

**FOLATE STATUS AND SUPPLEMENTATION IN THE HORSE**

Amy L. Ordakowski

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Approved by:

David S. Kronfeld, Co-chair  
Rhonda M. Hoffman, Co-chair  
Michael E. Houston  
Larry A. Lawrence  
Rebecca K. Splan  
Barbara A. Davis  
David Sklan

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Key Words: Folic acid, homocysteine, lactation, growth, exercise, anti-folate drugs

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### **(ABSTRACT)**

A series of studies were conducted to evaluate effects of lactation, exercise, and anti-folate drugs on folate status in the horse, and the bioavailability of supplement and feed folate in the horse. In the first study, mares and foals had adequate plasma folate, RBC folate, and plasma homocysteine concentrations during 6 mo of lactation and growth. Therefore, mares and foals maintained on quality grass/legume pastures and offered a pasture supplement did not require additional folate supplementation to maintain folate status during lactation and growth. In the second study, 25 mg of oral folic acid (FA) supplemented 5 times/wk to 11 mature horses engaged in routine submaximal exercise did not improve folate status, submaximal athletic performance, or combat the increase in oxidative stress during the 12 wk supplementation period compared to 11 horses not given supplemental folate. The common practice of supplementing horses with oral FA in vitamin supplements appears to be of little benefit to horses engaged in routine submaximal exercise. In the third study, daily oral administration of pyrimethamine (PYR) and sulfadiazine (SDZ) for 9 wk followed by 6 wk of coadministration of either Peptidoglycan or FA was associated with a decline in folate status resulting in moderate hyperhomocysteinemia, but not clinical signs of anemia. Peptidoglycan as a source of formylated folate and FA were not effective in improving folate status in horses coadministered PYR and SDZ, two anti-folate drugs commonly administered in equine veterinary practice. The last study assessed the bioavailability of oral and i.v. 5-methyltetrahydrofolate (5-mTHF), 5-formyltetrahydrofolate (5-fTHF), or FA, and the bioavailability of folate from concentrates fed to horses. The minimum efficiency of absorption for supplemental FA was 11 %. The low bioavailability of FA indicates a need for further research on the potential benefits of alternative sources of folate, including 5-fTHF, on increasing folate status in the horse.

Key Words: Folic acid, homocysteine, lactation, growth, exercise, anti-folate drugs

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## CHAPTER I

### Introduction

Folate has become one of the most heavily studied vitamins in human nutrition over the past two decades due to the fact that folate deficiency has been associated with numerous disorders including megaloblastic anemia, congenital abnormalities in infants, cardiovascular disease, Alzheimer's, and cancer. Folate deficiency in humans is caused by factors that include low dietary intake, increased requirements, and conditions or drugs that alter folate metabolism. In addition, genetic mutations in key enzymes in folate and homocysteine metabolism in humans increase the dietary intake of folate and thus increase the risk of certain folate related disorders. However, the daily supplementation of folic acid in humans has been shown to improve folate status and lower the incidence of folate related disorders. The implications of low folate status and disease states and the success associated with folic acid supplementation should prompt the reevaluation of folate nutriture in numerous other species.

With the exception of laboratory animal species, few studies have been conducted that investigate the role of folate status and supplementation in other animal species. This is partly due to the potentially large contribution and utilization of microbially derived folate in the foregut of ruminants and the hindgut of non-ruminants or monogastrics and partly due to the high forage diets containing adequate amounts of folate. However, species including dogs, cats, guinea pigs, swine, and poultry require supplemental folate to meet dietary folate requirements.

Studies assessing folate status and supplementation in the horse are limited. Horses are unique monogastrics in that they appear to be capable of utilizing folate derived from microbial synthesis in their hindgut and cecum (Carroll et al., 1949). However, horses kept in stalls denied ad libitum access to pasture and/or engaged in intense exercise regimens have lower serum and RBC folate concentrations compared to pastured horses (Seckington et al., 1967; Allen, 1978; Roberts, 1983). Exercise training in horses has also been shown to lower folate status (Allen, 1978; Allen and Powell, 1983). These earlier reports led to the recommendation for and routine supplementation of folic acid in equine concentrates and vitamin supplements. These

recommendations were made despite the lack of knowledge regarding the bioavailability and efficacy of synthetic folic acid supplementation in the horse.

More recently, three pregnant mares treated for Equine Protozoal Myeloencephalitis (EPM) with pyrimethamine (PYR), sulfonamides (sulfamethoxazole and sulfadiazine), folic acid, and vitamin E were reported to have had foals with congenital birth defects attributed to folate deficiency (Toribio et al., 1998). It appeared that the anti-folate effects of PYR and sulfonamides were potentiated by coadministration of folic acid, which has been shown to occur in other species (Kudo et al., 1988; Hayama et al., 1991; Chung et al., 1993). Therefore, coadministration of folic acid with PYR and sulfonamides used to treat EPM is not recommended in veterinary practice.

