of citrate contained in these foods was responsible; however, when tested independent of the foods, citrate produced a milder inhibition at the same concentrations than the foods in which it was found. The authors also wanted to determine if the inhibition of folate conjugase activity, by components of certain foods, was a result of a specific action on the conjugase activity, or a non-specific effect on the jejunal membrane in general. The effects of pinto beans, green lima beans, tomato, and orange juice on alkaline phosphatase and sucrase activity in the pig jejunum were determined, and no significant effect was observed. Worth mentioning is that all food extracts were neutralized before testing, thus the inhibitory effects caused by the foods was not the result of the enzyme reaction mixture pH. The authors concluded that although an inhibition of folate

conjugase activity by certain food components was shown, the exact mechanism exerting this effect had yet to be elucidated. The inhibition of brush border folate conjugase by these foods may be a factor affecting folate bioavailability. A comparison with rat jejunum previously fed polyglutamyl folate would have been beneficial since previous studies did not involve food sources of polyglutamates. As well, it would have been interesting to include intracellular conjugases as well as brush border conjugases. Finally, it must be recognized that *in vitro* studies do not parallel *in vivo* situations, and further studies are needed in this area. Bhandari and Gregory (1990) suggest the use of stable-isotopic protocols developed in their laboratory to further research diet composition effects on folate bioavailability.

2.3.4 Summary

Evidently there is a need for a new and more sensitive method for estimating the bioavailability of folate in foods. Clearly the rat is a useful model for folate bioavailability research, although some researchers argue that the rat is not suitable due to the claimed differences in folate digestion between humans and rats. Wang and colleagues (1985), stated that since brush border enzyme activity is negligible in the rat, and the intracellular conjugase in the rat exhibits a different pH dependence than in the human, the pig may be a more suitable model. However, these findings do not apply to the intestinal absorption of monoglutamyl folate because according to Said and colleagues (1987), transport in human intestinal brush border membrane vesicles is similar to the transport in the intestines of both rats and rabbits. Thus the use of the rat model in estimating the bioavailability of folates that are provided in the monoglutamyl

form is justified. Furthermore, Reisenhauer and Halsted (1987) provided evidence that the hydrolysis of polyglutamyl folate is not a limiting factor in folate absorption. Moreover, a recent study by Said and colleagues (2000) examining the effect of dietary folate deficiency on intestinal folate uptake in rats, tested for the presence of brush border membrane folate conjugase activity and found that it indeed exists, and is significantly upregulated in folate deficiency. Thus there is no cause to believe that the rat is not an effective and suitable model for folate bioavailability research.

Based on the research cited above, there are many considerations when using a rat model for the estimation of folate bioavailability. Generally, researchers use male, weanling rats (often Sprague-Dawley), housed individually in stainless steel, wire-bottom cages to prevent coprophagy. The depletion-repletion protocol typically requires 4 weeks for depletion and at least 2 weeks for repletion. To establish severe deficiency, the deficient diet must be amino acid-based, must be sufficient in all nutrients except folate, and must contain an antibiotic (typically 1% succinylsulfathiozole) to prevent bacterial synthesis of folate in the intestine. Outcome measures include growth, and liver and plasma or serum folate concentrations, although evidence suggests lack of sensitivity and specificity. Alternatively, a functional indicator would directly measure the nutritional status of the rat with respect to folate (Gregory, 1988). This has led to the possibility of using plasma homocysteine as a new and sensitive method of estimating folate bioavailability in rats. Low serum folate levels are often noted in conjunction with high homocysteine levels, which can be lowered by administration of folic acid (Boushey et al, 1995). In fact, there is an inverse correlation between the levels of folate and homocysteine in the blood (Wagner, 1995). Miller and colleagues (1994) showed that

folate deficient rats receiving supplementation of increasing levels of folate resulted in graded decreases in plasma homocysteine levels. Thus the sensitivity of homocysteine as a marker of folate status is introduced. Since plasma homocysteine has been shown to be a sensitive marker of folate status, the potential of its use in estimating folate bioavailability is probable; however, several confounding factors must be considered. As explained earlier, cobalamin and PLP are also important in the methionine cycle. A deficit in these vitamins, or a disruption in their corresponding enzymes, will also cause hyperhomocysteinemia. In addition, gender, and age affect homocysteine metabolism. Many studies have shown that women tend to have lower fasting homocysteine concentrations than men (Fukagawa et al., 2000). It is also known that plasma homocysteine levels increase with age (Selhub, 1999). Other conditions that are associated with high plasma homocysteine include impaired renal function, high plasma creatinine, smoking, coffee consumption, and drugs such as folate antagonists, nitrous oxide, and L-DOPA (Selhub, 1999). Consequently, with proper controls for confounding factors, the use of plasma homocysteine as a new and sensitive method for estimating the bioavailability of folate is plausible.

Chapter 3 – Hypotheses and Objectives

3.1 Hypotheses

Alternate Hypothesis: Folate bioavailability can be assessed in a rodent model using plasma homocysteine as an outcome measure.

Null Hypothesis: Folate bioavailability can not be assessed in a rodent model using plasma homocysteine as an outcome measure.

3.2 Objectives

The purpose of this research is to characterized and test a method for estimating folate bioavailability. This will be accomplished through the following objectives:

i) To characterize the time course of folate depletion in rats with respect to measures of folate status, including plasma and liver folate, plasma homocysteine, and plasma amino acid concentrations.

ii) To determine the responses of folate status determinants in folate depleted rats to folate repletion from either crystalline folic acid (CFA) or folate-enriched egg yolk.

iii) To develop a negative slope-ratio bio-assay for the determination of folate bioavailability in foods.

Chapter 4 –Characterization of Temporal Changes in Folate Status in a Rodent Model of Folate Deficiency

4.1 Introduction

The importance of achieving adequate folate status is well-established. The principle function of folate coenzymes is to accept or donate single-carbon units in key metabolic pathways (Bailey and Gregory, III, 1999). Folate status is dependent on both the amount and the bioavailability of the folate consumed (Abad and Gregory, 1987). The rat is a valuable model for folate bioavailability studies and has been used extensively. Rats experience rapid turnover of folate, are highly sensitive to depletion-repletion models, and are useful for estimating folate bioavailability based on plasma, and tissue folate concentrations (O'Leary and Sheehy, 2001).

Keagy and Oace (1982), utilized a folate depletion-repletion protocol, to demonstrate that the liver folate concentration in the rat is suitable as a response variable in a folate bioassay. Abad and Gregory (1987) found that fasting plasma folate concentration is a sensitive indicator of available dietary folate and that the prevention of coprophagy results in a significant reduction in folate status (Abad and Gregory, 1987). Research by Walzem and Clifford (1988) established that an amino acid-defined diet was more successful than a casein-based diet since casein has an endogenous folate content. As well, the authors state that the addition of 1% succinylsulfathiozole was necessary for maximum reduction of liver and whole blood folate levels (Walzem and Clifford, 1988). Additional research by Clifford and colleagues (1990) further supported the use of serum folate as an indicator of total body folate. In 1991, Rong and colleagues confirmed that folate synthesized by the microflora in the intestine is indeed incorporated into host

tissue folate pools, which highlights the use of antibiotics in establishing folate deficiency. The inverse relationship between folate and homocysteine led Miller and colleagues (1994) to demonstrate the sensitivity of plasma homocysteine levels to hepatic folate concentrations, introducing a potential new functional indicator of folate status.

It is generally agreed upon that an effective folate depletion rat protocol includes an amino acid-defined, folate deficient diet with antibiotics (most often 1% succinylsulfathiozole), for a period of 28 days, in a wire-bottom, stainless steel cage to prevent coprophagy. However, previous research has failed to provide detailed information regarding the time-course of plasma folate concentrations during depletion. In addition, the recent interest in homocysteine as a functional indicator of folate status has led to the need to develop the time-course for plasma homocysteine during folate depletion, as well as the amount of time needed to sufficiently elevate plasma homocysteine concentrations. Finally, plasma amino acid profiles during folate depletion have not been previously elucidated and may help to clarify the link between folate deficiency and disease.

The objectives of the present study are to characterize the time-course for measures of folate status during folate depletion, and to establish the appropriate time-period needed to achieve optimum folate depletion based on plasma homocysteine concentrations. Plasma and liver folate, plasma homocysteine, and plasma amino acid concentrations will all be examined with respect to folate status.

4.2 Materials and Methods

4.2.1 Animals:

Weanling, male, Sprague-Dawley rats were used. They were purchased from Central Animal Care (Winnipeg, MB). The rats were housed individually in wire-bottomed, stainless steel cages to minimize coprophagy. The room was kept at 21±2°C and 45±5% humidity, and 14 hours of light were provided daily. The rats had *ad libitum* access to water. Approval was obtained from the Protocol Management Review Committee in accordance with the Canadian Council of Animal Care.

4.2.2 Diets:

The diets were purchased from Harlan Teklad (Madison, WI) and consisted of a 17% amino acid-defined diet, either with folic acid or without (**Table 4.1**). PLP, which is also an important component of the methionine cycle, was provided at concentrations consistent with the AIN-76A diet (American Institute of Nutrition, 1980). The diets were in powder form and also contained 1% succinylsulfathiozole to inhibit the growth of folate synthesizing bacteria in the intestine.

Table 4.1 – Diet Composition

TD 01042 Folate-Free Diet	
	g/kg Diet
L-Alanine	3.5
L-Arginine	12.1
L-Asparagine	6
L-Aspartic Acid	3.5
L-Cytine	3.5
L-Glutamic Acid	40
Glycine	23.3
L-Hisitidine HCl.H2O	4.5
L-Isoleucine	8.2
L-Leucine	11.1
L-Lysine HCl	18
L-Methionine	8.2
L-Phenylalanine	7.5
L-Proline	3.5
L-Serine	3.5
L-Threonine	8.2
L-Tryptophan	1.8
L-Tyrosine	5
L-Valine	8.2
Sucrose	354.4
Corn Starch	150
Maltodextrin	150
Soybean Oil	80
Mineral Mix[1]	35
Calcium Phosphate	8.2
Succinylsulfathiazole	10
TBHQ	0.02
Choline Bitartrate	2.5
Nicotinic Acid	0.0339
Calcium Pantothenate	0.0181
Pyridoxine HCl	0.0079
Thiamine HCl	0.007
Riboflavin	0.0068
D-Biotin	0.0003
Vitamin B12 (0.1% in mannitol)	0.0283
DL-Alpha Tocopherol Acetate (500 IU/g)	0.1695
Vitamine A Palmitate (500 000 IU/g)	0.009
Vitamin D3 palmitate	0.0023
Vitamin K, Phylloquinone	0.001

[1]As per AIN-76A diet (American Institute of Nutrition, 1980)

4.2.3 Experiment:

Sixty-five rats were acclimatized to the environmental conditions and fed lab chow for 7 d. Five rats were terminated to obtain baseline data. In the fed state, the rats were anesthetized by intraperitoneal injection of 1.5mL/kg body weight of ketamine/xylazine (2:1). The abdominal and thoracic cavities were opened and blood was collected into heparinized vacutainers via direct cardiac puncture. Liver and kidneys were excised also. Blood was immediately chilled on ice and tissues were immediately frozen with liquid nitrogen (-70°C). Blood was then centrifuged, plasma was separated, and stored, along with the tissues, at -80°C for future analysis. The remaining 60 rats were randomly divided into two groups of 30 rats. The first group was the treatment group and they were fed the folate-free diet. The second group was the control group and they were fed the diet that included folic acid at 1mg/kg of diet. Each control rat was randomly assigned to be pair fed with a treatment rat to ensure that any differences in growth or biochemical values were due strictly to the absence of folic acid. The duration of the experiment was six weeks and data was obtained at weeks 1, 2, 3, 4, 5, and 6, by terminating five treatment rats and five control rats each week, as stated above, to obtain plasma, kidneys, and liver for analysis.

4.2.4 Analyses of Plasma and Tissues:

Plasma folate was determined by using a Quantaphase II B12/Folate Radioassay purchased from Bio-Rad (Hercules, CA). All reagents and samples were brought to room temperature prior to beginning the assay. The reaction tubes (12- x 75-mm) were

appropriately labeled and 200 μL of each standard, control or plasma samples were added to the corresponding tubes. All tubes received 1 mL of working tracer and were vortexed. Total count tubes were prepared by adding 1 mL of working tracer to the reaction tubes and were then set aside until further on in the assay. The test tube rack was covered with aluminum foil and placed in a boiling water bath at 100°C for at least 20 minutes, followed by a cold water bath for approximately 10 minutes to cool the tubes to room temperature. Next, 100 μL of blank reagent was added to the blank tubes and 100 μL of microbead reagent was added to all but the blank tubes. All tubes were vortexed and incubated at room temperature for 1 hour. The tubes were then centrifuged (Jouan, CR 3000) for 10 minutes at 1500 x g and immediately decanted, using an appropriate tube rack, to discard the supernatant. After decanting, the tubes were immediately returned to their upright position. Finally, all tubes were counted for 1 minute using a gamma counter (LKB Wallac, 1282 Compugamma). The folate concentrations contained in the samples were determined by using a standard curve that was prepared with the assay and mathematically fit to the curve.

Plasma homocysteine was determined by high pressure liquid chromatography (HPLC) using the method outlined by Vester and Rasmussen (1991). Firstly, a 0.1 mM D,L – homocysteine standard was made adding 27.04 mg D,L-homocysteine to 100 mL of 0.1M potassium borate buffer (see Appendix for recipe). Using the 0.1 mM solution as 100% concentration, a 1 mL standard curve of 0, 25, 50, 75, and 100% was prepared, with 0.1 M K-Borate buffer as the diluent. Next, 150 μL of each prepared standard curve and sample was pipetted into labeled microcentrifuge tubes in duplicate, 20 μL of tris (2-carboxyethyl)-phosphine hydrochloride (TCEP) (see Appendix for recipe) solution was

added, and the samples were vortexed (IEC, Micromax). The samples were incubated at room temperature for 30 minutes, followed by the addition of 0.6 M perchloric acid to cause protein denaturation, and then centrifugation for 10 minutes at 10,000 rpm. New microcentrifuge tubes were labeled and 100 μL of each standard and sample was transferred into the new tubes with the addition of 200 μL of 2 M potassium borate buffer (pH 10.5) (see Appendix for recipe) and 100 μL of 7-flurobenzo-2-oxa-1,3-diazole-4-sulfonic acid (SBDF) (see Appendix for recipe), followed by vortexing. The tubes were placed in a 60°C water bath for 1 hour, and then in a cold room to cool for 20 minutes. Finally, approximately 200 μL of the standard/sample was transferred to the HPLC vials and analyzed by HPLC. An isocratic elution system was used, consisting of 0.1 M Sodium Acetate, pH 5.0, with 2% methanol. The flow rate was 1.0 mL/min and the plasma homocysteine concentrations were determined by fluorescence detection (Shimadzu, Mantech, Guelph, ON) with an excitation of 385 nm and an emission of 515 nm with reference to an external standard curve.

Liver folates were extracted according to the method of Abad and Gregory (1987). Approximately 1 g of each liver sample was measured into centrifuge tubes and weights were recorded. Next, 10 mL of 0.05 M sodium acetate buffer (pH 4.9), containing 57 mM ascorbate, was added to the tubes, which were capped and immediately put on ice. The samples were then homogenized, topped with nitrogen gas, and placed in a 37°C hot water bath for 90 minutes, followed by a boiling water bath for 10 minutes and centrifugation at 12, 000 x g at 4°C for 30 minutes (Beckman, Model J2-21M). The resulting supernatant was poured into labeled 25 mL volumetric flasks. Another 10 mL ascorbate buffer was added to the centrifuge tubes and centrifuged again,

as stated above, for 15 minutes. The remaining supernatant was removed and added to a corresponding volumetric flask. The flasks were brought to volume with ascorbate buffer and inverted 5 times to thoroughly mix the samples. A small portion of the sample was transferred to microcentrifuge tubes and stored at -80°C until 5-methyl-THF analysis by HPLC. Liver 5-methyl-THF concentrations were determined by reverse phase-HPLC, using the method of Vahteristo et al. (1997). Initial solvent delivery conditions consisted of 90% 30 mM KH_2PO_4 (pH 2.2) and 10% acetonitrile delivered at a rate of 1 mL/min using a 4.5 X 25 cm C-18 column (Waters, Fisher Scientific, Nepean, ON). 5-methyl-THF was resolved by increasing, in a linear fashion, the acetonitrile concentration to 25% between 4.0 and 15.0 min. Initial running conditions were re-established between 15.0 and 20.0 min, with a total run time of 35.0 min. 5-methyl-THF was detected by fluorescence detection (excitation: 290 nm; emission 360 nm) with peak areas integrated using the Class VP software package (Shimadzu, Mantech, Guelph, ON). Concentrations were determined against an external standard curve generated using a 5-methyl-THF, barium salt standard (0 – 0.25 micromoles/L) in an ascorbate buffer.

Plasma amino acid concentrations were measured using the method of Blom and Huijmans (1985). In brief, 250 microlitres of plasma were mixed with 250 microlitres of a 5% sulfosalicylic acid solution containing 0.5 mmoles/L of norleucine as internal standard, in a 1.5 mL microcentrifuge tube. The mixture was placed at minus 20 degrees celcius overnight, thawed and centrifuged at 10, 000 x g for 10 min. An aliquot (40 microlitres) of the supernatant was filtered and injected onto a cation-exchange column contained within the LKB 4151 Alpha-Plus Amino Acid Analyzer. Amino acids were separated using a gradient elution system as previously described (Blom and Huijmans,

1985), reacted with ninhydrin and detected by 2-channel ultraviolet detection (440 nm and 570 nm). Peak areas were integrated using the Biochrome Integration Software Package.

4.2.5 Statistical analyses:

The experiments were completely randomized. Statistical analysis was performed by ANOVA using SAS Analyst (SAS Institute Inc., 1999). Differences between means were determined using the protected-LSD method. Differences with an α level of $P<0.05$ were considered to be statistically significant.

It was necessary to log transform the data for plasma folate and homocysteine, and liver folate, since a plot of residuals versus predicted values revealed heterogeneity in the variability.

4.3 Results

It was necessary to terminate 2 treatment rats before the final termination date. The first rat was terminated 2 days prior and the second rat was terminated 1 day prior to the scheduled termination date. Both rats had lost in excess of 10 % body weight in less than 1 week. They were also dehydrated, experiencing loss of appetite, and appeared very ill with signs of lesions forming around the mouth. Their pairs were terminated also. In general, there was a noticeable difference in appearance between the treatment and control groups. The treatment rats displayed fur that was sparse and had a yellowish colour compared to the control rats with fur that was thick and white. The skin on the ears and feet of the treatment rats appeared pale compared to the control rats whose skin

was still quite pink. In addition, the control rats were much more alert and active compared to the lethargic and subdued appearance of the treatment rats.

4.3.1 Growth:

The growth of the rats was not significantly different between the treatment and the control groups for the entire duration of the trial (P=0.55) (**Figure 4.1**). There was a significant time effect (P<0.001); however, there was no significant treatment by time interaction (P=0.9215).

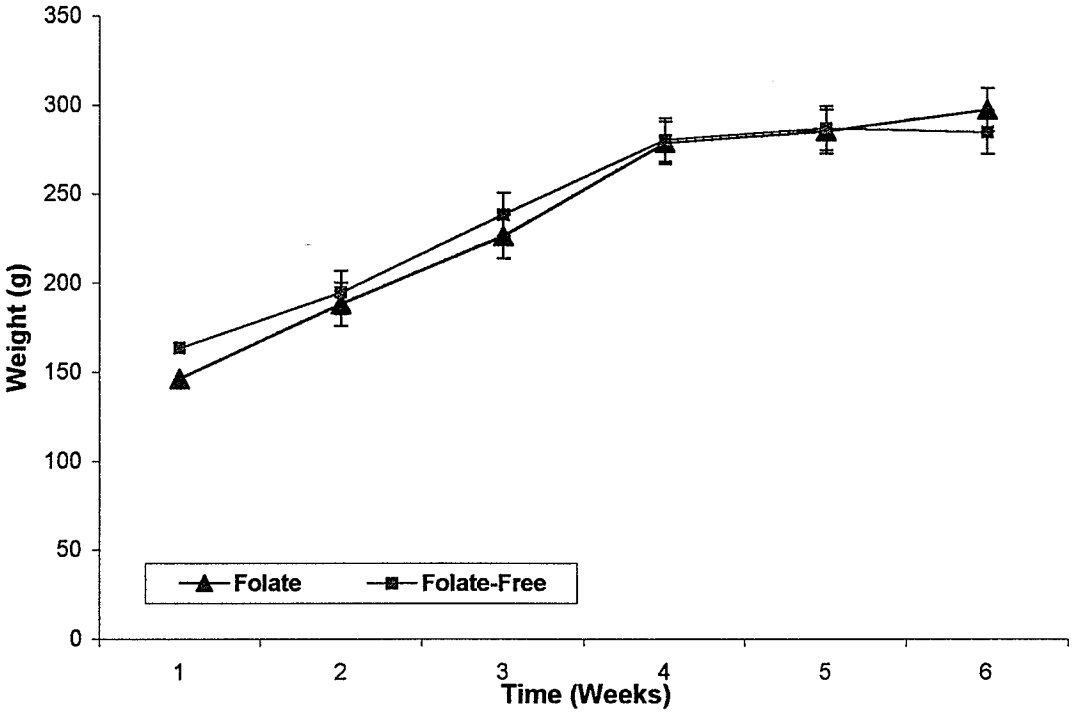
4.3.2 Feed Intake:

Feed intake was not significantly different between the treatment and control groups throughout the trial (**Figure 4.2**).