Given the importance of folate in health and disease in the human and the lack of understanding regarding the role of folate in the equine, the need for studies on folate status and supplementation in the horse has arisen. The objectives of our studies were to:

1. Assess the effects of lactation and growth on folate status in mares and foals
2. Assess the effects of long-term oral folic acid supplementation in moderately exercising horses
3. Assess the effects of long-term administration of pyrimethamine and sulfadiazine on folate status in mature geldings
4. Evaluate the bioavailability of oral and intravenously administered natural reduced folate sources and the synthetic oxidized folic acid in the horse

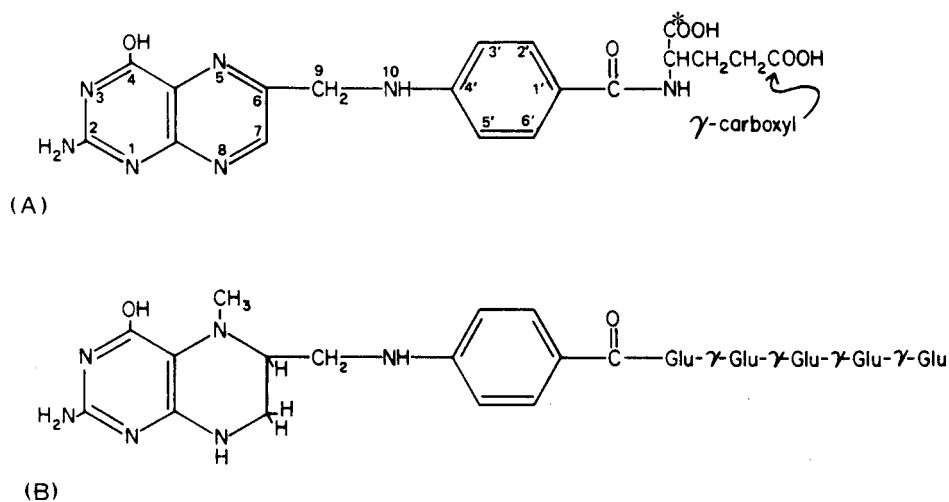
## CHAPTER II

### Literature Review

#### *Chemical structure and properties of folate*

Folate is a term used to describe a large group of similarly structured compounds that are classified as water-soluble B vitamins. Named after the Latin term “folium” meaning leaf, the term “folic acid” was originally assigned to the antianemic compound isolated from spinach (Mitchell et al., 1941). Since then, the term “folate” has been adopted and refers to all derivatives that share a common structural background including the oxidized folic acid (FA) commonly used for supplementation. The common structural background shared by the many folate derivatives consists of a 2-amino-4-hydroxypteridine (pterin) moiety linked via a methylene group at the C-6 position to a p-aminobenzoic acid (PABA) linked at the gamma carbon to a glutamate (Figure 1a) (Wagner, 1984; Herbert and Das, 1994). There are three main changes to that structural backbone that account for the numerous folate derivatives that can occur naturally. The first variation is the reduction state of pteridine moiety, which can be fully reduced, yielding a 5,6,7,8-tetrahydrofolate (THF) structure, partially reduced yielding 7,8-dihydrofolate (DHF) structure, or fully oxidized as in the pterin ring structure of FA. Second, the folate derivative may have additional glutamate residues attached at in  $\gamma$ -peptide linkage resulting in a polyglutamate structure instead of its original monoglutamate structure. Folate polyglutamates are more commonly found in nature, metabolically active tissue, and in tissue storage sites, whereas the monoglutamate form is more prevalent in blood and milk (Herbert and Das, 1994). Third, the N-5 and/or N-10 positions on the folate structure can serve as sites for attachment of one-carbon units including methane and formate, which plays a major role in one-carbon metabolism. The structures of FA and 5-methyltetrahydrofolate (5-mTHF) are shown in Figure 1A and 1B respectively (Brody, 1991). Metabolically significant forms of folate are DHF, THF, 5-mTHF, 5-formyltetrahydrofolate (5-fTHF), 10-formyltetrahydrofolate, 5,10-methylenetetrahydrofolate, 5-formiminotetrahydrofolate, and their respective glutamate conjugates. The 5-fTHF form has also been called citrovorum factor, leucovorin®, or folinic acid. Naturally occurring folate polyglutamates are predominately reduced derivatives, whereas the fully oxidized FA is found in much smaller amounts.

Folic acid (C<sub>19</sub>H<sub>19</sub>N<sub>7</sub>O<sub>6</sub>) is an odorless pale orange to yellow crystalline powder with a molecular weight of 441.4 (Herbert and Das, 1994). Folate derivatives have a low solubility in water and in most organic solvents, but solubility is increased in aqueous acids and bases with increases in temperature to 70°C (Temple and Montgomery, 1984).



**Figure 1.** Structure of folic acid (A) and 5-methyltetrahydrofolate (B). \* Denotes site of attachment of additional glutamate residues. Adapted from Brody (1991).