4.3.3 Plasma Folate:

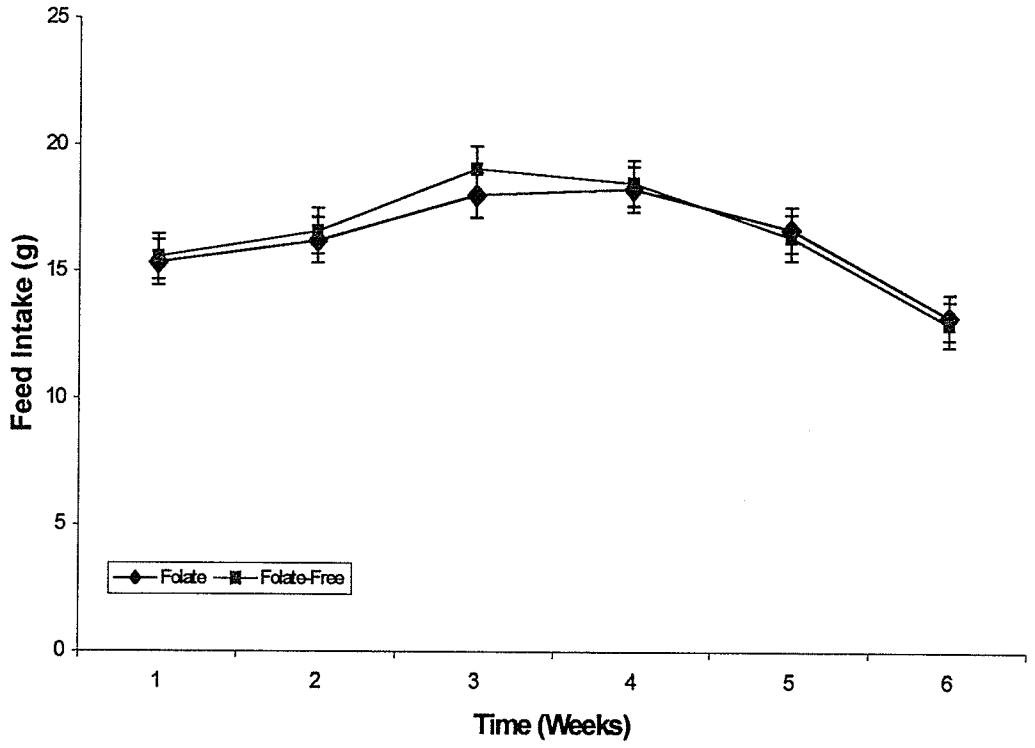
After 1 week of folate depletion, the rats receiving the folate-free diet had a significantly lower plasma folate concentration than the control rats receiving folate in their diet (P<0.0001), and by week 4 there was approximately a 50-fold difference between the 2 groups (P<0.0001) (**Figure 4.3**). There was no significant difference within the control group over time (P=0.72), and the folate-free group experienced no further significant decrease following week 4.

Figure 4.1 - Growth Performance in Rats During 6-Week Folate Depletion Period



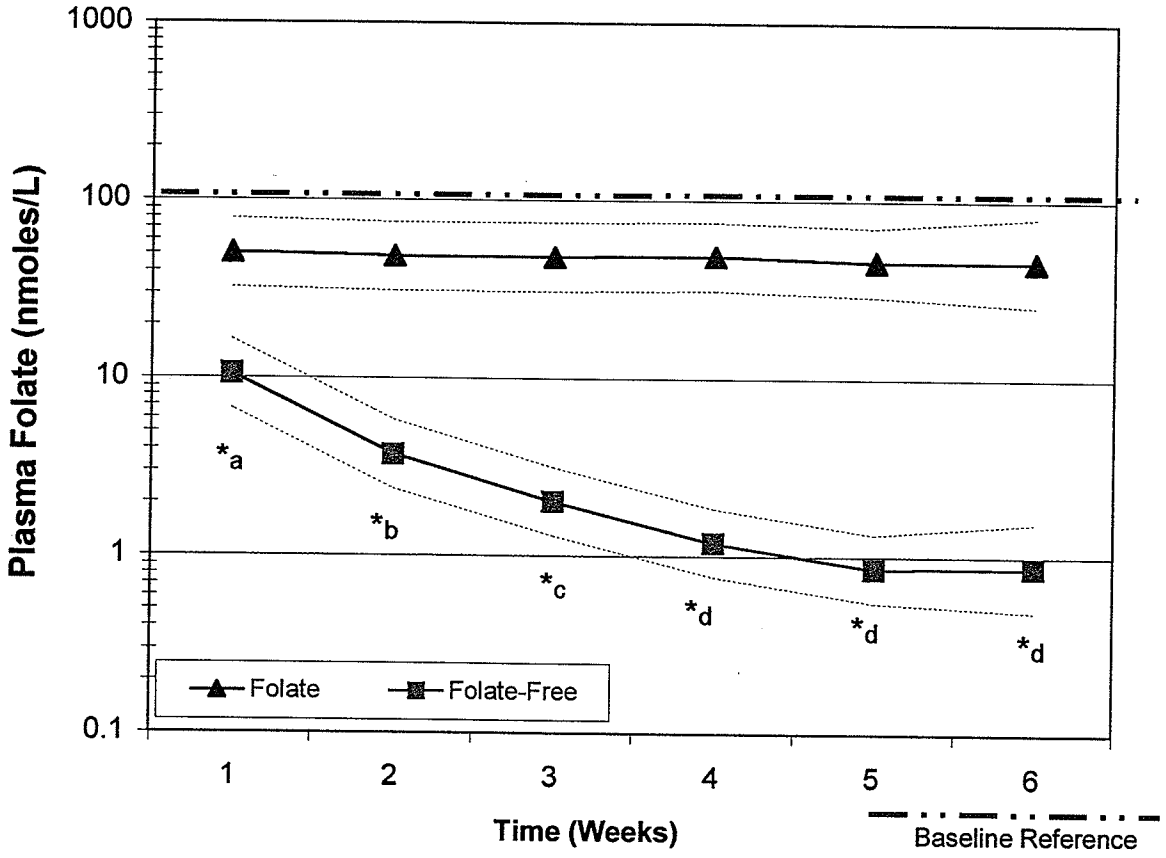
Data reported as LS Means +/- Standard Error
Treatment Effect P=0.5466
Week Effect P<0.0001
Treatment*Week Interaction P=0.9215

Figure 4.2 - Average Daily Feed Intake in Rats Following 6-Week Folate Depletion



Data reported as LS Means +/- Standard Error
Treatment Effect P=0.687
Treatment*Week Interaction P=0.9782

Figure 4.3 - Time Course for Plasma Folate Depletion in Rats during 6-Week Folate Depletion



Absolute differences are proportional to log units
Geometric means with 95% confidence intervals of Log10 transformed data
* Means significantly different ($P<0.05$) from corresponding time point in Folate group as determined by protected LSD
Means with different alphabetical notation within a treatment are significantly different ($P<0.05$) as determined by protected LSD

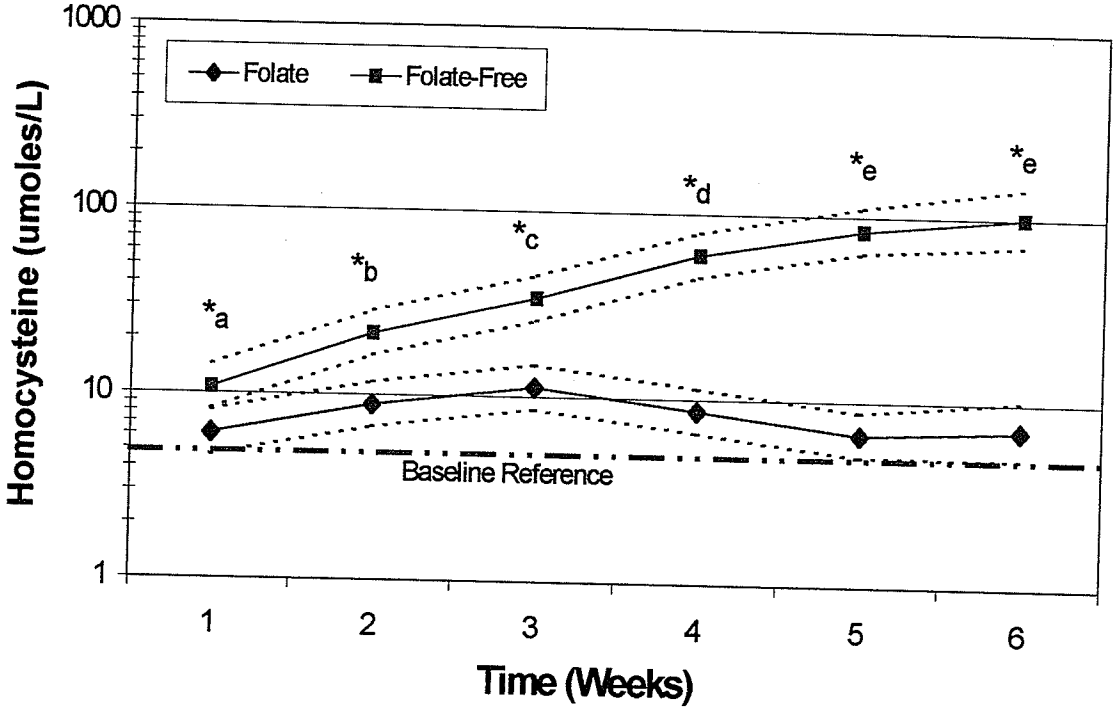
4.3.4 Plasma Homocysteine:

Plasma homocysteine concentrations nearly doubled after week 1 (P=0.0006), and increased almost 7-fold by the end of week 4 (P<0.0001) in the treatment group (**Figure 4.4**). There was a small but significant increase from week 4 to week 5 (P=0.03) though after week 5 there was no further significant increase in concentrations (P=0.32). Plasma homocysteine fluctuated somewhat within the control group; however, there was no overall significant difference from week 1 to week 6 (P=0.39). The two rats that were terminated early in the folate-free group had plasma homocysteine levels of 235.8 μmoles/L and 161.6 μmoles/L, respectively, whereas their pairs, also terminated early, had levels of 5.39 μmoles/L and 8.21 μmoles/L, respectively.

4.3.5 Liver Folate:

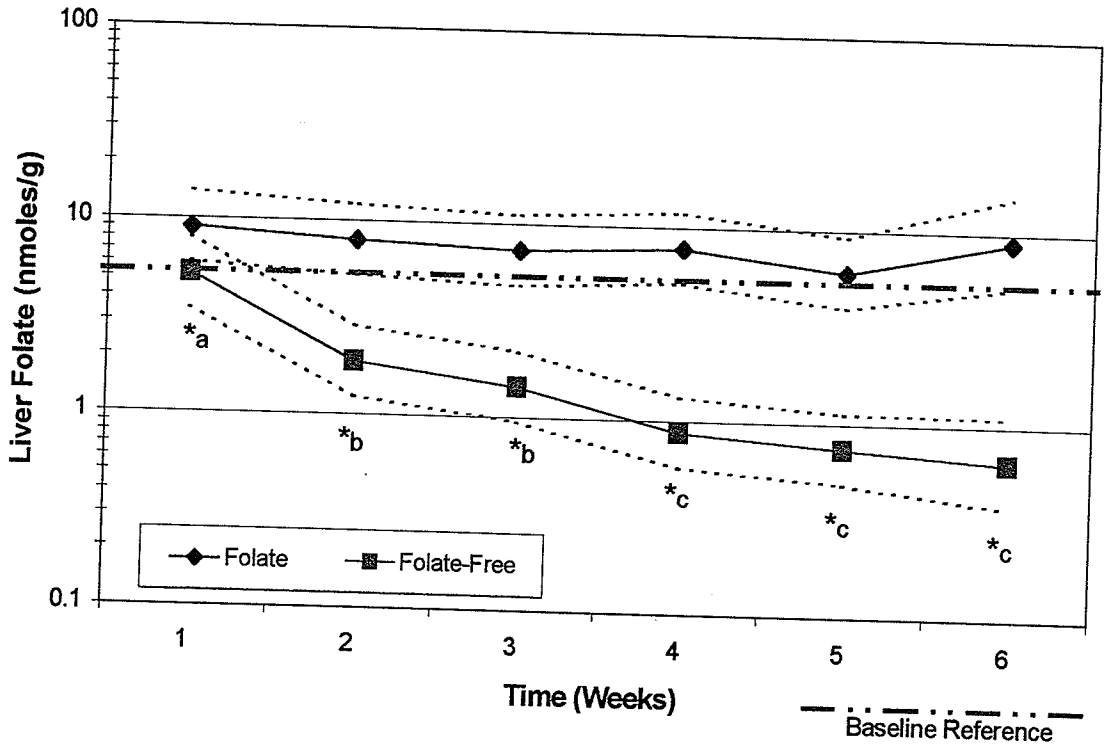
Liver folate concentrations were significantly lower in the folate-free group than in the control group after 1 week (P=0.02) (Figure 4.5). After week 4, there was approximately a 9-fold difference between the two groups (P<0.0001), and there was no further significant decrease in the folate-free group following week 4 (P=0.25). There was no significant difference within the control group over time (P=0.95).

Figure 4.4 - Time Course for Plasma Homocysteine in Rats During 6-Week Folate Depletion



Absolute differences are proportional to log units
Geometric means with 95% confidence intervals of Log10 transformed data
* Means significantly different ($P<0.05$) from corresponding time point in Folate group as determined by protected LSD
Means with different alphabetical notation within a treatment are significantly different ($P<0.05$) as determined by protected LSD

Figure 4.5 - Time Course for Liver Folate Depletion in Rats During 6-Week Folate Depletion



Absolute differences are proportional to log units
Geometric means with 95% confidence intervals of Log10 transformed data
* Means significantly different ($P<0.05$) from corresponding time point in Folate group as determined by protected LSD
Means with different alphabetical notation within a treatment are significantly different ($P<0.05$) as determined by protected LSD

4.3.6 Plasma Amino Acids:

Several of the plasma amino acid concentrations were altered during folate depletion (**Table 4.2**). Asparagine, alanine, serine, glycine, valine, methionine, isoleucine, leucine, tyrosine, and lysine, all had significant treatment effects, and, excluding serine and tyrosine, also had significant treatment by week interactions ($P<0.05$). In general, after 4 weeks of folate depletion, the plasma amino acid concentrations in the folate-deficient rats were significantly increased compared to the control group. For serine and tyrosine, there was a significant increase in the plasma amino acid concentrations in the folate-free group after weeks 1 and 2, respectively, but they returned to the same concentrations as the control group by week 6. Conversely, arginine had a significant treatment effect ($P<0.05$); however, the concentrations in the folate-free group were lower than the control group.

Glycine revealed the greatest difference between experimental groups by week six with a concentration of 694 μmoles in the control group compared to 5221 μmoles in the treatment group. See Appendix for graphical representation of the amino acid data.

Table 4.3 - Plasma Amino Acid Concentrations (μmoles/L) in Rats Following a 6-Week Folate Depletion Period

	Met (SE = ± 11.3)		**Ile** (SE = ± 12.4)		**Leu** (SE = ± 9.9)		**Tyr** (SE = ± 14.5)		**Lys** (SE = ± 53.4)	
	-†	+†	-	+	-	+	-	+	-	+
Week 1	68.2	40.8	111.4*	71.4*	125	112.4	136	128	784.4	734.2
Week 2	70.6	43.4	94	70.4	103.6	120.4	101.2	103.2	703.2	578
Week 3	70.6	69.2	85	104.2	120.6	119.6	135.2	100.4	580.2	648.8
Week 4	38.4	58.4	71	99.2	121.8	140.2	131	87.6	669.4	535.8
Week 5	101.2*	41*	95*	59.6*	123.6	103.2	104.8	69	613*	444.4*
Week 6	118.2*	50.8*	116.2*	71.6*	184.8*	122.6*	81	80.2	792.8*	382*
Treatment	P<0.05		P<0.05				P<0.05		P<0.05	
Week	-		-		-		-		-	
Interaction	P<0.05		P<0.05		P<0.05		-		P<0.05	

	Asp (SE = ± 6.8)		**Ser** (SE = ± 66.1)		**Gly** (SE = ± 464.7)		**Ala** (SE = ± 74.4)		**Val** (SE = ± 11.4)	
	-	+	-	+	-	+	-	+	-	+
Week 1	32.8	24.4	753.6	630.6	1479.6	964	943.8*	704.8*	154.2	131
Week 2	17.8	16.2	925.4	602.6	1639.8	741.8	761	576.6	166	151.6
Week 3	22.8	25.2	968	766	1822.4	985.2	774.2	667.4	146.4	149.4
Week 4	26.4	18.2	901.2	645.8	4618.6*	787.6*	703.4	703.6	151.8	158.2
Week 5	28.8	13.8	711.6	515	6070.8*	537.8*	820*	464.4*	150.2*	109.4*
Week 6	65*	18.8*	634.8	600.2	5220.8*	694.2*	944.2*	452.8*	217*8	138.4*
Treatment	P<0.05		P<0.05		P<0.05		P<0.05		P<0.05	
Week	-		-		-		-		-	
Interaction	P<0.05		-		P<0.05		P<0.05		P<0.05	

† + denotes inclusion of folic acid at 1 mg/kg diet; - denotes folate-free diet

* Means significantly different (P<0.05) between treatment groups within a time-point as determined by protected LSD

\- Denotes no significant week effect (P<0.05)

	Taur (SE = ± 32.1)		**OH Pro** (SE = ± 14.3)		**Thr** (SE = ± 138.1)		**Asn** (SE = ± 29.9)		**Glu** (SE = ± 31.4)		**His** (SE = ± 8.3)	
	-†	+†	-	+	-	-	+	+	-	+	-	+
Week 1	378.6	306.8	68.2	60.2	962.6	50.2	44.5	735.4	156.6	115.6	160.4	183
Week 2	322.2	282.6	54.2	58.6	1315	46	53.2	858	89	102.4	105	155
Week 3	277.2	227	70.8	93.8	1404	32.2	52.6	1335.2	150.6	125.8	134	158
Week 4	241.4	229.6	67.2	69.4	969	48	50.4	1005.6	176.4	127.6	109	160
Week 5	216.6	173	44.4	53.8	842.2	44.6	46	822.6	212.2	130.2	114.2	141
Week 6	259.6	243.2	92.4	91.8	671.2	63.4	46.6	784.2	144.2	145.6	222	166
Treatment	P<0.05		-		-		-		-		-	
Week	-		-		-		-		-		-	
Interaction	-		-		-		-		-		-	

	Gln (SE = ± 1853.7)		**Pro** (SE = ± 21.0)		**Cit** (SE = ± 10.1)		**Phe** (SE = ± 6.1)		**Orn** (SE = ± 9.5)		**Arg** (SE = ± 165.3)	
	-	+	-	+	-	-	+	+	-	+	-	+
Week 1	7235.2	8198.2	120.2	159.8	81.6	92.6	83.2	81.2	64.4	52.6	62.6	49.8
Week 2	6955.8	8785.2	119.4	104.2	56.4	87.8	124.8	59	47.6	62	53.4	53.2
Week 3	7919.2	9351	148	189.8	20.4	80.4	108	34.4	67.8	66	47.8	70
Week 4	7779	11834.2	140	147.2	31.8	78.6	173.4	31.2	71	70.6	32	58.8
Week 5	6388.8	10298.6	136	119.2	34.6	81.5	113.8	24.8	70.6	54.2	50.2	55.2
Week 6	13821.8	10858.2	142	153.4	34.6	97.6	133.4	50.4	91.6	79.2	89.2	68.4
Treatment	-		-		-		-		-		P<0.05	
Week	-		-		-		-		-		-	
Interaction	-		-		-		-		-		-	

† + denotes inclusion of folic acid at 1 mg/kg diet; - denotes folate-free diet; - Denotes no significant week effect (P<0.05)

* Means significantly different (P<0.05) between treatment groups within a time-point as determined by protected LSD

4.4 Discussion

The purpose of this experiment was to determine the time-course for plasma folate, homocysteine and amino acid concentrations during folate depletion. Previous folate depletion studies have reported plasma folate and homocysteine concentrations at the termination of the trial but they have failed to elucidate the concentrations at regular time points throughout. The appropriate parameters for folate depletion in a rat model in reference to plasma homocysteine concentrations needed clarification. It was also necessary to determine which biological components are most sensitive for estimating folate bioavailability. The data reported in this study clearly show the effects of folate deficiency throughout the depletion period. Interestingly, after 6 weeks of folate depletion, there was no indication of plasma homocysteine concentrations nearing a plateau. This was evident by the extremely high concentrations found in the rats that were terminated early (235.8 and 161.6 μmoles/L, respectively).

The rats were pair fed in this study because there was uncertainty regarding the effects of severe folate deficiency on appetite. Although a control group fed the control diet *ad libitum* was not incorporated into the study design, intake appears to decrease after week four. Feed consumption was reflected in the growth rates of the rats as well since there was no significant difference found between the treatment and the control groups, due to pair-feeding.