With the exception of FA, most folate in food and feedstuffs are easily oxidized under aerobic conditions or destroyed under conditions of high heat, light, and/or metal ions (Gregory, 1989; Combs, 1992a). Folic acid, on the other hand, has an oxidized pterin ring making it the most stable folate derivative, and has thus been used in the fortification of foods and feedstuffs and inclusion in vitamin supplements. Stability is greater with the addition of one-carbon units such as a methyl group compared to the stability of THF, but the size of the glutamate residue has not been shown to influence stability (Gregory, 1989). The relative ease of oxidation and destruction of folate requires attempts to keep samples away from light, heat, and oxygen during collection, processing, and analysis. To prevent loss of folate during sample storage, reducing agents such as ascorbate, 2-mercaptoethanol, and dithiothriol should be added to all samples and samples should be kept at freezing temperatures with ultralow temperatures being most favorable (Gregory, 1989).

## *Sources*

Humans and animals cannot synthesize folate and therefore rely on exogenous sources to meet their folate requirements. Folate derivatives are widely distributed in various concentrations in plant and animal tissues. The folate content in some food and feedstuffs has been published (Brody, 1991; Bailey, 1995; McDowell, 2000). Examples of human foodstuffs high in folate include organ meats (e.g., liver and kidney), raw green leafy vegetables (e.g., broccoli and spinach leaves), and mushrooms, whereas fruits and dairy foods are poor to moderate sources of folate. In animal diets, fresh forages, oilseed meals (e.g. soybean meal) and animal by-products are good sources of folate. The predominate folate derivatives in foodstuffs are polyglutamated THF's with 5-methylTHF and 5- and 10-formylTHF derivatives predominating (Combs, 1992a).

In addition to meeting folate requirements by consumption of food, humans and animals are capable of absorbing and utilizing folate synthesized by gut microbes (Miller and Luckey, 1963; Klipstein and Samloff, 1966; Rong et al., 1991). Unlike the humans and animals, microbial species in the gut can synthesize folate from PABA and other necessary precursors. The site of microbial folate production and amount of folate produced vary depending on the species. By using infusions of tritiated PABA in rat intestine, it was shown that the newly synthesized radiolabeled folate by gut microbes were absorbed and deposited as mainly penta and hexa glutamated folate in liver and kidney (Rong et al., 1991). It has also been shown that humans are capable of absorbing bacterially synthesized radiolabeled folate in the upper small intestine (Camilo et al., 1996). In animals that contain large fermentation vats like the rumen or cecum, microbial synthesis of folate is most likely produced in greater amounts and provides a greater contribution to the meeting folate requirements. The mechanisms and site of absorption of microbially derived folate is not fully understood.

The horse is a unique monogastric species in that it has a cecum that contains microbes that can synthesize folate (Carroll et al., 1949). Horses fed low folate diets (< 0.1 mg/kg BW) had higher folate concentrations in the duodenum, ileum, cecum, large colon, small colon and feces compared to the folate content in the diet. The highest values of folate occurred in the cecum and large colon indicating microbial synthesis of folate was greatest in those sections. Because

microbial synthesis appears to contribute to the folate status of the horse (NRC, 1989b), it is likely that folate transport mechanisms are present in the hindgut of the horse.

### *Bioavailability of folate*

Bioavailability as it pertains to folate has been described as the “overall efficiency of utilization, including physiological and biochemical processes involved in the intestinal absorption, transport, metabolism, and excretion” (Gregory, 1995). Several factors can influence bioavailability of folate including the form of folate in the diet, composition of the diet, and nutritional status, age, and health of the individual. The three main forms of folate ingested in the body are natural food folate, FA fortified foods, and dietary supplements containing FA. From experimental data in humans, the bioavailability of FA fortified in foods is 85 %, nearly twice as much as food folate alone (FNB, 1998; Gregory, 2001). Bioavailability of tritiated forms of FA, 5-mTHF, and 5-fTHF were found to have similar absorption, metabolism, and in vivo kinetics in rats (Bhandari and Gregory, 1992). Therefore, it appears that the determining factor in bioavailability is how the folate source is supplied to the body. Numerous reviews have discussed the bioavailability of food folate and synthetic FA fortified in foods or as an oral dietary supplement in humans (Gregory, 1995; Gregory, 2001).

### *Absorption and transport*

Dietary folate entering the intestinal lumen are primarily in the reduced polyglutamated form and must first have the glutamate residues hydrolyzed to yield mono and diglutamate folate prior to absorption. The hydrolysis of the glutamate tail is catalyzed by an exocarboxypeptidase enzyme called folate  $\gamma$ -glutamylhydrolase, which is commonly known as folate conjugase. Folate conjugase is located at the luminal surface and has an optimal pH between 6.5 and 7.0 (Rosenburg and Selhub, 1986). Active folate conjugases have also been found in bile, pancreatic juice, kidney, liver, placenta, bone marrow, leukocytes, and plasma, although the importance and function of the enzyme in these tissues is uncertain (Combs, 1992a). Once hydrolyzed to the monoglutamate form, folate is ready to be absorbed by cells.

The major site of folate absorption in the human occurs in the proximal jejunum with very little being absorbed in the distal jejunum and ileum (Hepner et al., 1968; Rosenburg and Selhub,

1986). In addition, folate has been shown to be absorbed in colonic epithelium in the human (Dudeja et al., 1997; Kumar et al., 1997).

There are two main types and one minor type of transport processes involved in the intracellular internalization of folate. The first type of transport process is by a Na<sup>+</sup>-coupled saturable carrier-mediated process binding to a transporter at the membrane surface to mediate internalization through membranes (Sirotnak and Tolner, 1999). Carrier-mediated processes are present in the intestinal and colonic epithelium exhibiting an optimal pH of 5.0 and K<sub>m</sub> value between 2 and 4 μM with equal affinity for FA, 5-mTHF, and 5-fTHF (Selhub et al., 1984; Blakeborough and Salter, 1988; Sirotnak and Tolner, 1999). Studies in the rat indicate that expression of the RFC-1 gene regulates folate absorption in the small intestine (Chiao et al., 1997), and its expression is increased and folate uptake is increased during folate deficiency (Said et al., 2000). In the colon, dietary absorption of folate in humans appears to occur via a carrier-mediated system, which is temperature, pH, and energy dependant, and appears to be under the regulation of cAMP and protein tyrosine kinase (Kumar et al., 1997). A saturable transport system for folate in the basolateral membrane of the rat small intestinal enterocyte was found to have similar attributes except that its not affected by either sodium or potassium (Said and Redha, 1987).

The second type of transport method is via a receptor-mediated processes that utilizes high-affinity binding of folate at the membrane surface to a receptor-like protein, which mediates unidirectional flux following internalization of the receptor-folate complex (Sirotnak and Tolner, 1999). Folate receptors are glycosyl-phosphatidylinositol (GPI) anchored and are expressed on numerous tissues including the placenta, renal tubular cells, and the blood-brain barrier/blood-cerebrospinal fluid barrier (Antony, 1996). The membrane associated binding proteins are 38 to 44 kD, which bind to physiological folate with high affinity in the nanomolar range (Antony, 1996).

The third type of transport process is via simple diffusion, which has been documented at pharmacological doses of folate and during transplacental folate transport occurring in concert with folate receptors (Antony, 1996). The nonsaturable process proceeds linearly related to

luminal folate concentrations and accounts for 20 to 30 % of folate absorption at high folate intakes (Combs, 1992a). When pharmacological doses of the monoglutamate form of folate are consumed, it is also absorbed by a nonsaturable mechanism involving passive diffusion. The overall efficiency of folate absorption in the human appears to be about 50 % (10 to 90 %) and can be affected by malabsorption syndromes (Gregory, 2001).

After uptake of folate into the enterocyte, reduction of oxidized folate monoglutamate derivatives takes place. In the case of FA, it is reduced to DHF by DHF reductase and then can be further reduced to THF in order to be methylated or formylated (Rosenberg and Selhub, 1994). Reduced derivatives, mainly 5-mTHF, are transported into the portal circulation (Whitehead, 1994). Administration of non-physiological concentrations will saturate the system allowing a greater proportion of absorbed folate to pass through the enterocyte without reduction or methylation (Mason and Rosenberg, 1994).

In addition to the release of folate into the circulation, a larger percentage of the folate monoglutamates are also incorporated into bile and excreted into the small intestine (Steinberg et al., 1979; Whitehead, 1994). Biliary folate including methylated, formylated, and oxidized forms (Shin et al., 1995), are reabsorbed and transported to tissues within the body. As much as 50 % of the folate ultimately reaching peripheral tissues may be accounted for by this recirculation processes (Steinberg, 1984). Interruption of the enterohepatic cycle results in a drastic decline in serum folate levels indicating the importance of the enterohepatic cycle in folate homeostasis (Steinberg et al., 1979).

After absorption, reduced folate monoglutamates are transported in the portal circulation to the liver where they can be polyglutamated and retained or released in blood or bile. In most species, 5-mTHF is the main folate derivative released from the liver and transported to tissues in the plasma. However, swine have THF as the main form of folate in circulation (Natsuhori, et al., 1991).

Once in circulation, 50 % of plasma folate is bound to low affinity folate binding proteins (FBP) including albumin for transport to tissues (Whitehead, 1994; Shane, 1995). It has been

suggested that protein binding may facilitate folate transported by tissues such as liver (Combs, 1992a). Plasma also contains low levels of high affinity folate binder, which appears to be the same protein as the cellular high affinity folate binding protein, which can be released from cells after hydrolysis of its glycosylphosphatidylinositol anchor. Therefore, FBPs are also called folate receptors. The binding affinity of swine binding proteins was found to be much greater than that of other animals including sheep, goat, cattle, horse, rabbit, dog, rat, guinea pig, and chicken (Mantzios et al., 1974), which led to their use in a folate radioassay a year later (Mantzios, 1975). In addition to plasma FBP's, milk FBP's have also been identified in humans and animals (Metz et al., 1968; Weitman et al., 1994; Wigertz et al., 1997). The regulation of FBP expression is not well understood but it is clear that extracellular folate concentration plays an important role in the process, serving as an inverse stimulus to FBP expression (Combs, 1992a).