The amino acid-defined folate-free diet chosen for this trial was effective in establishing folate deficiency as evidenced by the significant decrease in plasma folate and the significant increase in plasma homocysteine. The plasma folate concentrations achieved after five weeks of folate depletion are similar to those reported by Walzem and

Clifford (1988) for the same time period in a protocol that also included an amino acid-based diet containing 1% succinylsulfathiozole. Liver folate concentrations were comparable to results found by both Walzem and Clifford (1988) after five weeks of depletion, and Miller and colleagues (1994) after four weeks of depletion using a similar protocol. The high plasma concentrations of homocysteine reached in our study were again comparable to the results of Miller and colleagues after four weeks of depletion. A similar depletion/repletion protocol was used by O'Leary and Sheehy (2001); however they sought only to induce moderate folate deficiency and accordingly used casein as a protein source, which is known to have an endogenous folate content. As a result, a much greater concentration of plasma homocysteine was attained after four weeks of folate depletion in our study compared to theirs (60.4 μmol/L vs. 26.6 μmol/L). This comparison clearly accentuates the necessity of incorporating an amino acid-defined diet into a folate depletion protocol when aiming to achieve severe folate deficiency defined by high plasma homocysteine concentrations. Interestingly, physical signs of deficiency, such as lesions and lethargy, were noted during the fourth week of their study, whereas lesions weren't apparent in our study until the sixth week of folate depletion. Based on the low concentrations of plasma folate and the high concentrations of plasma homocysteine following four weeks of folate depletion, evidently there is no need to extend the deficiency period beyond four weeks. In addition, growth began to decline after week four, and two of the folate deficient rats necessitated termination shortly after week five, indicating that a deficiency period longer than four weeks is not recommended.

Several plasma amino acid concentrations were altered in the folate-deficient rats. In the majority of cases, there was an increase in plasma levels in the folate-deficient rats compared to the control rats. This could be a result of increased protein breakdown, decreased protein synthesis or a combination of both. Noteworthy is that arginine displayed an opposite effect with concentrations in the folate-deficient rats being lower than the control, the reason for which is uncertain.

Glycine was notably the amino acid that was most affected by folate depletion. The major route for glycine catabolism is the glycine cleavage system. Glycine synthase, the enzyme that converts glycine to CO_2 and NH_4^+, is dependent on both pyridoxal 5' phosphate (PLP)-and tetrahydrofolate (THF) (Rodwell, 1996). The high concentrations of glycine found in the folate-deficient rats may have resulted from the inability to catabolize glycine due to lack of THF. Nonketotic hyperglycinemia (NKH) is a disorder of glycine metabolism resulting in elevated glycine concentrations (Applegarth and Toone, 2001). NKH is caused by a deficiency in the glycine cleavage system and results in a severe neurological disease (Applegarth and Toone, 2001). In most patients, the deficiency is caused by mutations in specific protein genes involved in the glycine cleavage system (Toone et al., 2000). However, our research suggests a possible link between folate deficiency and the inability of the glycine cleavage system to catabolize glycine.

The reaction which interconverts serine and glycine involves the folate-dependent serine hydroxymethyltransferase (SHMT). Serine is important as both a direct and indirect source of one-carbon units (Gregory et al., 2000). SHMT transfers the 3-carbon from serine to tetrahydrofolate to form glycine and 5,10-methylenetetrahydrofolate

(Bailey and Gregory, 1999). Thymidilate synthase uses 5,10-methylenetetrahydrofolate as its C1 donor for the conversion of the uracil-type base in RNA into the thymine-type base in DNA (Scott, 1999). Therefore, a deficiency of folate in the SHMT reaction could lead to a serious impairment of cell division, including the production of proteins crucial for normal metabolism. Additionally, 5,10-methylenetetrahydrofolate reductase converts a portion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, of which the N-5 methyl group can only be used in the methylation of homocysteine to form methionine (Bailey and Gregory, 1999). S-adenosylhomocysteine can only be hydrolyzed when adenosine and homocysteine are metabolized, thus excess homocysteine causes a surplus of S-adenosylhomocysteine which consequently inhibits most methyltransferases (Finkelstein, 1990). The disruption of homocysteine metabolism, and thus countless methylation reactions, due to insufficient folate is apparent.

This study was successful in revealing the time-course for plasma folate, homocysteine, and amino acid concentrations using a well-established folate depletion-repletion protocol. It was established that the appropriate time-period for folate depletion should not exceed four weeks so that plasma homocysteine concentrations are sufficiently elevated to allow for a potentially significant decrease once folate repletion commences, without severely debilitating the health of the rats. Possible relationships between folate deficiency and amino acid concentrations were also exposed. Most importantly, the sensitivity of plasma homocysteine concentrations as a functional indicator of folate status was made known. This leads to the plausibility of utilizing

plasma homocysteine in a folate depletion-repletion protocol to estimate folate bioavailability in rats.

Chapter 5 – Determination of the Bioavailability of Folate in Enriched Eggs Using Plasma Homoctsteine as an Outcome Measure

5.1 Introduction

Folate bioavailability research is of great interest due to the important role of folate in achieving and maintaining optimum health. Convincing evidence demonstrates the potential for folate supplementation and fortification to significantly decrease the incidences of both neural tube defects (NTDs) and coronary artery disease (CAD) (Boushey et al., 1995). Certain types of cancer have also been linked to poor folate intake (Bailey and Gregory, 1999).

Folate is very important in the remethylation of homocysteine to methionine (Brouwer et al., 1999a). Thus, lack of folate can result in hyperhomocysteinemia. Consequently, high plasma homocysteine is considered a risk factor for such conditions as cardiovascular disease and neural tube defects (Brouwer, 1999a). Plasma amino acid concentrations of several other amino acids are also altered during folate deficiency, as was clearly shown previously in our laboratory.

In recent years, the Food and Nutrition Board of the Institute of Medicine (IOM), and scientists from Canada, have joined forces to create a comprehensive set of reference values for nutrient intakes of healthy Canadian and United States populations (IOM, 1998). This newly established set of reference values replaces the previously published Recommended Nutrient Intakes (RNIs) for Canada and the Recommended Dietary Allowances (RDAs) for the United States (IOM, 1998). Bioavailability and nutrient-nutrient interactions were considered when developing the DRIs for folate and consequently, Dietary Folate Equivalents (DFEs) were established (IOM, 1998). The

poor bioavailability resulting from the poor chemical stability of the natural folates profoundly influences recommendations (Scott, 1999). DFEs are units that account for differences in absorption between food folate and synthetic folic acid from dietary supplements or folic acid-fortified food (Suitor and Bailey, 2000). Therefore, 1 μg DFEs = 1 μg of food folate = 0.5 μg of folic taken on an empty stomach = 0.6 μg of folic acid taken with meals (IOM, 1998). The DFEs are based on the assumption that food folates are approximately 50% bioavailable. Unfortunately, the methods established to date for estimating bioavailability of folate have produced variable results (Prinz-Langenohl et al., 1999). Plasma folate and plasma homocysteine are inversely related (Wagner, 1995) and homocysteine levels are a sensitive marker of folate status (Minet et al., 2000). Accordingly, the main objective of this study was to use plasma homocysteine to estimate folate bioavailability in a rat model. To test this model, folate-enriched egg-yolks, developed by House and colleagues (2002), were incorporated into the basal diet. Previously in our laboratory we characterized the time course of folate depletion pertaining to several measures of folate status. We also established the appropriate time-period necessary for folate depletion in a rat model. Homocysteine was shown to be more sensitive than plasma or liver folate concentrations. The alternate hypothesis is that based on plasma homocysteine as an outcome measure, egg folate is highly available in a rodent model.

5.2 Materials and Methods

5.2.2 Animals:

Weanling, male, Sprague-Dawley rats were used. They were purchased from Central Animal Care (Winnipeg, MB). The rats were housed individually in wire-bottomed, stainless steel cages to prevent coprophagy. The room was kept at 21±2°C and 45±5% humidity, and 14 hours of light were provided daily. The rats had *ad libitum* access to water. Approval was obtained from the Protocol Management Review Committee in accordance with the Canadian Council of Animal Care.

5.2.3 Diets:

The diets were purchased from Harlan Teklad (Madison, WI) and consisted of a 17% amino acid-defined diet, either with folic acid or without. The diets were in powder form and also contained 1% succinylsulfathiozole (**see Table 4.1**).

5.2.4 Generation of Materials:

The folate-enriched egg yolk powder was generated following the procedures of House and colleagues (House et al., 2002). Laying hens were fed a basal diet, with the addition of 4 mg/kg diet of folic acid, for 2 wks before collecting the eggs. The eggs were hard-boiled for 10 min, and the yolks were removed, freeze-dried, and ground into powder. The dried egg yolk powder was stored at -20°C until analyzed for 5-methyl-THF content. The extraction of 5-methyl-THF from the folate-enriched egg yolk powder, using the method of Vahteristo and colleagues (1997) was accomplished by first measuring 0.5 g of the powder into a glass tube with a lid. Ten mL of the extraction buffer (20 g/L sodium ascorbate; 12.1 g/L Trizma base; pH 7.8) were added to the tube.

The tube was topped with N_2 gas, vortexed, and then placed in a boiling water bath for 60 min. Next the tube was centrifuged at 4000 x g for 30 min and the supernatant was decanted into a 25 mL volumetric flask. Ten mL of the extraction buffer was again added to the tube followed by vortexing and centrifugation as detailed previously. The supernatant was again decanted into the volumetric flask and the final volume was brought to 25 mL. An aliquot of the sample was transferred to a microcentrifuge tube and stored at -20°C until analyzed. The process was repeated until ten samples, representative of the total amount of powder, were obtained. 5-methyl-THF content of the folate-enriched egg yolk powder was determined according to the procedures outlined by Vahteristo and colleagues (1997), as described in Chapter 4.2.4. The desired amount of powder was then incorporated into the basal rat diet which was the equivalent of 32.35 g of folate-enriched egg yolk powder in 967.65 g of the basal diet.

5.2.5 Experiment:

Fifty-four rats were acclimatized to the environmental conditions and fed lab chow for 7 d. The rats were then subjected to a 4 wk folate depletion period as described in experiment 1; however, since all rats underwent the folate depletion, pair-feeding was not necessary. Following the depletion period, six rats were terminated to obtain baseline data. The terminations proceeded as in the first experiment. The remaining 48 rats were randomly divided into 2 groups of 24 rats. The first group was the treatment group, and they were fed a diet consisting of the basal diet with the addition of folate-enriched egg yolk powder to achieve a total folate content of 250 μg/kg diet. The second group was the control group and they were fed a diet consisting of the basal diet with the addition of

250 μg/kg diet of crystalline folic acid (CFA). Pair-feeding was not necessary for this phase since close observation for 2 weeks did not reveal any differences in intake. The duration of the experiment was 4 weeks and data was obtained at weeks 1, 2, 3, and 4, by terminating 6 treatment rats and 6 control rats each week, as stated above, to obtain plasma, kidneys, and liver for analysis.

5.2.6 Analyses of Plasma and Tissues:

Plasma folate, plasma homocysteine and liver 5-methyl-THF were determined as outlined in Chapter 4.2.4.

5.2.7 Statistical analyses:

The experiment completely randomized. Statistical analysis was performed by ANOVA using SAS Analyst (SAS Institute Inc., 1999). Differences between means were determined using the protected-LSD method. Differences with an α level of $P<0.05$ were considered to be statistically significant.

Folate bioavailability was estimated using a negative slope-ratio bio-assay of the data of both treatments from wk 0 to wk 2, using the equation: % bioavailability = [$\Delta_{t2\text{-}t0}$ Homocysteine(Egg)/ $\Delta_{t2\text{-}t0}$ Homocysteine(Control)] * 100%. The plasma homocysteine concentrations from wk 0 to wk 2 were used in the bio-assay because of the dramatic decrease of the concentrations in the treatment group within that time period.

5.3 Results

5.3.1 Growth:

The growth of the rats was not significantly different between the treatment and the control groups for the entire duration of the trial (P=0.27) (**Figure 5.1**). There was a significant time effect (P<0.001); however, there was no significant treatment by time interaction (P=0.89).

5.3.2 Feed Intake:

There was no significant difference in feed intake between the treatment and control groups (**Figure 5.2**).

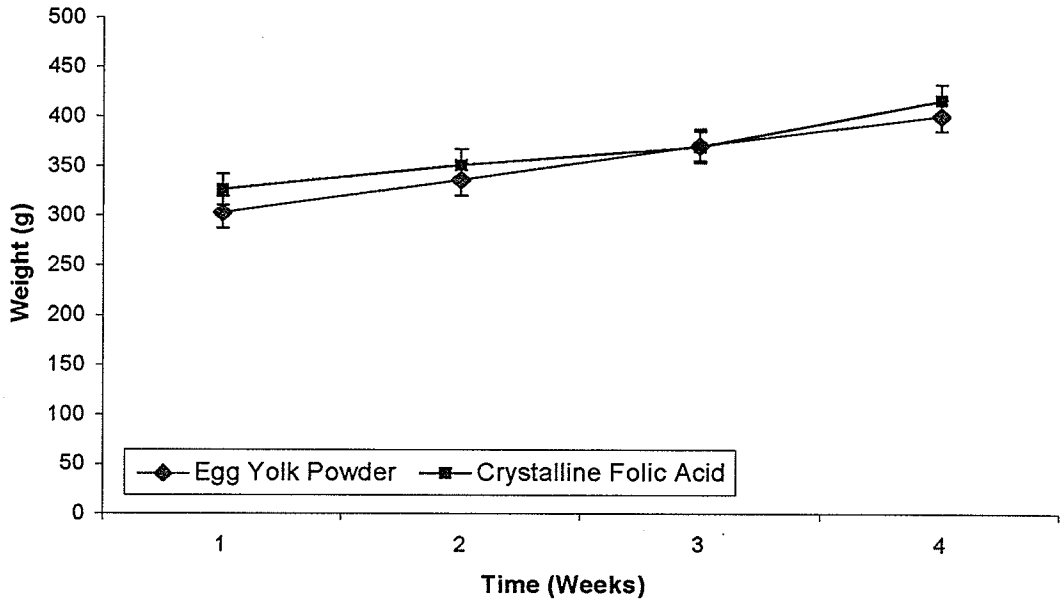
5.3.3 Plasma Folate:

There was no significant difference in plasma folate concentrations between the treatment group being supplemented with folate-enriched egg yolk powder and the control group being supplemented with CFA (P=0.18) (**Figure 5.3**). There was no significant effect of time on plasma folate though there was a significant treatment by time interaction (P=0.04).

5.3.4 Plasma Homocysteine:

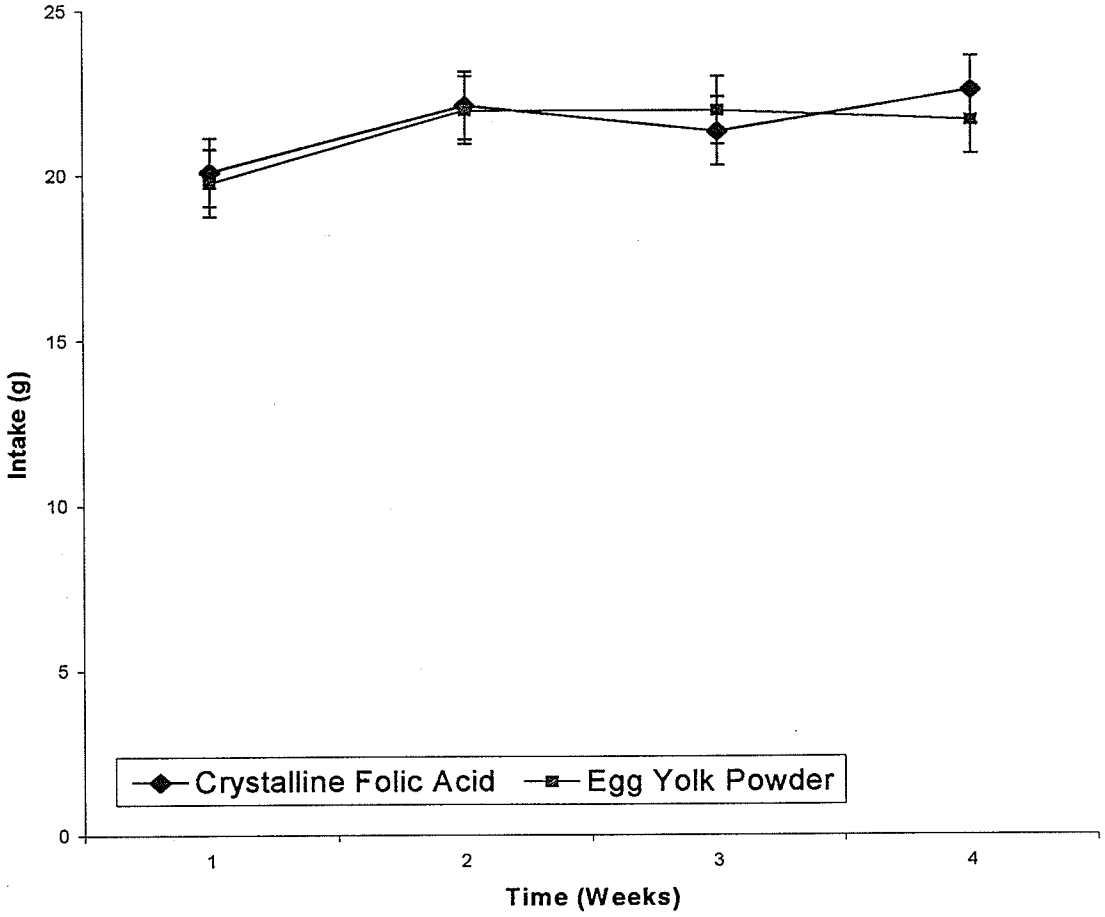
Plasma homocysteine concentrations between groups were significantly different after only one week of folate repletion, 51.63 ± 2.76 μmoles/L and 36.08 ± 2.76 μmoles/L in the control and treatment groups, respectively (P<0.0001) (**Figure 5.4**). There were significant treatment (P<0.0001) and time (P<0.0001) effects but the effect of treatment by time interaction was not significant (P=0.2421).

Figure 5.1 – Average Weekly Growth in Rats Following a 4-Week Folate Repletion Period



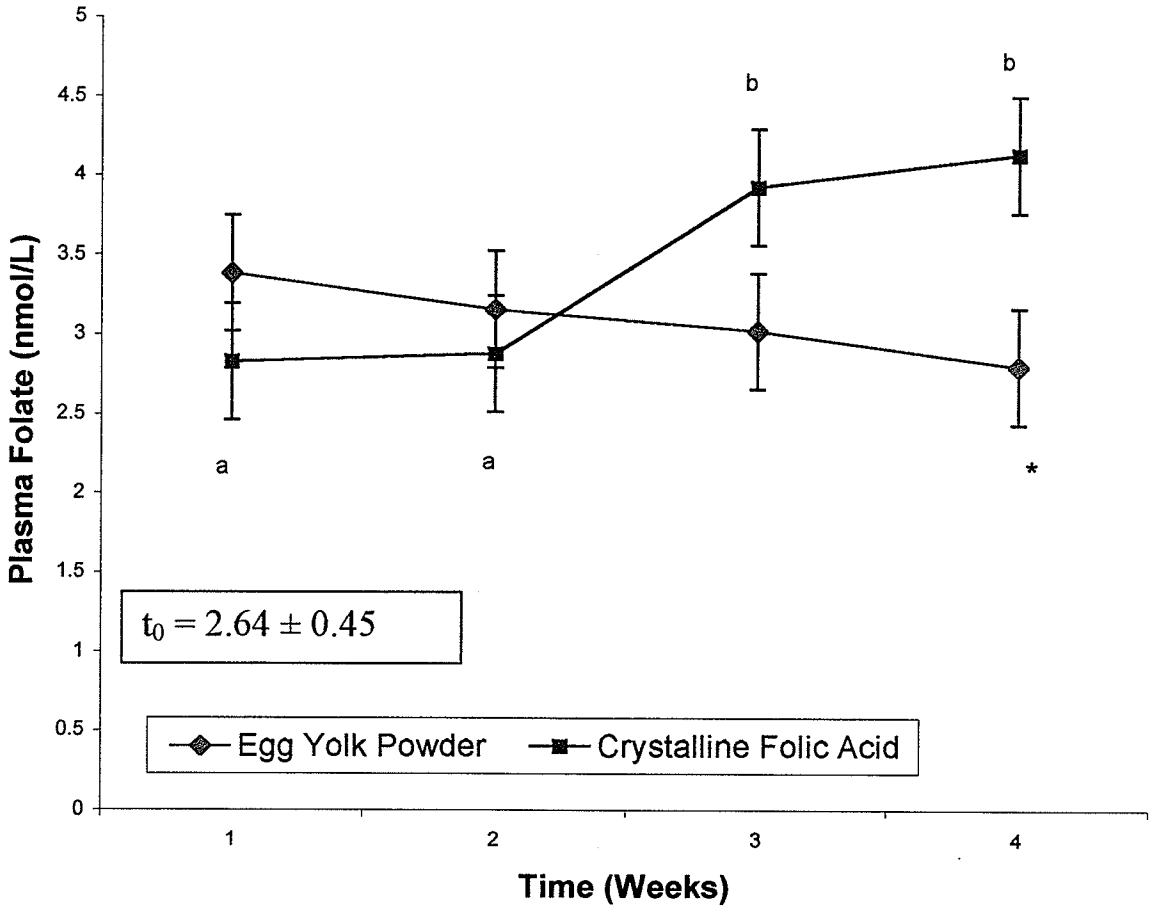
Data reported as LS Means +/- Standard Error
Treatment Effect P=0.2655
Week Effect P<0.001
Treatment *Week Effect P=0.8873

Figure 5.2 - Average Daily Feed Intake in Rats Following 4-Week Folate Repletion Period