#### *Cellular uptake and distribution*

Cellular uptake of folate monoglutamates occurs either by a folate receptor via a carrier mediated process requiring energy and sodium, or by a reduced folate carrier anion-exchange system (Combs, 1992a). The folate receptor is a glycoprotein, has bound fatty acids and is attached to the membrane via a glycosylphosphatidylinositol anchor (Weitman et al., 1994). The regulation and distribution of folate receptors has been reviewed (Weitman et al., 1994). These transport systems are not saturated by folate under physiological conditions, and folate influx into tissues would be expected after any elevation in plasma folate after supplementation.

Once transported inside the cell, monoglutamates must be converted to polyglutamates by  $\gamma$ -glutamate synthetase after demethylation for the retention and storage inside the cell (Steinberg, 1984). Folate that are polyglutamated are better substrates for folate-dependent enzymes and folate-related cell functions are related to polyglutamate reserves (Steinberg, 1984).

Within the cell, there is nearly equal compartmentalization of folate polyglutamates between the cytosol and the mitochondria (Shane, 1995). Within these compartments, folate derivatives are associated with FBPs. In the cytosol, major FBPs are glycine N-methyltransferase and 10-formylTHF dehydrogenase, whereas in the mitochondria, major FBPs have been identified as

sarcosine and dimethylglycine (Shane, 1995). The mitochondrial and cytosolic folate pools are distinct from each other and also have a distinct one-carbon metabolism.

### *Tissue Distribution*

The estimated body folate content in the human is between 12 and 28 mg (FNB, 1998). Within the body, nearly half of the folate derivatives are concentrated in the liver, with the remaining folate accumulating in greater amounts in rapidly dividing tissue (e.g. intestinal mucosa, regenerating liver, carcinoma) (Combs, 1992a). The type of folate derivatives, length of glutamyl residues and concentration varies within different tissues (Cossins, 1984; Whitehead, 1994). Folate deficiency lowers the folate concentrations in tissues with longer polyglutamates predominating (Varela-Moreiras and Selhub, 1992). However, folate concentrations in the brain are unresponsive to changes in dietary folate intake (Shane, 1995).

### *Turnover and excretion*

Catabolism of folate occurs by cleavage of the folate molecule at the C-9 and N-10 bonds resulting in the pteridine and p-aminobenzoylpolyglutamate (pABG) moieties (Scott, 1984; Combs, 1992a). The resulting PABA polyglutamates are hydrolyzed to a monoglutamate, which is N-acetylated in liver prior to excretion (Shane, 1995). The rate of folate catabolism appears to be related to the rate of intracellular folate and is therefore greatest during conditions of high cell turnover (e.g. rapid growth, pregnancy) (Wang et al., 1994) and intake (Von den Porten et al., 1992; Gregory, 1998). The turnover of folate in the human is less than 1 % of their total body folate per day (Von den Porten et al., 1992). A model for whole body turnover has been reported (Gregory, 1998). Recently, ferritin purified from rat liver was observed to have catabolizing activity against 5-fTHF in vitro and that heavy chain ferritin may be involved in maintenance of folate levels within the cell (Suh et al., 2000).

The primary excretory site of folate catabolism products is through the urine (Scott, 1984). Folate is freely filtered at the glomerulus, but is reabsorbed in the proximal renal tubule by folate receptors along the brush border membrane resulting in low losses of folate in the urine (Combs, 1992a; Antony, 1996). The total urinary excretion of folate and metabolites is small and is estimated to be less than 1 % of total body stores per day (Combs, 1992a). Fecal folate excretion

is variable and is not a measure of folate availability due to the confounding contribution from the enterohepatic cycle, lysed intestinal cells, and microbially synthesized folate in the gut (Scott and Weir, 1986).

### *Metabolic Functions*

The primary function of folate derivatives is to exchange one-carbon units from various sources in what is commonly called one-carbon metabolism. The one-carbon units exist in various levels of oxidation including those that are reduced as in methane to those that are more oxidized including carbon dioxide. The one-carbon units are generated during metabolism, with the exception of carbon dioxide, and are carried from one reaction to another as folate derivatives (Wagner, 1984). When the one-carbon units are incorporated as folate derivatives, they must be converted from one oxidation level to another by gain (reduction) or loss (oxidation) of electrons. The single carbon exchange by folate coenzymes in one carbon metabolism plays an essential role in amino acid metabolism, DNA and purine synthesis, and the generation of formate. The major metabolic functions of folate are summarized below and a basic schematic of folate derivatives involved in one-carbon metabolism is shown in Figure 2.

The reduced THF derivative is the basic coenzyme for that serves as a single carbon acceptor and donor in metabolism (Wagner, 1984). Therefore, prior to being a metabolically active substance, oxidized forms of folate including FA must first be reduced to THF. Folic acid is enzymatically reduced by DHF reductase to 7,8-dihydrofolate (DHF) and then further reduced by the same enzyme to THF (Brody, 1991). In both steps of this reversible reaction, NADPH serves as the donor of hydrogen atoms. The reduction of FA to THF is essential for utilizing the synthetic form of the vitamin, and administration of DHF reductase inhibitors can prevent its use in the body (Herbert and Das, 1994).