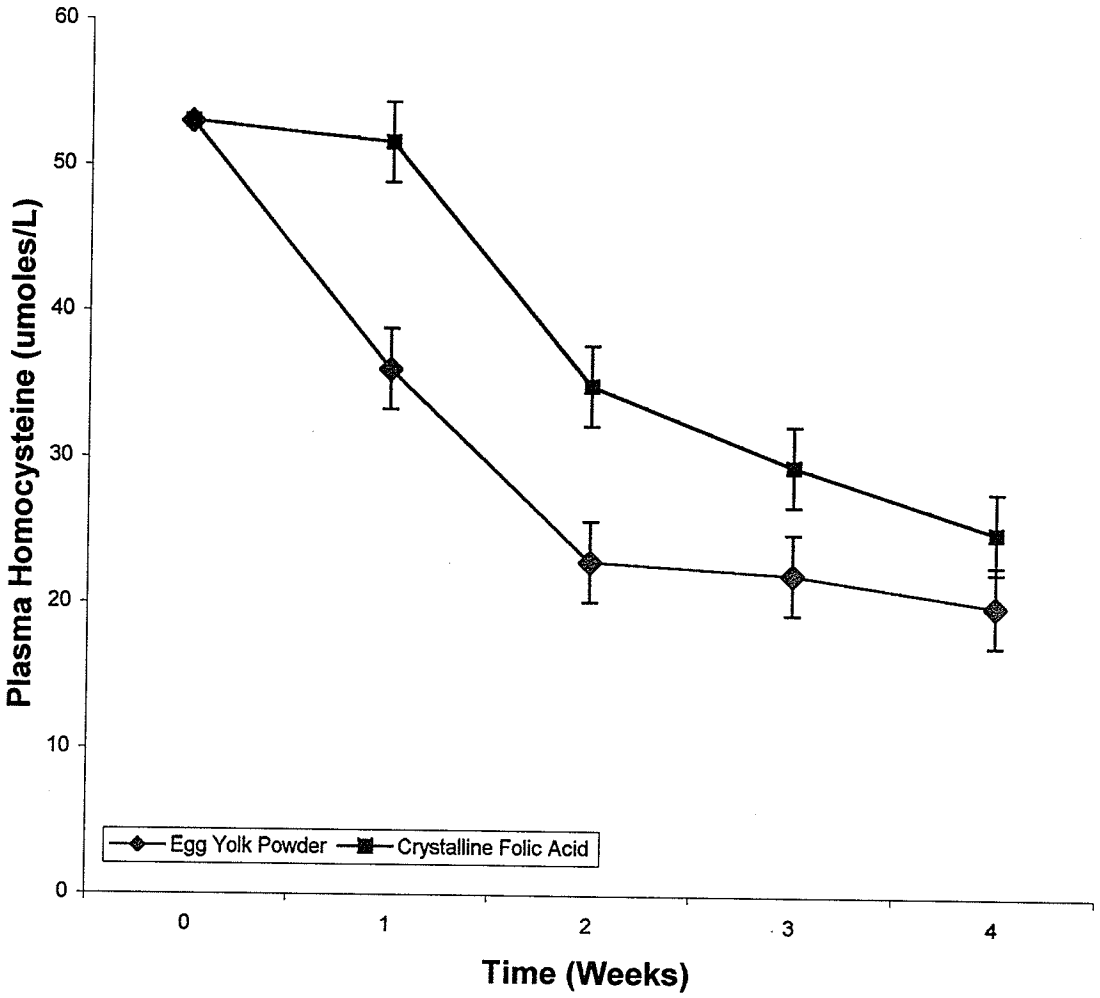
Data reported as LS Means +/- Standard Error
Treatment Effect P=0.8009
Week Effect P<0.001
Treatment *Week Effect P=0.907

Figure 5.3 - Plasma Folate in Rats During 4-Week Folate Repletion



Data reported as LS Means +/- Standard Error
Treatment*Week Interaction P=0.0367
Means with different alphabetical notation within a treatment are significantly different (P<.05) as determined by Protected LSD
Means denoted by an asterisk are significantly different (P<.05) from corresponding time point in crystalline folic acid group as determined by protected LSD

Figure 5.4 - Plasma Homocysteine in Rats During 4-Week Folate Repletion



Data reported as LS Means +/- Standard Error
Treatment Effect P<0.0001
Week Effect P<0.0001
Treatment*Week Effect P=0.2421

5.3.5 Liver Folate:

There was no significant difference in liver folate concentrations between the treatment and control groups (P=0.9370), as well as no significant effect of treatment by week interaction (P=0.9630) (**Figure 5.5**). A significant effect of time was observed (P=0.0003).

5.3.6 Assessment of Bioavailability:

The relative bioavailability of the folate in the folate-enriched egg yolks compared to the CFA, based on plasma homocysteine concentrations was estimated by negative slope-ratio assay. The slope for the homocysteine concentrations during the first two weeks of repletion was chosen:

% bioavailability = [$\Delta_{t2\text{-}t0}$ Homocysteine(Egg)/ $\Delta_{t2\text{-}t0}$ Homocysteine(Control)] * 100%.

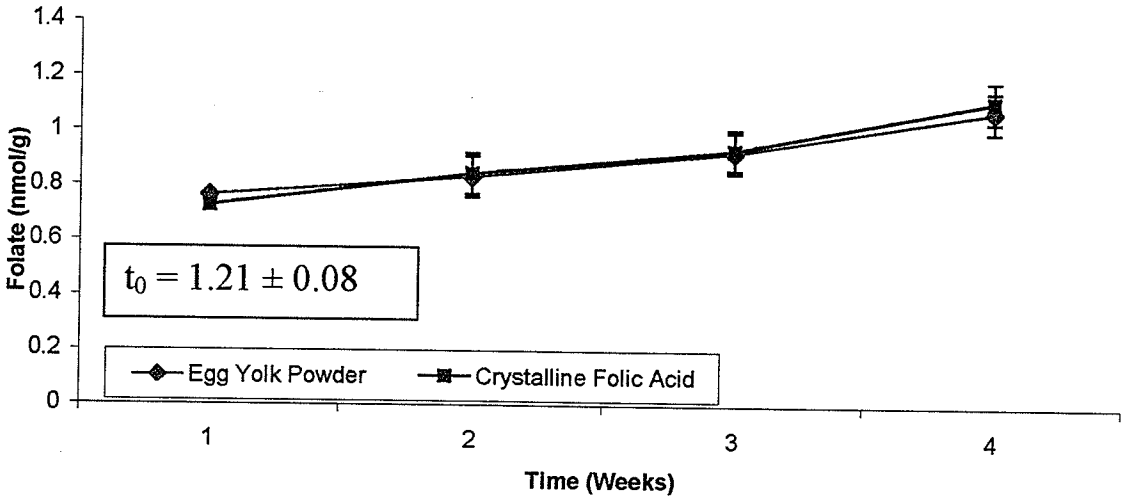
$$\% \text{ bioavailability} = \frac{22.92-53.05}{34.97-53.05} * 100\%$$

% bioavailability = 167

5.4 Discussion

The purpose of this study was to use plasma homocysteine, a functional indicator of folate status, to estimate the bioavailability of folate from folate-enriched eggs in a rat model. The length of the depletion period was chosen based on previous research in our lab. The repletion period was chosen based on evidence from other researchers (Miller et al., 1994; O'Leary and Sheehy, 2001). The folate depletion/repletion protocol was used

Figure 5.5 - Liver Folate in Rats During 4-Week Folate Repletion Period



Data reported as LS Means +/- Standard Error
Treatment Effect P=0.9370
Week Effect P=0.0003
Treatment*Week Effect P=0.9630

because of its proven effectiveness in establishing severe folate depletion in a relatively short period of time, as demonstrated by our previous study, as well as those of others (Walzem and Clifford, 1988; Miller et al., 1994). The level of folate used in the repletion trial (250 μg/kg diet) was selected since it is well below the requirement of 1 mg/kg diet and would not cause a rapid decrease in plasma homocysteine. It is in close agreement with O'Leary and Sheehy (2001) who chose 200 μg/kg diet. In addition, it is a physiologically relevant dose. Miller and colleagues (1994) showed that diets providing 250 and 500 μg of folate/kg diet were successful in significantly reducing plasma homocysteine concentrations in depleted rats. After three weeks of repletion, mean plasma homocysteine concetrations were 17.3 ± 5.5 μmoles/L and 8.0 μmoles/L, respectively, with latter not being significantly different from the control (6.4 μmoles/L).

The inclusion of folate-enriched egg-yolk powder to attain 250 μg/kg diet amounted to 3% of the total feed consisting of egg-yolk powder. We are confident that such a low level of inclusion did not significantly alter the overall nutrient content of the diet (see Comparison of Basal and Egg Yolk Powder Enriched Diets in Appendix). Specifically, the nutrients relevant to the methionine cycle were as follows: methionine was 8.2 g/kg diet in both the control and treatment diets, pyridoxine was 6.5 mg/kg of diet and 6.6 mg/kg of diet in the control and treatment diets, respectively, and cobalamin was 28.3 μg/kg of diet and 29.1 μg/kg in the control and treatment diets, respectively. The requirements outlined by the National Research Council (NRC) (1995) for growing rats are 9.8 g/kg of diet for methionine and cystine combined, 6 mg/kg of diet for pyridoxine, and 50 μg/kg diet for cobalamin. All requirements were met except for cobalamin; however, the levels of cobalamin were similar between the treatment and the

control diets. In addition, the results in Chapter 4 clearly showed that the levels of cobalamin in the diet did not impact homocysteine levels since they were normal in the control group. It would be advised to update the inclusion of cobalamin to meet the NRC requirements in future studies.

Pair-feeding was considered since there was uncertainty regarding differences in palatability between the two diets. Intake was closely monitored for two weeks and no difference was apparent; accordingly we continued to feed both groups *ad libitum*.

Some researchers argue that the rat is not suitable due to the claimed differences in folate digestion between humans and rats (Gregory, 2001). Wang and colleagues (1985), stated that since brush border enzyme activity is negligible in the rat, and the intracellular conjugase in the rat exhibits a different pH dependence than in the human, the pig may be a more suitable model. It must be noted that the optimum pH range in humans was reported as 4.5-5.0 and in rats, 4.0-7.5, thus the optimum pH of humans is represented in the rat (Wang et al., 1985). Additionally, these findings do not apply to the intestinal absorption of monoglutamyl folate because according to Said and colleagues (1987), transport in human intestinal brush border membrane vesicles is similar to the transport in the intestines of both rats and rabbits. Thus the use of the rat model in estimating the bioavailability of folates that are provided in the monoglutamyl form is justified. Seyoum and Selhub (1998) state that the folate contained in egg yolks is strictly in the monoglutamate 5-methyl-THF form, thus the presence, or absence, of folate conjugase in our study is inconsequential . Furthermore, Reisenhauer and Halsted (1987) provided evidence that the hydrolysis of polyglutamyl folate is not a limiting factor in folate absorption. Moreover, a recent study by Said and colleagues (2000)

examining the effect of dietary folate deficiency on intestinal folate uptake in rats, tested for the presence of brush border membrane folate conjugase activity and found that it indeed exists, and is significantly upregulated in folate deficiency. Thus there is no cause to believe that the rat is not an effective and suitable model for folate bioavailability research. It may be of interest to assess the applicability of our model for other forms of folate with varying glutamate chain lengths, perhaps with a comparison to a swine model.

There was no significant difference between the treatment and control groups for growth, feed intake, or liver folate concentrations, and there was only a significant treatment by week interaction for plasma folate concentrations. Alternatively, there was a significant treatment effect and a significant week effect for plasma homocysteine concentrations. Although not certain, the sensitivity of plasma homocysteine to folate status likely results from the importance of folate to the methionine cycle. When folate is lacking, there is an increase in plasma homocysteine. Conversely, in folate deficiency, any folate consumed will be channeled first to the tissues where it will be utilized in metabolic reactions. Thus, a rise in plasma folate will not likely be seen until the tissues are receiving adequate folate to meet metabolic demands. Plasma folate was signficantly higher in the crystalline folic acid group at week four, therefore it could be argued that based on plasma folate, crystalline folic acid is more bioavailable than the folate in the eggs. However, when comparing to the control group in Chapter 4, the plasma folate levels reported here are much lower. At week, plasma folate concentrations were 4.13 and 2.80 (SE = 0.37) whereas in the control group in Chapter 4, plasma folate concentrations were 45.4 (SE = 1.25) nmoles/L. Additionally, the liver, a storage site for folate, will not store folate when there are deficits in tissues throughout the body. There

was a signficant week effect for liver folate indicating that liver concentrations were increasing over time, but there was no significant difference between the treatment groups. The level of folate provided in the repletion period (250 μg/kg of diet) was well below the requirement for growing rats (1mg/kg of diet), and was certainly not meeting metabolic demands, but since the only nutrient lacking for the methionine cycle was folate, the amount of folate provided was enough to affect remethylation of homocysteine.

The bioavailability of folate in folate-enriched egg yolks, estimated using plasma homocysteine concentrations in a negative slope-ratio bio-assay is reported as 167 % bioavailable relative to crystalline folic acid. The slope of the plasma homocysteine concentrations after two weeks was chosen for the negative slope-ratio assay because there did not appear to be any further decrease in the treatment group. The greatest decrease in the control occurred in the first two weeks as well, although not to the same extent as the treatment group. Additionally, it is unlikely that the slope for the control group would have been much different after four weeks than it was after two. Thus, two weeks of folate repletion is recommended in future studies.

Folic acid is considered the gold standard to which all other estimates are compared since it is deemed 100% bioavailable. According to our research the folate in folate-enriched egg yolks is more available than crystalline folic acid. Seyoum and Selhub (1998) have demonstrated *in vitro* that the 5-methyl-THF in egg yolks is not only highly available, but also highly stable. Consequently, it is not surprising that the 5-methyl-THF in the folate-enriched egg yolks is also highly available, although a certain explanation for the increased bioavailability over CFA is not available at this time. The

answer may lie in the fact that the folate in eggs is strictly in the 5-methyl-THF monoglutamate form (Seyoum and Selhub, 1998), and this form of folate is absorbed in the intestine rapidly and virtually unchanged (Steinberg, 1984). Other monoglutamates, such as folic acid, must first be converted to the formyl- or methyl-THF forms by the mucosal cells in the small intestine before absorption can occur, although a small quantity of folic acid may be transported to the portal circulation unaltered (Steinberg, 1984). Another possible explanation is that the presence of folate binding proteins in the egg yolk increase folate bioavailability, as postulated in the study performed by Swiatlo and colleagues (1990) in which folate bioavailability of milk-containing (human and bovine) diets was significantly enhanced over milk-free diets. Babu and Srikantia (1976) found the bioavailability of certain foods to be greater than folic acid as well, and they questioned whether there were certain factors in foods that either facilitated better absorption or promoted excretion of stored folate. More research in this area is needed before any firm conclusions can be made.

This is the first study to report using plasma homocysteine concentrations in a negative slope-ratio assay to estimate folate bioavailability in rats. O'Leary and Sheehy (2001) conducted a similar study in which they investigated the ability of selected folic acid-fortified foods to increase the folate status of folate-deficient rats. They measured plasma homocysteine as a functional indicator of folate status; however, they were not interested in assessing folate bioavailability (O'Leary and Sheehy, 2001). Our results show that plasma homocysteine appears to have increased sensitivity to folate status compared to plasma folate, liver folate, or growth, and that it is a viable method for estimating folate bioavailability in rats. At week two, there was no significance

difference between corresponding time points for growth, plasma folate and liver folate. As such, for each of the outcome measures mentioned, the bioavailability of folate in the enriched eggs, as determine by slope-ratio assay, would be 100% relative to crystalline folic acid. It is evident why a functional indicator of folate status, such as plasma homocysteine, may be a sensitive outcome measure. The sensitivity of plasma homocysteine concentrations to folate status, compared to all other outcome measures, is clearly demonstrated. Further investigation into the scopes and limitations of this method are needed.

Chapter 6 – General Discussion

Folate bioavailability research has been of interest for decades. Various experimental approaches have been employed to estimate the bioavailability of food folates, including changes in hematologic values, tissue folate levels, urinary folate excretion, and growth, in response to the consumption of known amounts of folic acid or folate-containing foods (Clifford et al., 1990). The models used most often are humans, rats and chicks. There is considerable variability in reported estimates of folate bioavailability which likely results from the variety of protocols used, as well as the controversy concerning the appropriate response criterion (Clifford et al., 1991). Miller and colleagues (1994) revealed a sensitive, inverse relationship between plasma homocysteine and folate status in the rat which led to the possibility of using plasma homocysteine to estimate folate bioavailability.

The results obtained here using plasma homocysteine, a functional indicator of folate status, are very positive. A more sensitive relationship was observed between plasma homocysteine and folate status than between plasma folate, liver folate, or growth.

The bioavailability of the folate in folate-enriched egg yolks was reported as 167% which indicated that the folate in these eggs is more bioavailable than crystalline folic acid. The next step is to examine possible components of egg yolks that might increase folate bioavailability, such as folate binding proteins, or the influence of the form of folate. Since betaine, a metabolite in choline metabolism, can also remethylate homocysteine to some extent (Green and Jacobsen, 1995), the amount of choline provided by the egg yolk powder deserves consideration; however, the basal diet also

provides choline. If choline is a confounding factor then the differences observed in plasma homocysteine concentrations may be independent of dietary folate.

The negative slope-ratio assay is the assay most often used in recent bioavailability studies and was thus used with confidence (Littell et al., 1995). The area under the curve method has also been used and is generally considered to be a valid technique but only for studies performed within an eight to 24 hour period in which sufficient blood samples are taken (Gregory, 1988). Since plasma homocysteine was the desired outcome measure in this study, it was unlikely that a significant decrease would be apparent within a 24-hour period. Additionally, the restricted amount of blood available from the rat coupled with the physiological stress of repeated blood sampling on an animal of that size, would have confounded the results.

Rats have been used extensively in folate bioavailability research. The suitability of the rat model for folate bioavailability studies has been criticized (Gregory, 2001). The differences in folate deconjugation between rats and humans previously reported question the extrapolation of results in the rat model to use in humans (Wang et al., 1985). More recent findings suggest that folate deconjugation is not a limiting factor, and that the intestinal uptake does not differ between rats and humans (Reisenhauer and Halsted, 1987; Said et al., 2000), thus providing sufficient evidence to support its use. In addition, folate in egg yolks is in the monoglutamate 5-methyl-THF form and, as such, does not require folate conjugase activity for digestion (Seyoum and Selhub, 1998).

The folate depletion/repletion protocol is often used; however, there are many considerations. The weanling rats must be housed individually in wire-bottom cages to prevent coprophagy. An amino acid-defined, folate free diet which is adequate in all

nutrients is the most effective in producing folate deficiency, and the inclusion of an antibiotic, such as succinylsulfathiozole, is necessary to prevent intestinal bacterial synthesis of folate. The diet can also easily be manipulated to include various test foods or substances.

The plasma amino acid profiles of rats during folate depletion provided useful information linking folate deficiency to a plethora of metabolic disturbances. In general, an increase in several amino acid concentrations in the folate deficient rats was noted in comparison to the control rats. Glycine was most affected by folate depletion, and was likely due to a breakdown in the THF-dependent glycine cleavage system. Nonketotic hyperglycinemia (NKH), typically a genetic disorder of glycine metabolism, results in elevated plasma and cerebral spinal fluid glycine concentrations, and ultimately severe neurological disorders (Applegarth and Toone, 2001). The extremely high plasma glycine concentrations found in the folate deficient rats suggest an association between folate deficiency, excessive glycine concentrations, and all of its manifestations, including neurological disorders. Interestingly, in neonatal NKH, cerebral spinal fluid glycine concentrations can be greater than 30X the upper limit while plasma glycine concentrations, although elevated, may remain in the normal range (Applegarth and Toone, 2001). Unfortunately, cerebral spinal fluid amino acid concentrations were not assessed for this research. However, it can be hypothesized, that increased plasma glycine due to folate deficiency may be exerting detrimental effects to neurological integrity, especially if cerebral spinal fluid concentrations are also elevated. Obviously, subsequent research focusing on the affected amino acids is necessary to gain a better understanding in this area.

The importance of folic acid is evident when comparing growth in the folate depletion experiment to growth in the depletion-repletion experiment. At week five in the folate depletion trial, the average body weights of the rats in the group are 6% lower than those in the corresponding time point in the repletion trial, and after week six, bodyweights are 15% lower than in the repletion trial. Interesting is that the level of folic acid provided in the repletion period (250 μg/kg diet) is only one quarter of the folate requirement for rats (1 mg/kg diet), yet it resulted in a marked difference in growth between the two trials. Evidently, 250 μg/kg diet was sufficient to affect the weight gains of the rats.

The results obtained in these studies support the use of plasma homocysteine for estimating folate bioavailability in rats. The amount of time appropriate for both folate depletion and repletion, using an amino acid-defined diet has been established. Further testing of different food sources of folate can now be performed. A recent study by O'Leary and Sheehy (2002) claims that homocysteine is a poor response variable for folate status when very low levels of folate are included in the diet. It must be pointed out that the inclusion levels of folate in their study did not exceed 25 μg folate/kg of diet. The level of folate used in this study was 10-fold the highest level used in their study and we found plasma homocysteine to be a sensitive response variable when 250 μg folate/kg of diet is incorporated into the repletion period.

The implications of developing reliable estimates of folate bioavailability in foods are that conversion factors for specific foods could be established to accurately reflect a food's contribution to folate status. The potential impact on nutrient databases is that folate content of foods, as well as the folate bioavailability, could be reported. For

example, based on the results obtained by this research, the bioavailability of folate in folate-enriched egg yolks is 167% relative to crystalline folic acid. According to the DFEs, if an egg has 50 μg of folate, then 50 μg of folate is consumed. However, if the bioavailability is truly 167%, then 50 μg of folate would translate into 167 μg DFEs. This would provide the consumer with the knowledge to determine the amount of folate they are obtaining from food. The current dietary folate equivalents assume a folate bioavailability of 50% which likely underestimates many foods. As a result, folate recommendations and folate labeling are affected by this assumption. The method of estimating folate bioavailability in rats presented here may help to develop appropriate recommendations that can be accurately reported to the consumer, resulting in improved folate status and reduction of related illnesses.

A limitation to our study is that the HPLC method utilized to determine the folate content of the egg yolk powder could only measure the 5-methyl-THF content. Although this is the only form of folate in egg yolks (Seyoum and Selhub, 1998), and as such, our methods for determining folate content were adequate, a more suitable assay for determining folate content of other foods would be necessary. A modification to the current method that includes the hydrolysis of the polyglutamate forms, and the ability to detect different forms of folate would be suitable. Additionally, the microbiological assay using lactobacillus casei can determine the folate activity from different forms of folate, both in the monoglutamate and polyglutatmate forms.

Another limitation is that a second functional indicator of folate status was not used to validate plasma homocysteine. Although the results showed that folate-enriched egg yolks were more effective in reducing plasma homocysteine than crystalline folic

acid, there is uncertainty as to whether these difference were strictly related to the folate in the diet or whether there is another component of egg yolks impacting plasma homocysteien levels that is independent of folate, such as betaine. Testing a second funtional indicator would provide a stronger argument. The results of the plasma amino acid analysis in Chapter 4 showed that glycine is significantly affected in folate deficiency. As such, plasma glycine levels could also be used as a functional indicator of folate status, to either support, or contradict, the use of plasma homocysteine for estimating folate bioavailability.

There is much knowledge to be gained in relation to the data reported here. Firstly it will be important to compare different dosage levels of the same test foods to determine any differences associated with different levels of folate. Said and colleagues (2000) reported that folate deficiency results in upregulation of intestinal folate uptake in rats and this should be further explored in the rat model using the folate depletion-repletion protocol. Also, the folate contained in egg yolks is almost strictly 5-methyltetrahydrofolate in the monoglutatmate form, thus the suitability of our model in testing different forms of folate, as well as the polyglutamate forms, necessitates evaluation. Once the parameters and reliability of this model are tested, it will be possible to test other animal models, such as the weanling pig, in which repeated blood sampling is possible. The scope and limitations of the current model necessitate further elucidation.

Based on the results, the alternate hypothesis that folate bioavailability can be assessed in a rodent model using plasma homocysteine as an outcome measure was accepted.

Chapter 7 - References

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Chapter 8 - Appendices

Calculations of Nutrients in Basal Diet

TD 01042 Folate-Free Diet	g/kg Diet	g/967.65g Diet*
L-Alanine	3.5	3.387
L-Arginine	12.1	11.709
L-Asparagine	6	5.806
L-Aspartic Acid	3.5	3.387
L-Cytine	3.5	3.387
L-Glutamic Acid	40	38.706
Glycine	23.3	22.546
L-Hisitidine HCl.H2O	4.5	4.354
L-Isoleucine	8.2	7.935
L-Leucine	11.1	10.741
L-Lysine HCl	18	17.418
L-Methionine	8.2	7.935
L-Phenylalanine	7.5	7.257
L-Proline	3.5	3.387
L-Serine	3.5	3.387
L-Threonine	8.2	7.935
L-Tryptophan	1.8	1.742
L-Tyrosine	5	4.838
L-Valine	8.2	7.935
Sucrose	354.4	342.93516
Corn Starch	150	145.1475
Maltodextrin	150	145.1475
Soybean Oil	80	77.412
Mineral Mix[1]	35	33.86775
Calcium Phosphate	8.2	7.93473
Succinylsulfathiazole	10	9.6765
TBHQ	0.02	0.019353
Choline Bitartrate	2.5	2.419125
Nicotinic Acid	0.0339	0.032803335
Calcium Pantothenate	0.0181	0.017514465
Pyridoxine HCl	0.0079	0.007644435
Thiamine HCl	0.007	0.00677355
Riboflavin	0.0068	0.00658002
D-Biotin	0.0003	0.000290295
Vitamin B12 (0.1% in mannitol)	0.0283	0.027384495
DL-Alpha Tocopherol Acetate (500 IU/g)	0.1695	0.164016675
Vitamine A Palmitate (500 000 IU/g)	0.009	0.00870885
Vitamin D3 palmitate	0.0023	0.002225595
Vitamin K, Phylloquinone	0.001	0.00096765

*The amount to which the folate-enriched egg yolk powder will be added to achieve a folate content of 250 ug/kg of diet

[1]As per AIN-76A diet (American Institute of Nutrition, 1980)

Calculations of Nutrients in Folate-Enriched Egg Yolk Powder

Dried Egg Yolk (Folate-Enriched)	/100 g portion	/32.35 g portion*
L-Alanine (g)	1.759	0.569
L-Arginine (g)	2.444	0.791
L-Asparagine (g)	0.000	0.000
L-Aspartic Acid (g)	3.348	1.083
L-Cytine (g)	0.614	0.199
L-Glutamic Acid (g)	4.34	1.404
Glycine (g)	1.058	0.342
L-Hisitidine (g)	0.888	0.287
L-Isoleucine (g)	1.732	0.560
L-Leucine (g)	3.009	0.973
L-Lysine (g)	2.718	0.879
L-Methionine (g)	0.849	0.275
L-Phenylalanine (g)	1.463	0.473
L-Proline (g)	1.43	0.463
L-Serine (g)	2.926	0.947
L-Threonine (g)	1.819	0.588
L-Tryptophan (g)	0.4	0.129
L-Tyrosine (g)	1.523	0.493
L-Valine (g)	1.907	0.617
Protein (g)	34.25	11.080
Total Lipid (g)	55.8	18.051
CHO (g)	3.6	1.165
Calcium (mg)	284	91.874
Iron (mg)	5.42	1.753370
Magnesium (mg)	13	4.205500
Phosphorus (mg)	920	297.620000
Potassium (mg)	244	78.934000
Sodium (mg)	135	43.672500
Zinc (mg)	4.93	1.594855
Copper (mg)	0.012	0.003882
Manganese (mg)	0.119	0.038497
Selenium (ug)	86.8	28.079800
Vitamin C (mg)	0	0
Thiamin (mg)	0.29	0.093815
Ribofalvin (mg)	1.88	0.60818
Niacin (mg)	0.095	0.0307325
Pantothenic Acid (mg)	7.765	2.5119775
Vitamin B6 (mg)	0.66	0.21351
Folate (ug)	772	250
Vitamin B12 (ug)	5.33	1.724255
Vitamin A (IU)	1315	425.4025
Vitamin A (ug RE)	395	127.7825
Vitamin E (mg ATE)	6.42	2.07687

*32.35 g of folate-enriched egg yolk powder provides 250 ug/kg of diet

Adapted from www.nal.usda.gov

Comparison of Basal and Egg Yolk Powder-Enriched Diets Used in Manuscript #2

	TD 01042 Folate-Free Diet	TD 01042 + Folate-Enriched Egg Yolk
	g/kg Diet	g/kg Diet
L-Alanine	3.5	4.0
L-Arginine	12.1	12.5
L-Asparagine	6	5.8
L-Aspartic Acid	3.5	4.5
L-Cytine	3.5	3.6
L-Glutamic Acid	40	40.1
Glycine	23.3	22.9
L-Hisitidine HCl.H2O	4.5	4.6
L-Isoleucine	8.2	8.5
L-Leucine	11.1	11.7
L-Lysine HCl	18	18.3
L-Methionine	8.2	8.2
L-Phenylalanine	7.5	7.7
L-Proline	3.5	3.8
L-Serine	3.5	4.3
L-Threonine	8.2	8.5
L-Tryptophan	1.8	1.9
L-Tyrosine	5	5.3
L-Valine	8.2	8.6
Cobalamin	0.0000283	0.0000291
Pyridoxine	0.0065	0.0066
Total Lipid	80	95.5
Total CHO	654.4	634.3

Recipes

2 M Potassium Borate Buffer (pH 9.5) – *Solution A*

1. Add 112.22 g potassium hydroxide (KOH) to ~900 mL of deionized water. Bring to a final volume of 1 L using the deionized water.

2. Add 123.66 g boric acid to ~900 mL of deionized water followed by 300 mL of the KOH solution.

3. Stir and heat on low-medium heat for approximately 40 minutes or until the boric acid is solubilized.

4. Cool the solution only until it can be handled since cooling too long will cause the boric acid to precipitate.

5. Bring the boric acid to a final volume of 1 L using deionized water. Note: since 300 mL of KOH was added, the final volume will be 1.3L. Next, add KOH until a pH of 9.5 is achieved.

6. Filter.

2 M Potassium Borate Buffer (pH 10.5, containing 5mM ethylenediaminetetracetic acid (EDTA)) – *Solution B*

1. Using Solution A, continue adding KOH until a pH of 10.5 is achieved.

2. Calculate and add the appropriate amount of EDTA to make a 5 mM EDTA solution (5 mM EDTA = 1.46 g/L).

3. Filter.

0.1 M Ptassium Borate Buffer (pH 9.5, containing 2 mM EDTA) – *Solution C*

1. Using Solution A, make a 1:20 dilution using 50 mL of solution A and 950 mL of deionized water.

2. Calculate and add the appropriate amount of EDTA to make a 2 mM EDTA solution (2 mM EDTA = 0.585 g/L)

3. Filtering is optional.

Tris (2-Carboxyethyl)-Phosphine Hydrochloride (TCEP) – Reducing Agent

1. Weigh 100 mg TCEP solution into a microcentrifuge tube and add 1 mL of deionized water to make a 10% solution.
2. Vortex.

7-Fluro-2-Oxa-1,3-Diazole-4-Sulfonic Acid (SBDF)

1. Measure 1 mg of SBDF into a glass test tube and add 1 mL of Solution A.
2. Vortex.
3. May need to put in 60°C water bath to dissolve SBDF.

0.1 M Acetate Buffer (pH 5, containing 2% methanol) – Buffer A

1. Add 13.608 g sodium acetate trihydrate to ~900 mL deionized water and bring to a final volume of 1 L to make a 0.1 M sodium acetate solution.
2. Add 5.75 mL of glacial acetic acid to ~900 mL deionized water and bring to a final volume of 1 L to make a 0.1 M glacial acetic acid solution.
3. Slowly begin adding the 0.1 M glacial acetic acid solution to the entire amount of sodium acetate solution until a pH of 5 is achieved.
4. Measure the amount of solution in a graduated cylinder. Calculate and add the appropriate amount of methanol to achieve a 2% methanol solution.
5. Filter.

Dietary Reference Intakes (DRIs)

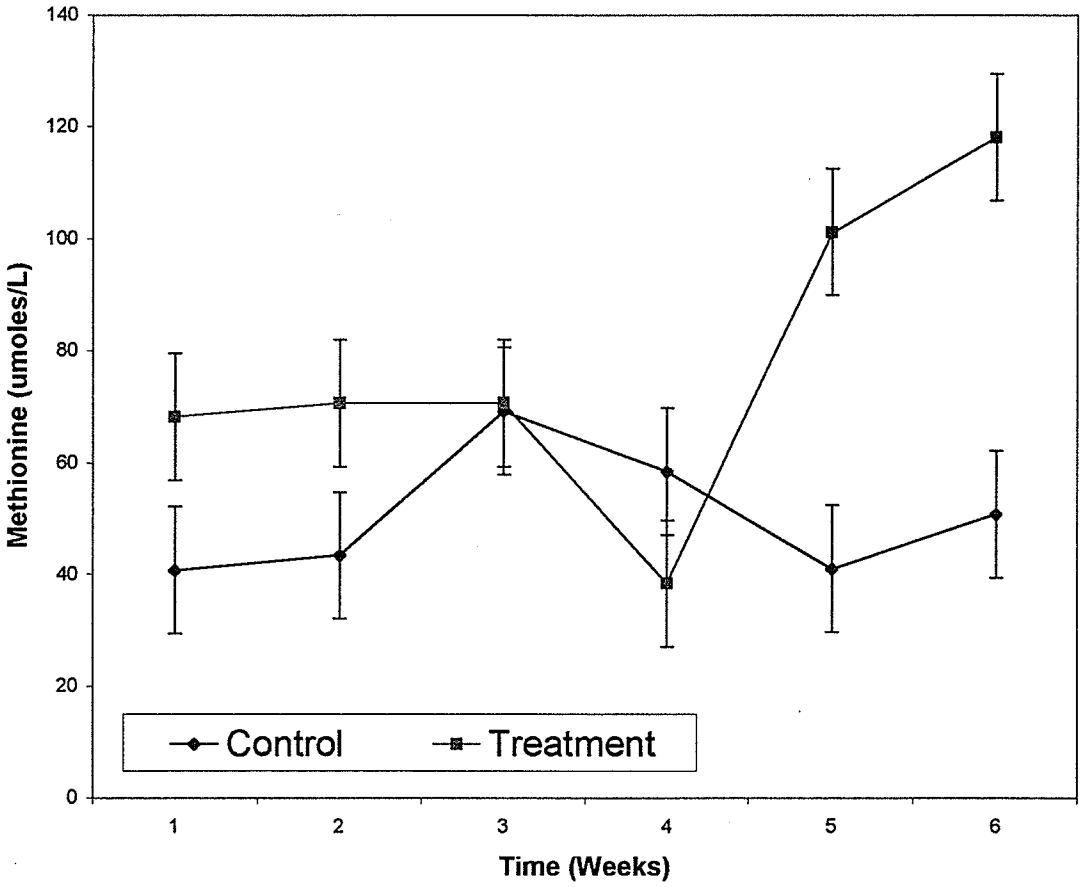
Age	Sex	Adequate Intake (AI) µg/day DFE*	Estimated Average Intake µg/day DFE*	Recommended Daily Intake (RDA) µg/day DFE*
Months				
0-6	Both	65		
7-12	Both	80		
Years				
1-3	Both		120	150
4-8	Both		160	200
9-13	M		250	300
	F		250	300
14-18	M		330	400
	F		330	400
19-50	M		320	400
	F		320	400
51-70	M		320	400
	F		320	400
>70	M		320	400
	F		320	400
Pregnancy				
14-50	F		520	600
Lactation				
15-50	F		450	500

*DFE = Dietary Folate Equivalents

**Special Considerations: An increased RDA may be required for mothers carrying more than one fetus or nursing more than one baby and for those with chronic heavy alcohol intake and chronic anticonvulsant or methotrexate therapy.

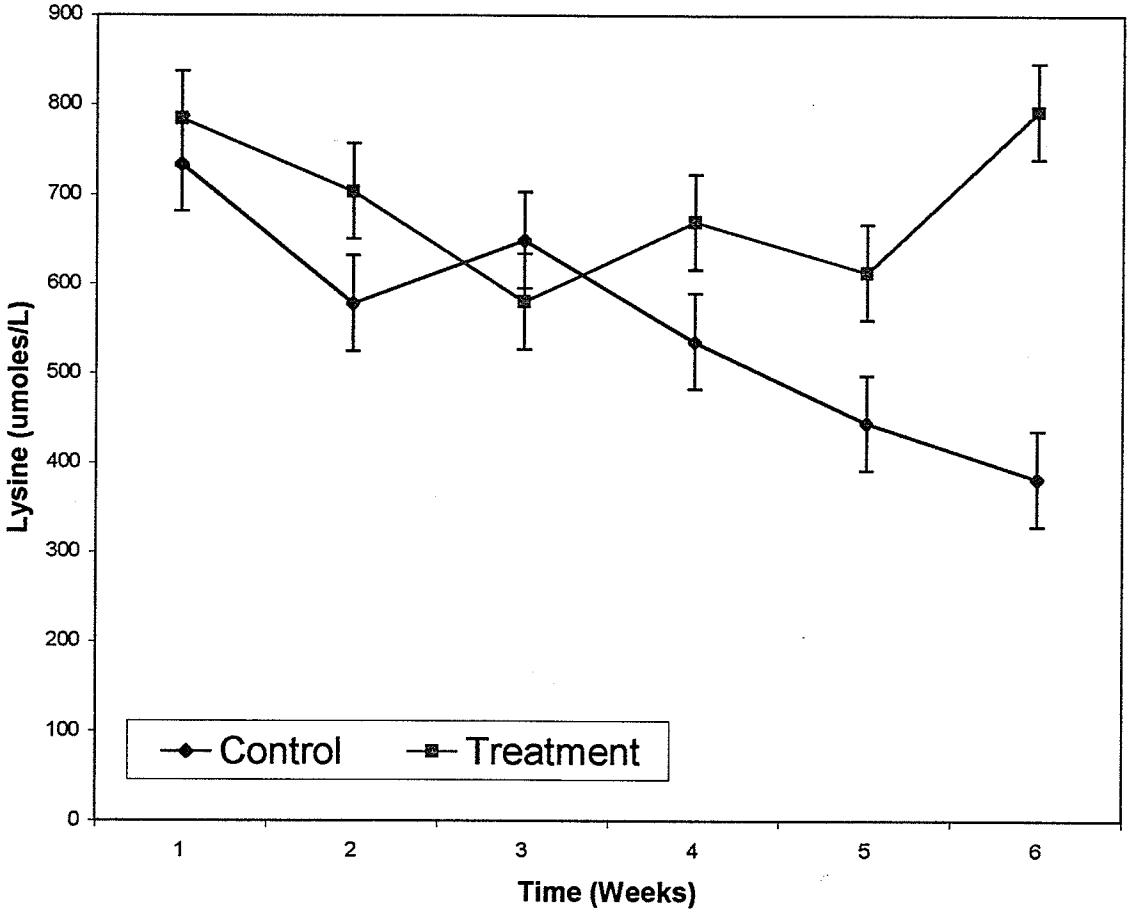
Adapted from Institute of Medicine, 2000

Plasma Methionine Levels in Rats Following a 6-Week Folate Depletion Period



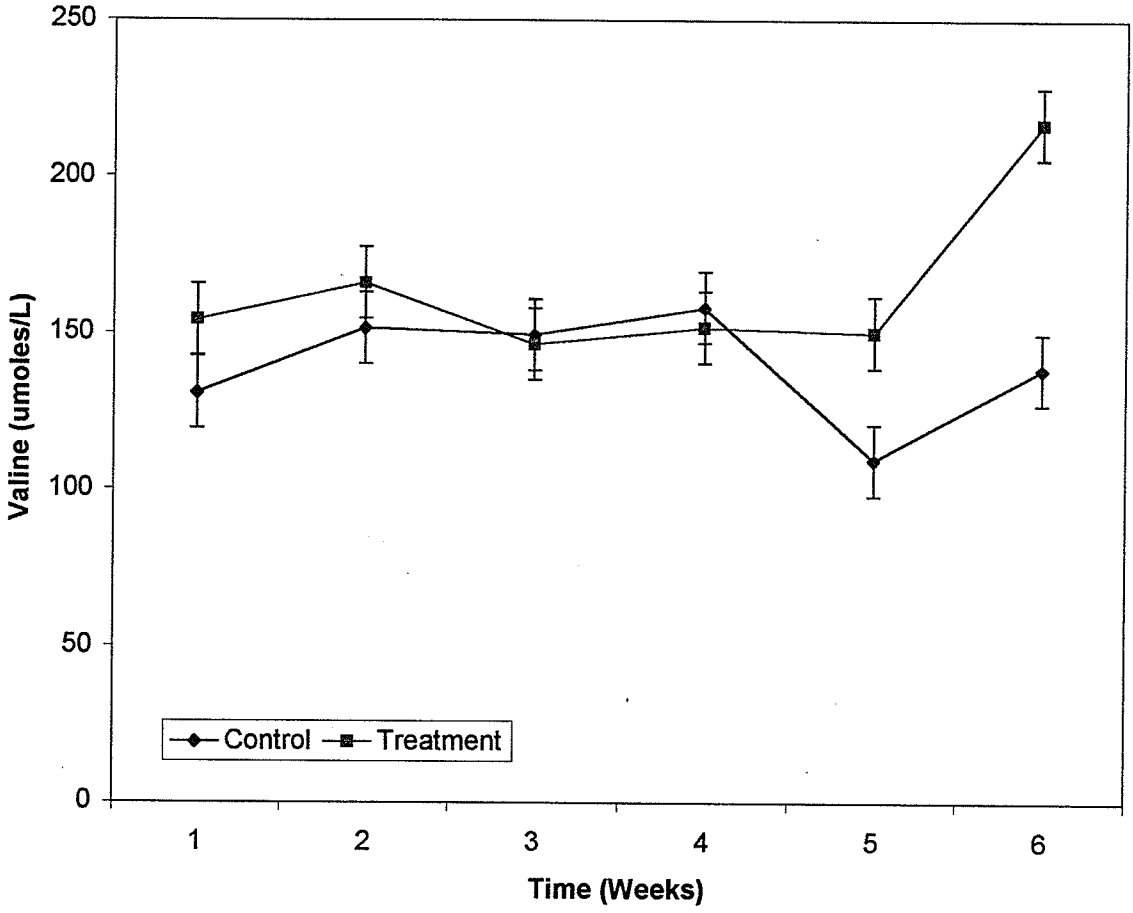
Data reported as LS Means +/- Standard Error
Treatment Effect P<0.0001
Treatment*Week Interaction P=0.0024