*Glycine-serine interconversion.* The  $\beta$ -carbon of serine is the major source of one-carbon units used in folate metabolism. The B<sub>6</sub> requiring enzyme, serine hydroxymethyltransferase (SHMT), catalyzes the transfer of serine to THF to produce 5,10methyleneTHF, glycine and water (Brody, 1991). The reaction is freely reversible. In the cytosol, the enzyme catalyzes the

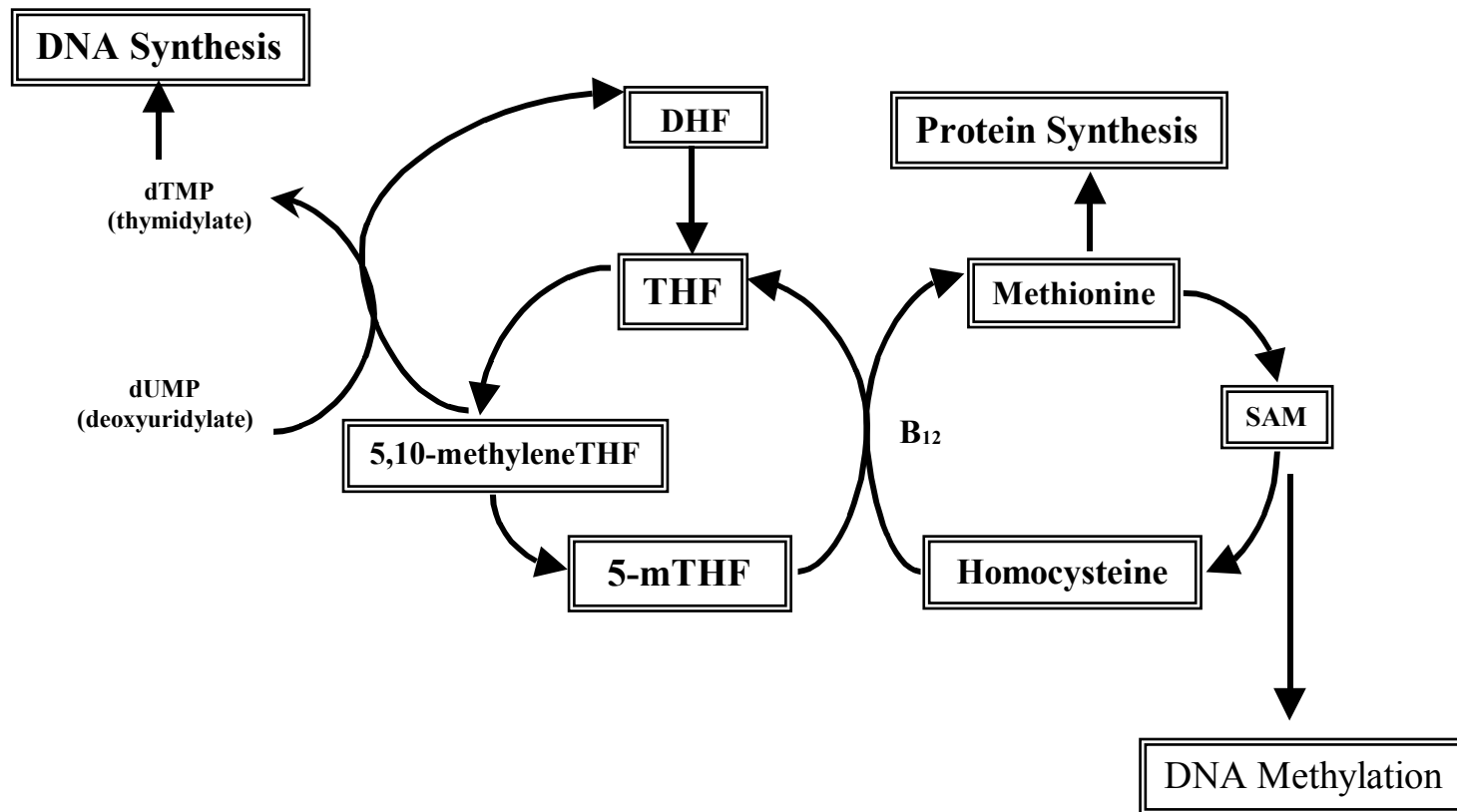
conversion of serine to glycine, whereas the opposite is believed to occur in the mitochondria (Bailey and Gregory, 1999a).

Folate is also involved in the glycine cleavage system located in the mitochondria involving the donation a methyl group from glycine to THF, which regenerates 5, 10-methyleneTHF (Brody, 1991).

The two reactions that generate 5,10-methyleneTHF are very important due to its source of methyl groups for homocysteine methylation and DNA synthesis. The enzyme that catalyzes the reaction of 5,10-methyleneTHF to 5-mTHF is 5,10-methyleneTHF reductase (MTHFR) (Brody, 1991). The MTHFR enzyme utilizes NADPH and the reaction is reversible although it favors formation of 5-mTHF. The MTHFR enzyme is also very important in human nutrition because of genetic variants in the gene that encodes for the enzyme that leads to inborn errors of metabolism and increases the risks of certain folate-related disorders (Bailey and Gregory, 1999b).