Plasma Lysine in Rats Following 6-Week Folate Depletion Period



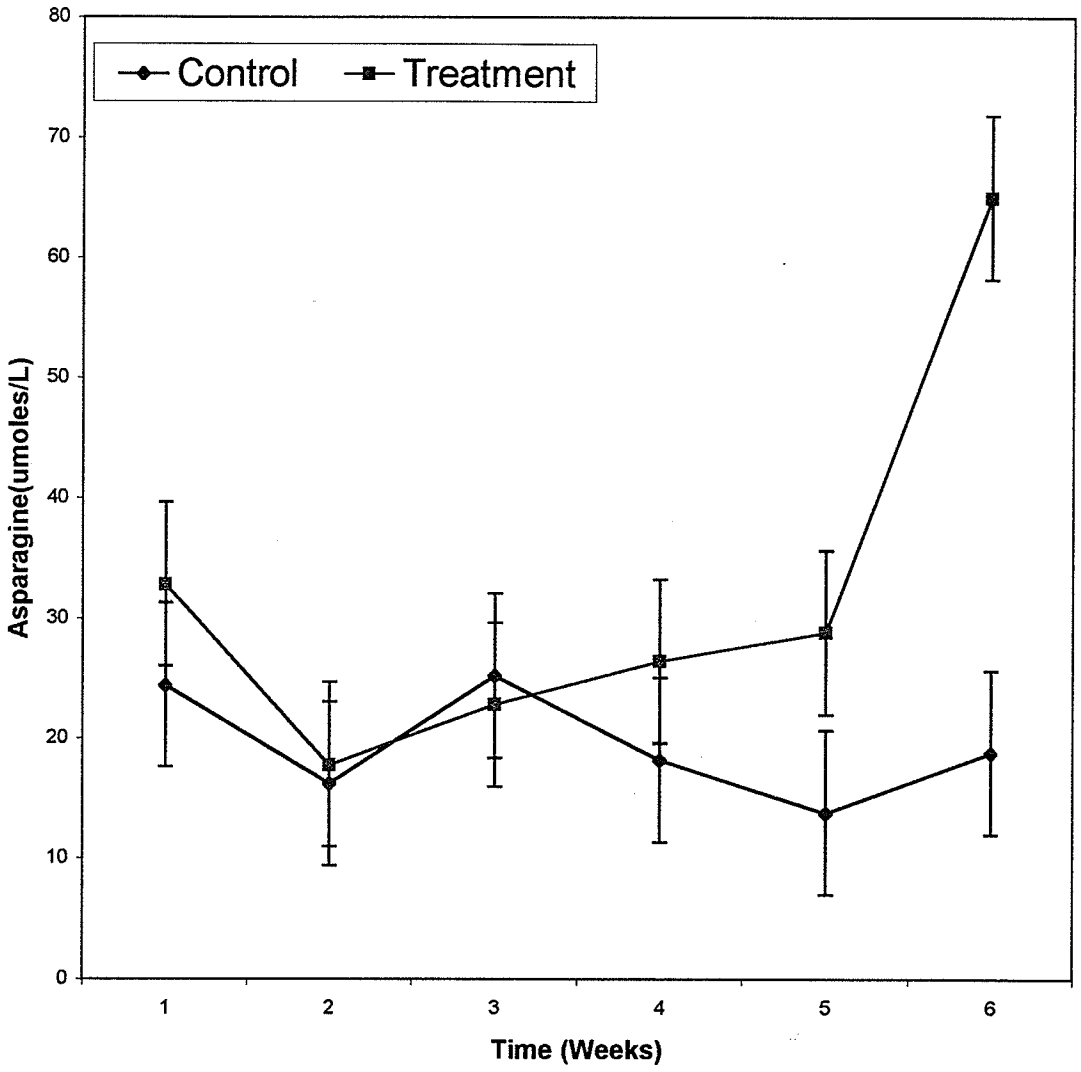
Data reported as LS Means +/- Standard Error
Treatment Effect P<0.0001
Treatment*Week Interaction P=0.0022

Plasma Valine in Rats Following 6-Week Foalte Depletion Period



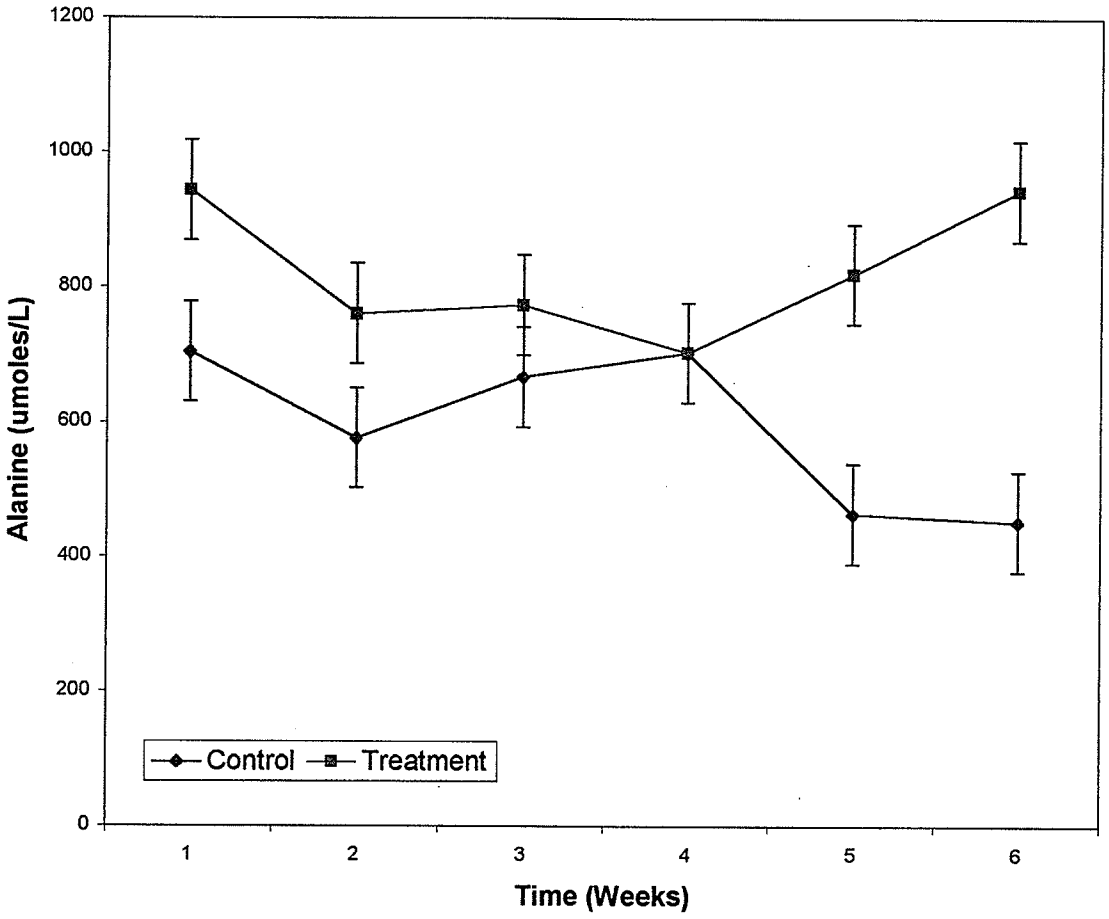
Data reported as LS Means +/- Standard Error
Treatment Effect P=0.0005
Treatment*Week Interaction P=0.0052

Plasma Asparagine in Rats Following a 6-Week Folate Depletion Period



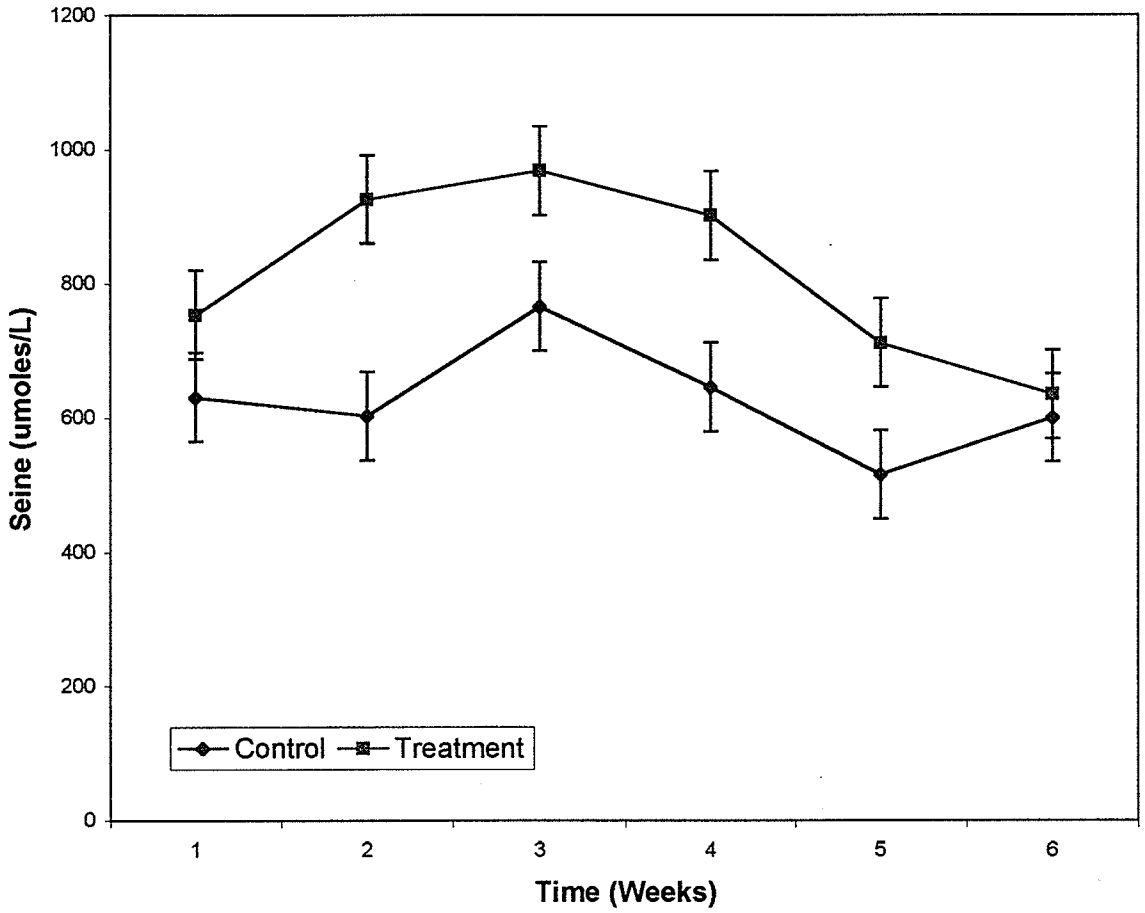
Data reported as LS Means +/- Standard Error
Treatment Effect P=0.0021
Treatment*Week Interaction P=0.0131

Plasma Alanine in Rats Following 6-Week Folate Depletion Period



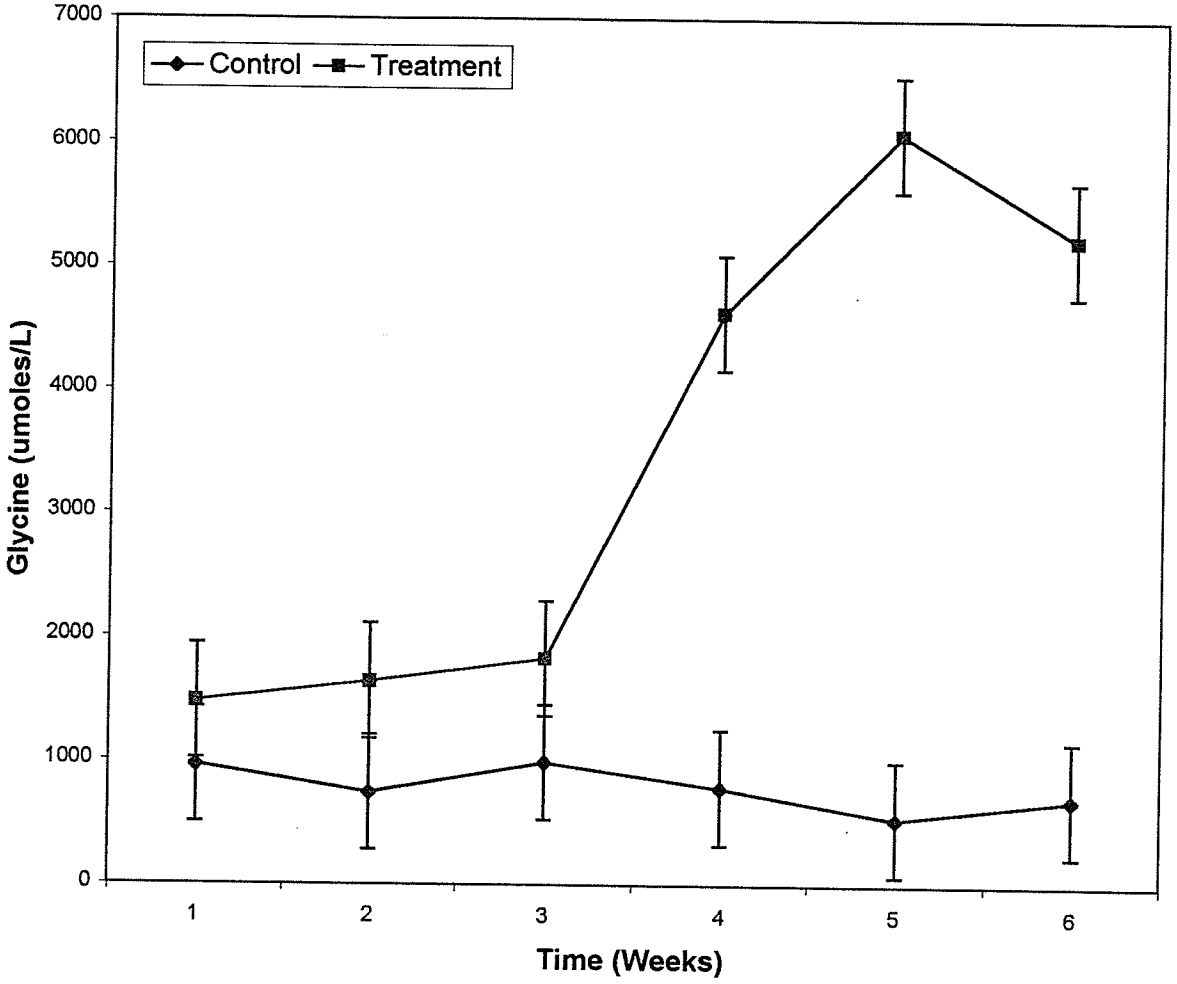
Data reported as LS Means +/- Standard Error
Treatment Effect P<0.0001
Treatment*Week Interaction P=0.0271

Plasma Serine in Rats Following 6-Week Folate Depletion Period



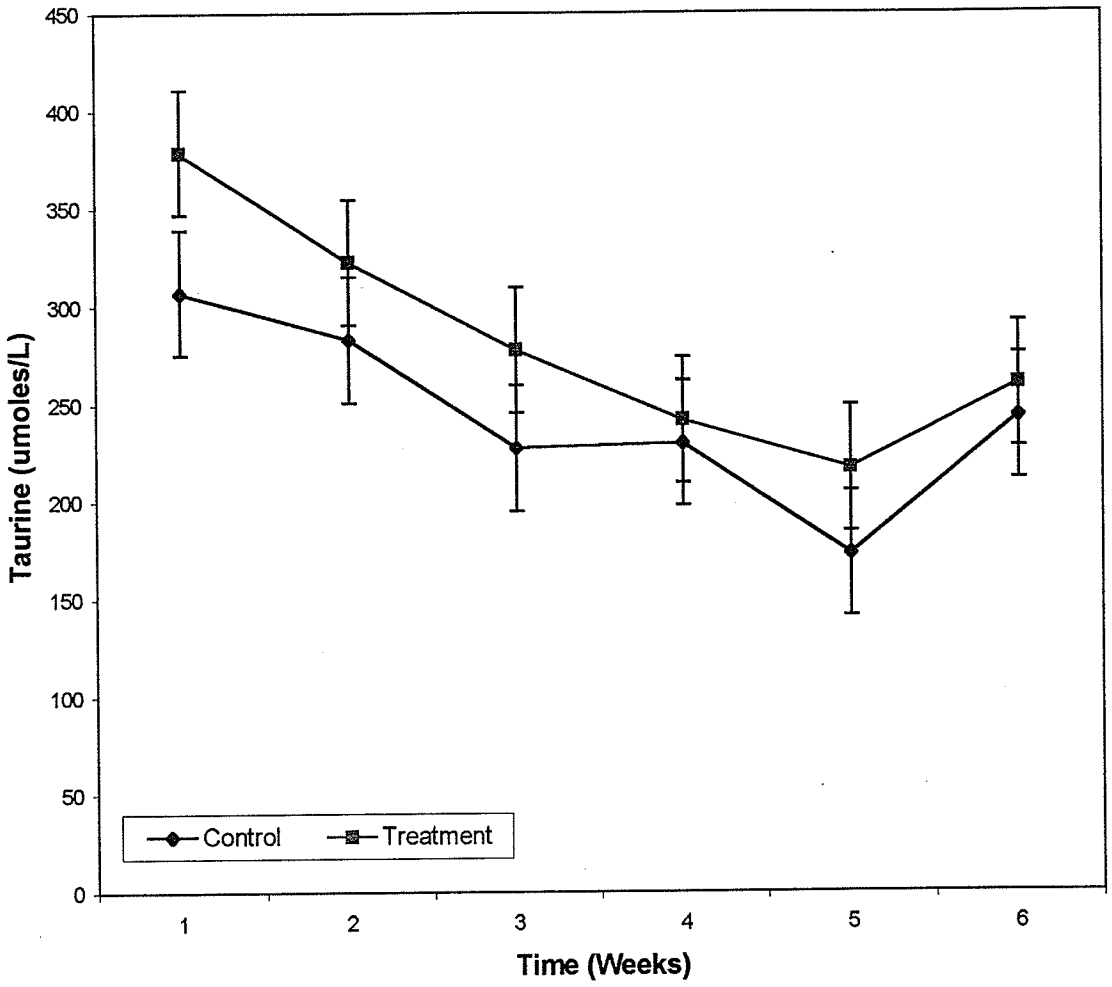
Data reported as LS Means +/- Standard Error
Treatment Effect P=<0.0001
Treatment*Week Interaction P=0.3422

Plasma Glycine in Rats Following 6-Week Folate Depletion Period



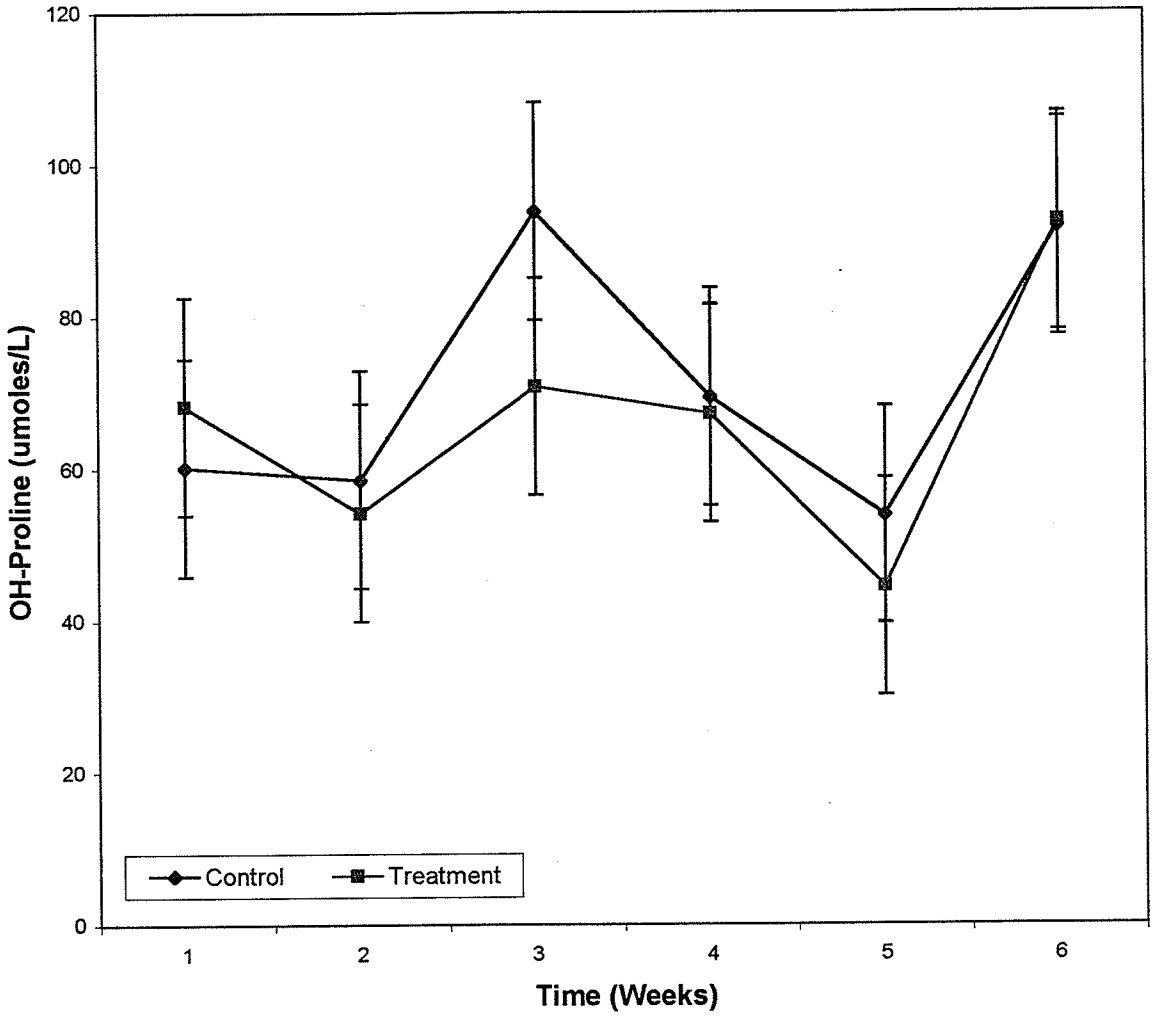
Data reported as LS Means +/- Standard Error
Treatment Effect P<0.0001
Treatment*Week Interaction P<0.0001

Plasma Taurine in Rats Following a 6-Week Folate-Depletion Period



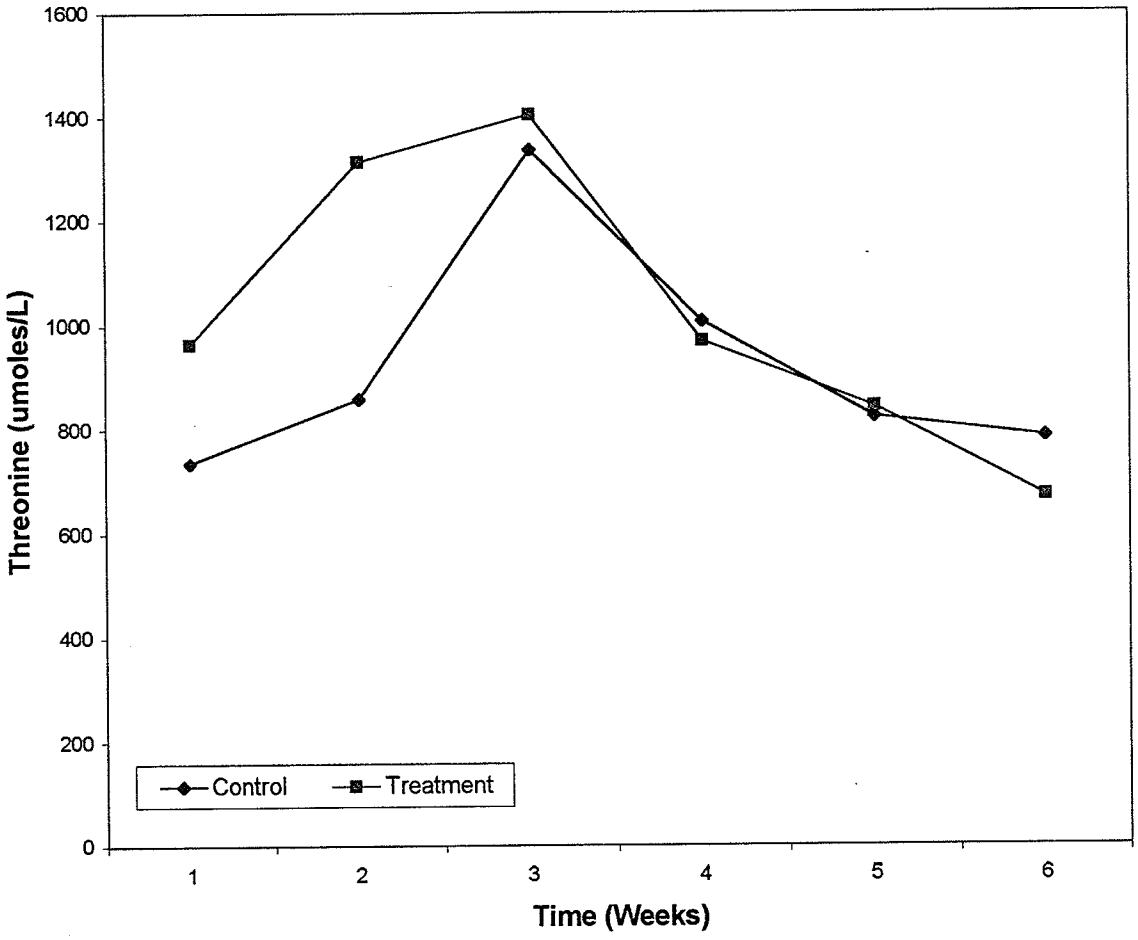
Data reported as LS Means +/- Standard Error
Treatment Effect P=0.0408
Treatment*Week Interaction P=0.9424

Plasma OH-Proline in Rats Following a 6-Week Folate Depletion Period



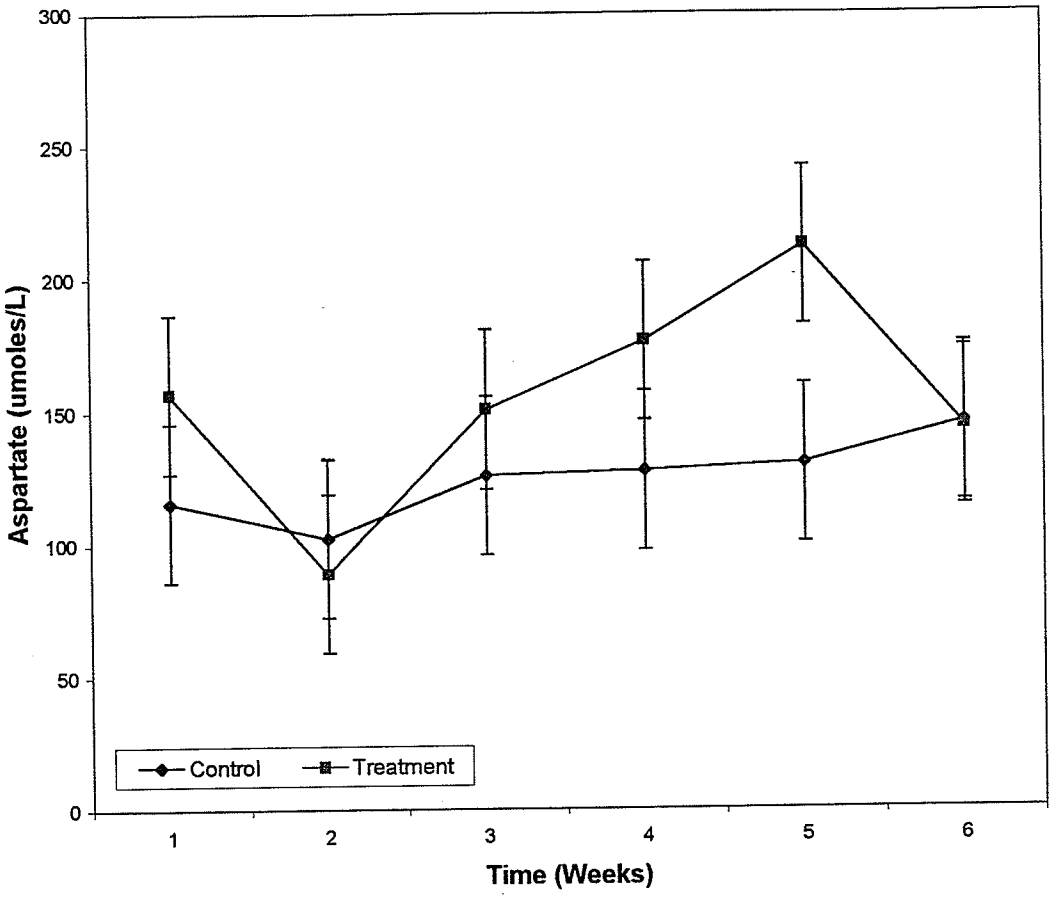
Data reported as LS Means +/- Standard Error
Treatment Effect P=0.5427
Treatment*Week Interaction P=0.9276

Plasma Threonine in Rats Following 6-Week Folate Depletion Period



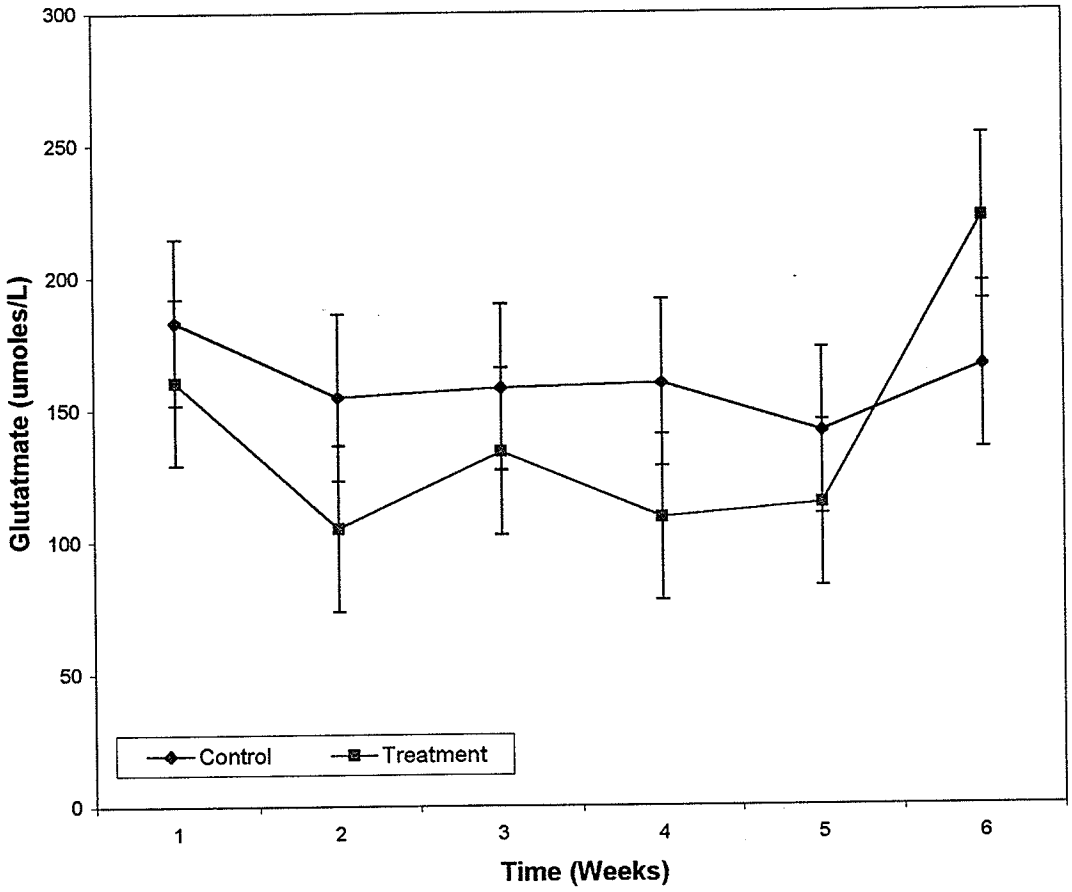
Data reported as LS Means +/- Standard Error
Treatment Effect P=0.1990
Treatment*Week Interaction P=0.3586

Plasma Aspartate in Rats Following a 6-Week Folate Depletion Period



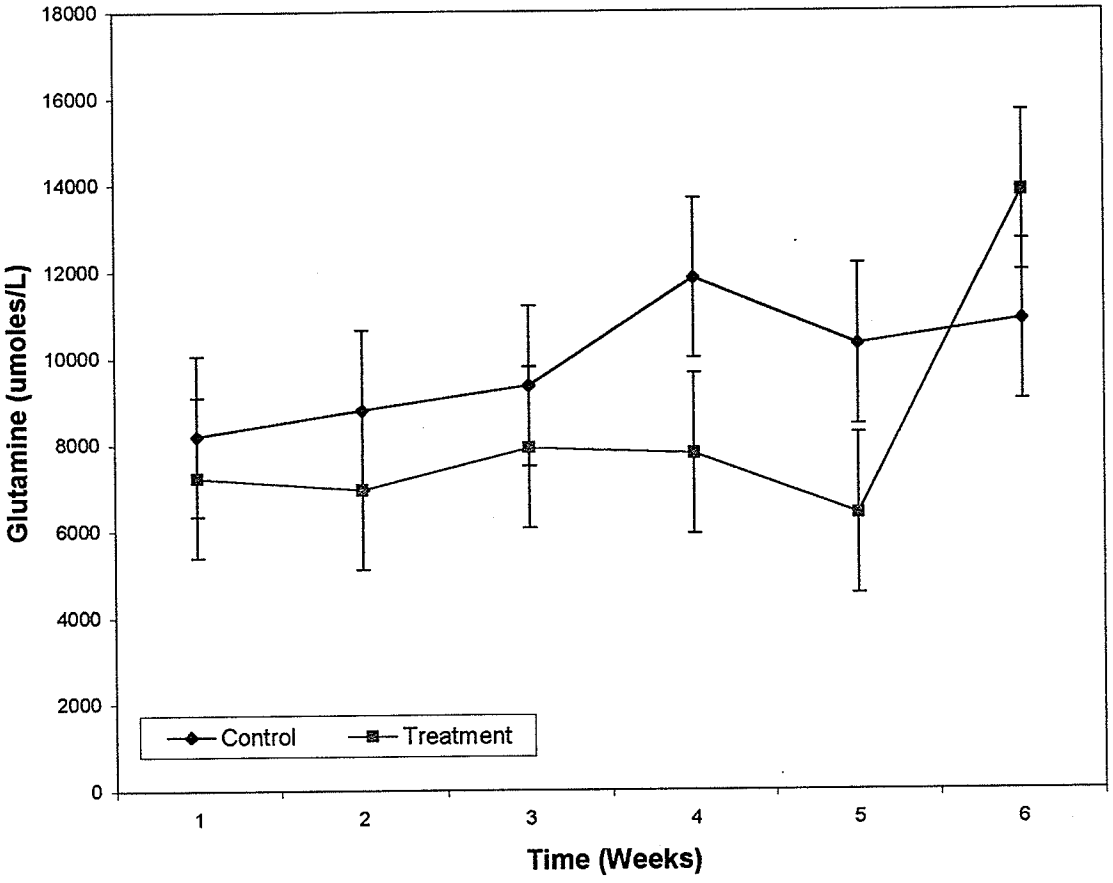
Data reported as LS Means +/- Standard Error
Treatment Effect P=0.0854
Treatment*Week Interaction P=0.6404

Plasma Glutamate in Rats Following 6-Week Folate Depletion Period



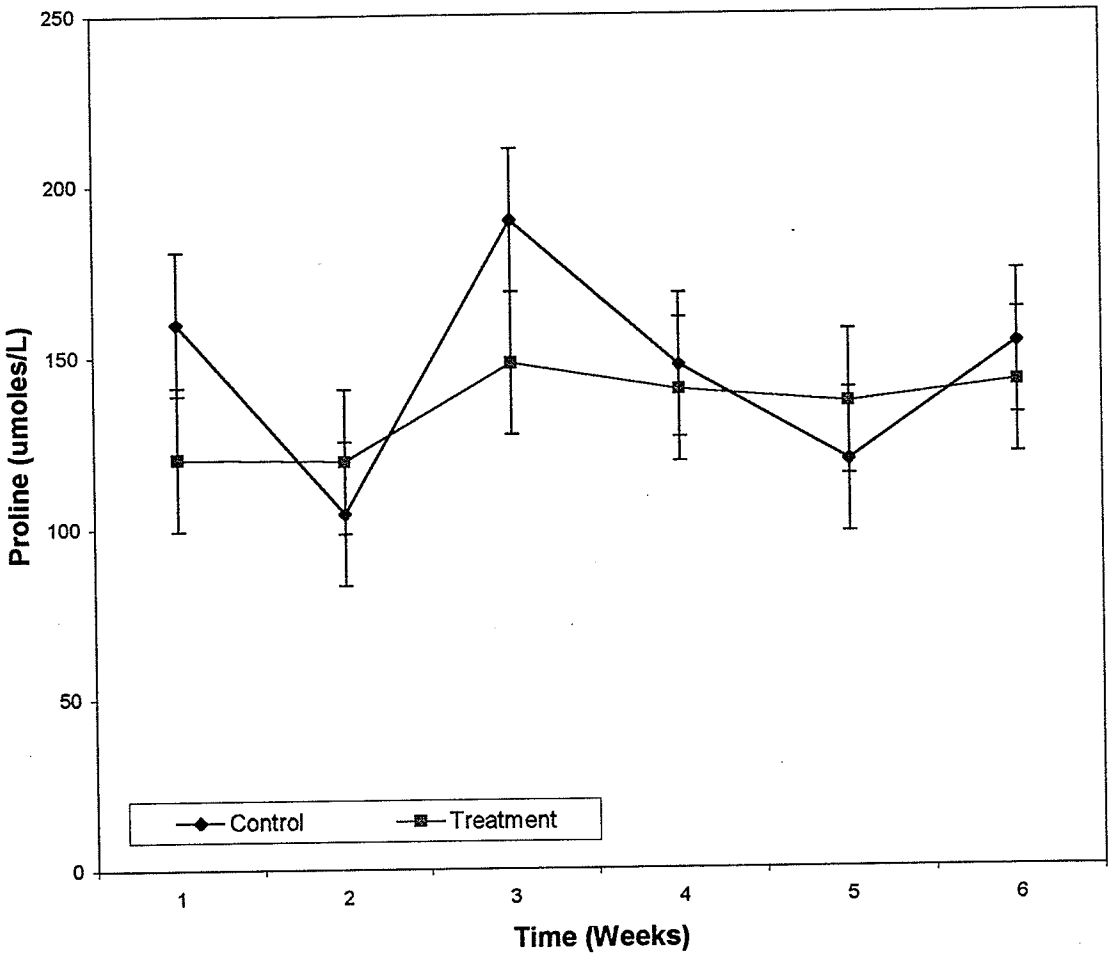
Data reported as LS Means +/- Standard Error
Treatment Effect P=0.2813
Treatment*Week Interaction P=0.5706

Plasma Glutamine in Rats Following 6-Week Folate Depletion Period



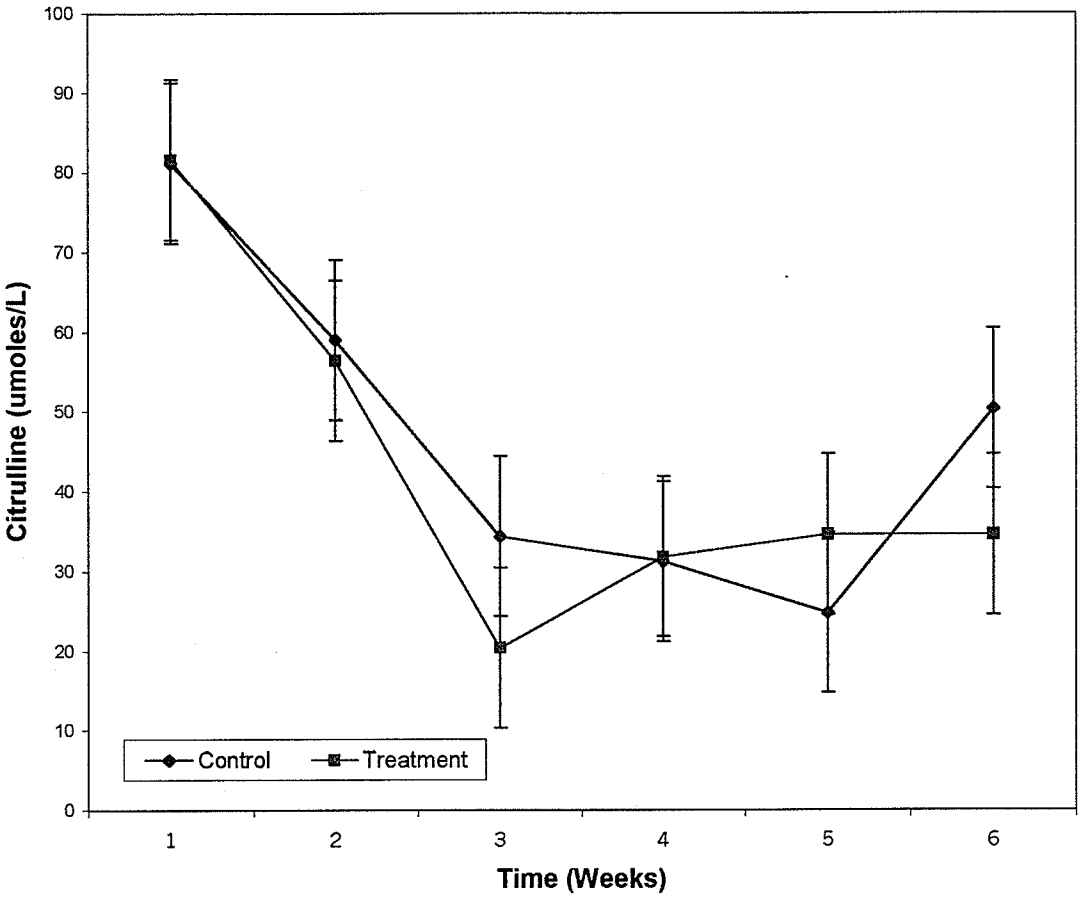
Data reported as LS Means +/- Standard Error
Treatment Effect P=0.1573
Treatment*Week Interaction P=0.4575