*Homocysteine methylation.* Homocysteine is a non-essential sulfur containing amino acid that can be methylated to form the essential amino acid methionine. In order to regenerate methionine, the B<sub>12</sub> dependent enzyme methionine synthetase catalyzes the transfer of a methyl group from 5-mTHF to homocysteine, resulting in methionine and THF (Brody, 1991). This reaction depends heavily on the regeneration of 5-mTHF from 5,10-methyleneTHF by the MTHFR enzyme and from adequate dietary intake. Low concentrations of 5-mTHF or B<sub>12</sub> results hyperhomocysteinemia, a condition associated with many clinical disorders. As a consequence of this B<sub>12</sub>-folate relationship, a deficiency in B<sub>12</sub> may cause a build up of 5-mTHF at the expense of other folate derivatives and thereby produce an accompanying folate deficiency. This condition has been termed the “methylfolate trap” (Herbert and Das, 1976).

The formation of methionine from homocysteine is very important because methionine is incorporated into proteins and serves as a methyl group donor through s-adenosylmethionine (SAM). The formation of SAM is very important methylating agent in over 100 reactions in the body. In addition, SAM is very important in the regulation of folate metabolism.



**Figure 2.** Basic schematic of one-carbon metabolism involving folate derivatives. Abbreviations: dUMP, deoxyuridine momphosphate; dTMP, deoxythymidylate monophosphate; DHF, dihydrofolate; THF, tetrahydrofolate; 5-mTHF, 5-methyltetrahydrofolate; SAM, s-adenosyl methionine.

High concentrations of SAM may inhibit the conversion of 5-mTHF to homocysteine, and concentrations of SAM may upregulate the enzymes involved in the trans-sulfuration pathway increasing the degradation of homocysteine (Bailey and Gregory, 1999a).

*Pyrimidine biosynthesis.* The availability of folate coenzymes is essential for normal DNA synthesis. Perhaps one of the most important functions of folate is to serve as a methyl donor in the synthesis of the DNA base pair, thymine (T). This reaction is catalyzed by thymidylate synthase and involves the methylation of dUMP by 5,10-methyleneTHF producing deoxythymidylate monophosphate (dTMP) and DHF (Brody, 1991). Following this reaction, DHF is reduced to THF by DHF reductase. The formation of thymidylate is essential because it produces an important precursor of DNA and it is the rate-limiting step in cellular DNA synthesis (Herbert and Das, 1976). In the absence of dTMP, uracil will be incorporated into DNA instead of thymidylate leading to ineffective DNA synthesis. Ineffective DNA synthesis can lead to DNA strand breakage and impaired cell division, which has been associated with numerous clinical disorders (FNB, 1998; Bailey and Gregory, 1999a).

*Purine nucleotide biosynthesis.* Another major function of folate metabolism is to provide the carbon groups of the 2 and 8 positions of the purine ring (Rowe, 1984; Brody, 1991). De novo synthesis of purines begins with a series of 11 reactions of which folate plays a role in the fourth and tenth. In the fourth reaction, 10-formylTHF donates its formyl group to glycinamide ribonucleotide catalyzed by glycinamide ribonucleotide (GAR) transformylase to produce formyl-GAR and THF. In the eleventh reaction, aminoimidazolecarboxamide ribonucleotide (AICAR) transformylase catalyzes the formylation of AICAR by 10-formylTHF to formyl-AICAR and THF. The formyl-AICAR will proceed through further reactions until inosinemonophosphate (IMP) is synthesized. The *de novo* synthesis of IMP is very important because it serves as a precursor to adenosine monophosphate (AMP) and guanosine monophosphate (GMP) (Rowe, 1984; Brody, 1991).

*Histidine synthesis and catabolism.* The folate derivative 10-formylTHF also plays a role in histidine synthesis since a precursor of its synthesis is formylated-AICAR (Shane and Stokstad, 1984). Of greater importance is the involvement with folate in histidine catabolism. Histidine is

catabolyzed to glutamate in a series of reactions. During those reactions, formiminoglutamic acid (FIGLU) is produced as an intermediate degradation product. The degradation of FIGLU to glutamate can occur by means of a number of reactions including one involving THF. In the liver, the enzyme formimino glutamic acid transferase catalyzes transfer of the formimino group from FIGLU to THF, which produces 5-formiminoTHF and glutamic acid. The 5-formiminoTHF can enter the formylTHF pool and the glutamic acid is excreted in the urine (Shane and Stokstad, 1984). When concentrations of THF are low as in folate deficiency, excess FIGLU will appear in urine. These findings led to the development of the histidine loading test, which measures concentration of urine FIGLU in individuals compared to controls as an assessment of folate status.