Plasma Proline in Rats Following a 6-Week Folate Depletion Period



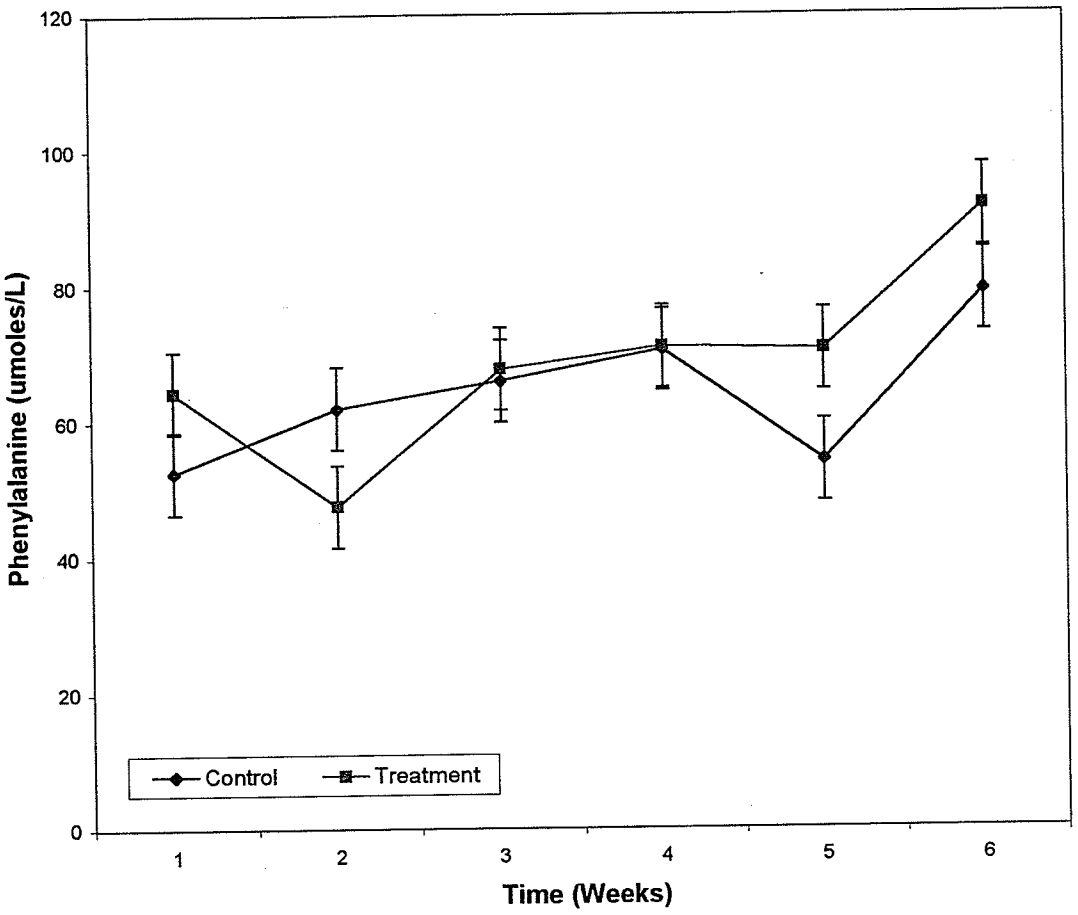
Data reported as LS Means +/- Standard Error
Treatment Effect P=0.3549
Treatment*Week Interaction P=0.6017

Plasma Citrulline in Rats Following a 6-Week folate Depletion Period



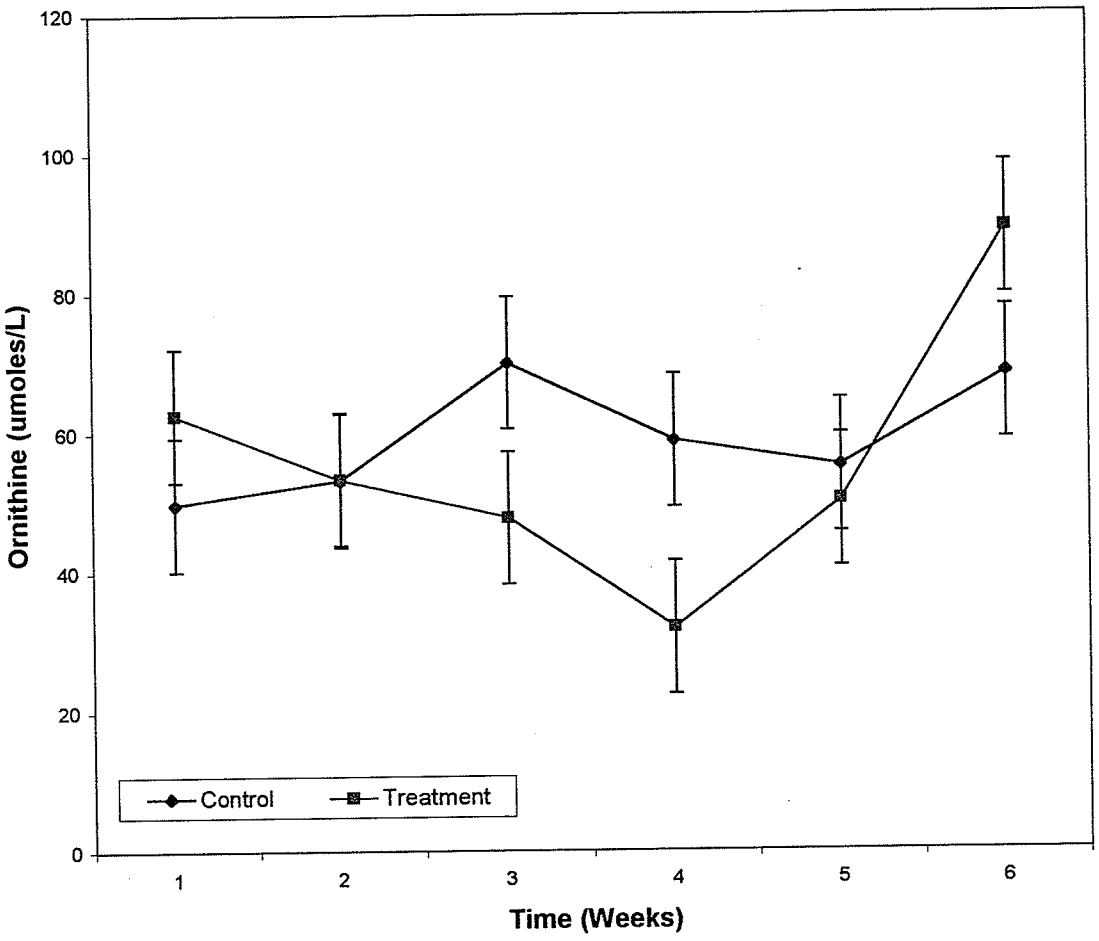
Data reported as LS Means +/- Standard Error
Treatment Effect P=0.5386
Treatment*Week Interaction P=0.8005

Plasma Phenylalanine in Rats Following a 6-Week Folate Depletion Period



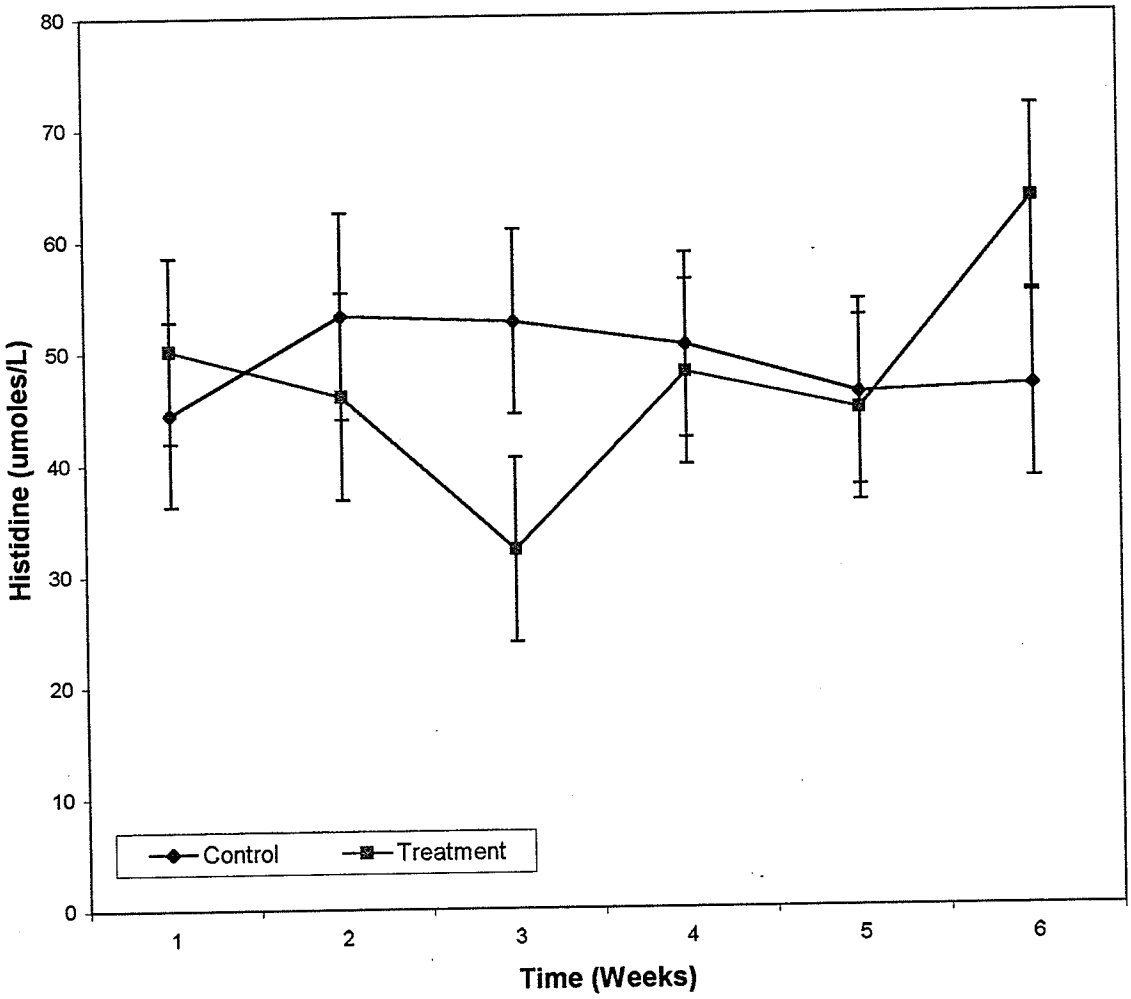
Data reported as LS Means +/- Standard Error
Treatment Effect P=0.1828
Treatment*Week Interaction P=0.1447

Plasma Ornithine in Rats Following a 6-Week Folate Depletion Period



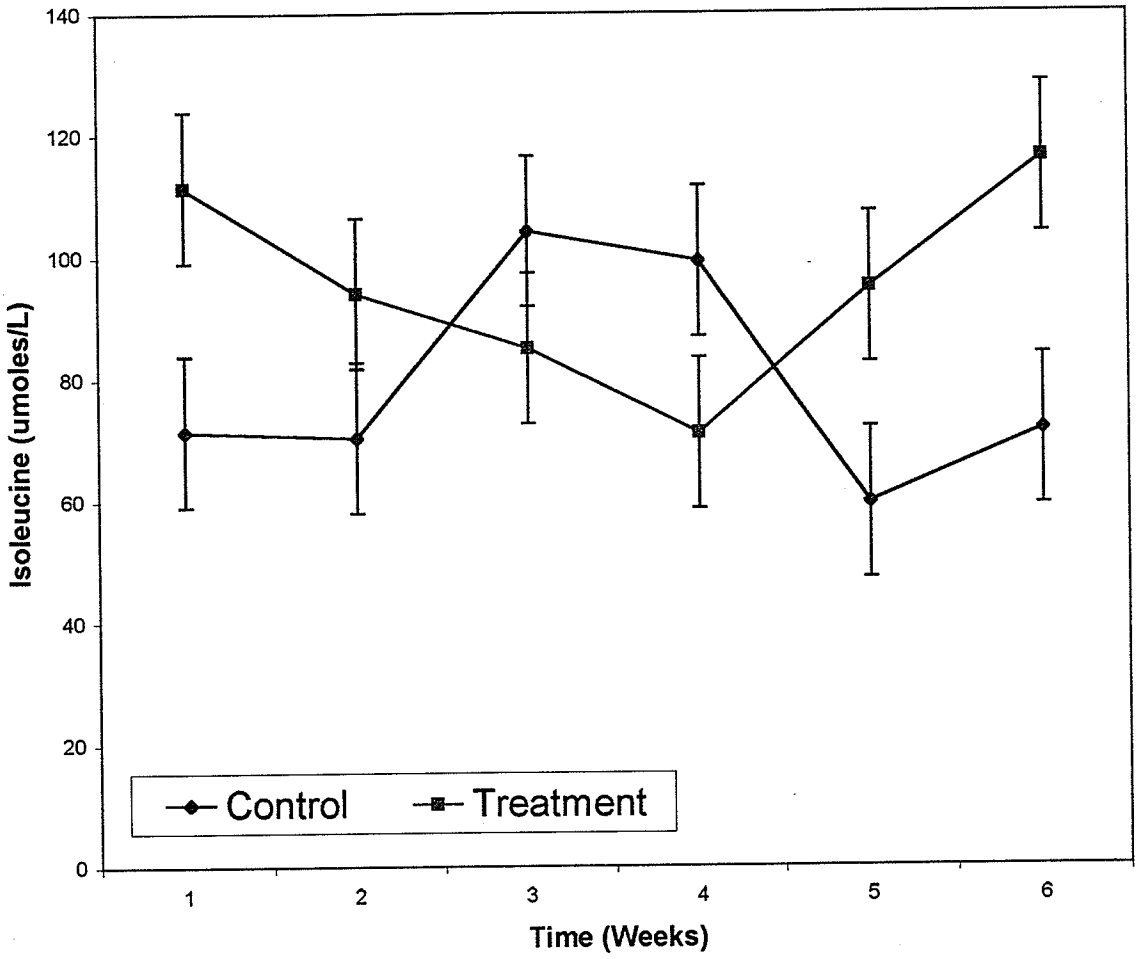
Data reported as LS Means +/- Standard Error
Treatment Effect P=0.5434
Treatment*Week Interaction P=0.1043

Plasma Histidine in Rats Following a 6-Week Folate Depletion Period



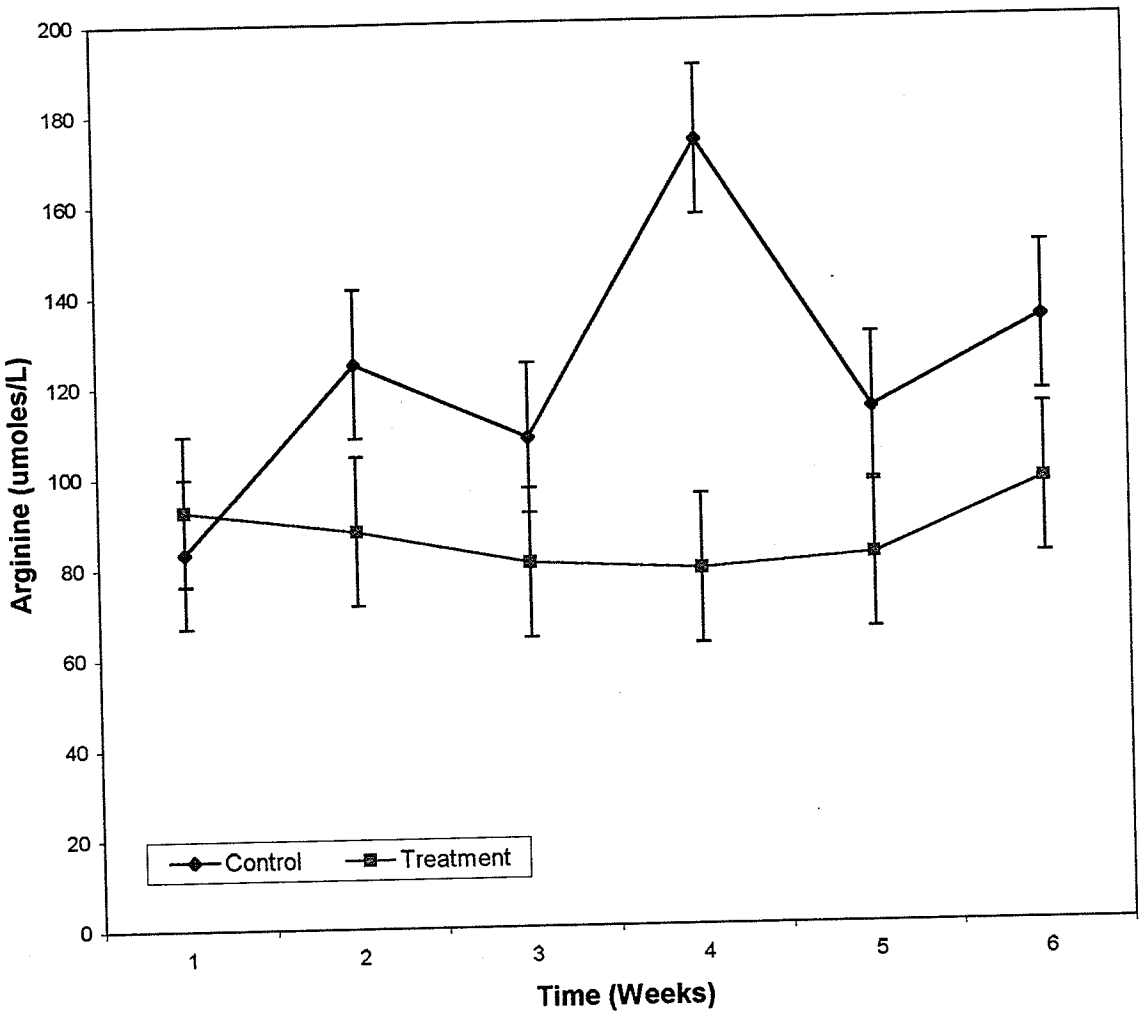
Data reported as LS Means +/- Standard Error
Treatment Effect P=0.7461
Treatment*Week Interaction P=0.3648

Plasma Isoleucine in Rats Following 6-Week Folate Depletion Period



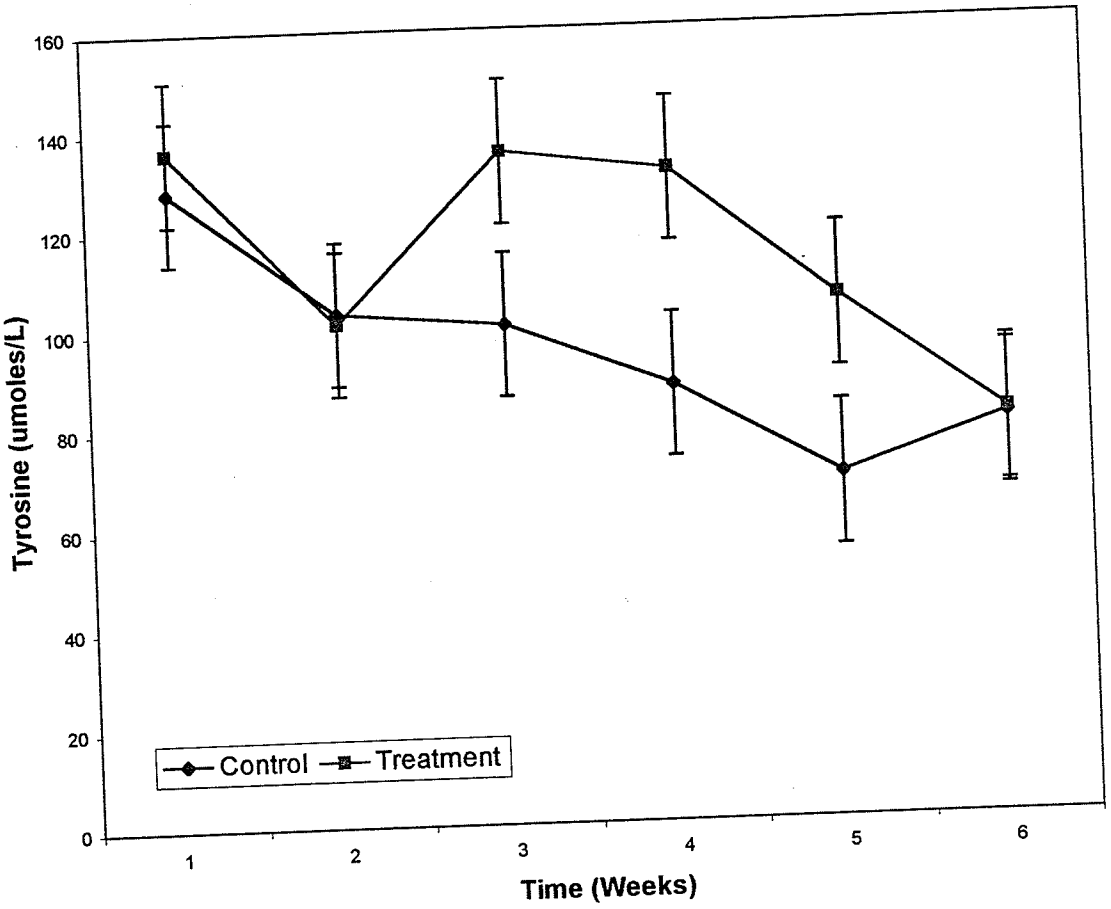
Data reported as LS Means +/- Standard Error
Treatment Effect P=0.0292
Treatment*Week Interaction P=0.0124

Plasma Arginine in Rats Following a 6-Week Folate Depletion Period



Data reported as LS Means +/- Standard Error
Treatment Effect P=0.0005
Treatment*Week Interaction P=0.0885

Plasma Tyrosine in Rats Following 6-Week Folate Depletion Period



Data reported as LS Means +/- Standard Error
Treatment Effect P=0.0198
Treatment*Week Interaction P=0.4508