#### *Assessment of Folate Status*

Assessment of folate status may involve a combination of dietary evaluation, clinical signs and laboratory analysis. Dietary evaluation is important to assess whether individuals are consuming enough folate in their diet to meet daily requirements. In humans, dietary evaluation is a useful tool that sometimes can underestimate folate intake due to lower estimates of total food intake by the individual. In grazing animals, assessment of folate intake is nearly impossible under field conditions and marred by the fact that no estimation of microbial synthesis can be determined.

A number of direct and indirect clinical tests have been developed for the assessment of folate status. Direct measures of folate levels include folate concentration in plasma/serum, red blood cells, and tissues, whereas indirect functional indicators of folate status include plasma homocysteine, the histidine loading test, and the dU suppression test. Laboratory tests and methods used to assess folate status have been reviewed (Gibson, 1990; Eitenmiller and Landen, 1998) and will only be summarized below.

*Plasma/serum folate.* Analysis of serum or plasma folate has been one of the most extensively used to measure folate status. In most species, 5-mTHF is the main folate derivative released into circulation from the liver and tissues, while swine have THF as the main circulating form of folate (Natsuhori, et al., 1991). Normal ranges of folate vary with species, but are

usually between 6 and 20 ng/ml (Anonymous, 1992; Herbert and Das, 1994). Plasma folate levels less than 3 ng/ml may indicate an ensuing folate deficiency, whereas folate concentrations between 3 to 6 ng/ml denote marginal folate status, and levels above 6 ng/ml are indicative of adequate folate status (Herbert and Das, 1994).

There are numerous reasons why the sole use of plasma folate as an indicator of folate status is strongly discouraged. Plasma folate fluctuates with recent dietary intake, alcohol consumption, anticonvulsant medication, and with temporary changes in folate metabolism even when body stores are stabilized (Gibson, 1990). For instance, studies assessing the bioavailability of food folate utilize fluctuating plasma folate concentrations after consumption of food (Gregory, 2001). Also, individuals with megaloblastic anemia due to folate deficiency may not have drastically lowered plasma folate levels or individuals with very low plasma folate have adequate folate stores in the body (Lindenbaum and Allen, 1995). Lastly, the physical act of blood collection can often lead to hemolysis of red blood cells which may produce misleadingly elevated serum folate values as the folate content of erythrocytes is much higher than plasma.

*Red blood cell folate.* The analysis of red blood cell (RBC) folate, unlike plasma folate, is a more widely accepted test for folate status because RBC folate concentrations are less sensitive to short-term fluctuations in folate status than serum folate levels, and decrease only after several mo of folate deprivation (Herbert and Das, 1994). The determination of RBC folate is related to body stores of folate at the time of RBC synthesis. The primary folate derivative in RBC's is 5-mTHF with a concentration approximately 30 times that of plasma folate. Clinical signs of megaloblastic anemia in humans was associated with RBC folate levels less than 140 ng/ml, and thus this concentration has been used as an indicator of ensuing folate deficiency (Herbert and Das, 1994). Red blood cell folate concentrations between 140 to 160 ng/ml suggest marginal folate status and levels above 160 ng/ml indicate adequate folate status (Herbert and Das, 1994).

The major difference between RBC folate and plasma folate is that folate in the RBC are polyglutamated and require deconjugation with folate conjugase prior to analysis by microbial assay. Several methods have been established for deconjugating polyglutamates prior to folate

analysis. One of the simplest is by lysing RBC's and allowing the natural folate conjugase present in plasma to hydrolyze the polyglutamate derivatives. Other methods employ the use of extracted folate conjugase from rat and human plasma, hog kidney or chicken pancreas. A review of extraction procedures for erythrocytes and other tissues has been published (Eitenmiller and Landen, 1998).

Red blood cell folate is a more useful indicator of folate status than plasma folate because it reflects body folate stores and is not influenced by dietary intake. However, the use of RBC folate as an indicator of long-term folate status is not without its shortcomings. Like plasma folate, RBC folate has been adequate in times when anemia was present or low when individuals did not have anemia (Hoffbrand et al., 1966; Lindenbaum and Allen, 1995). Also, concentrations of RBC folate are associated with a larger variation within run and between laboratories (Gunter et al., 1996). The development and validation of reference methods for serum and RBC analysis are needed.

*Milk folate.* Milk folate is not often considered an indicator of folate status; however it is useful in the dietary evaluation of nursing humans and other animals. In addition, concentration of folate in milk and the amount produced is essential for evaluating maternal folate needs (Picciano, 1995). Estimates of the concentration of 5-mTHF compared to other forms of folate have been between and 21 and 60.5 % (Selhub, 1989; O'Conner et al., 1991). Evidence for polyglutamated folate in milk exists due to notable increase in folate concentration after incubation of the milk with folate conjugase (O'Conner et al., 1991). Estimates for the percentage of folate polyglutamates in milk range from 42 to 60 % (Smith et al., 1983; Eitenmiller et al., 1984; Tamura et al., 1980). However, other investigators observed no increase in folate concentrations after incubation with folate conjugase (Cooperman et al., 1982). In addition to difference in estimates of the amount of polyglutamated forms, there is a large range of reported milk folate concentrations. A review of recent literature revealed an average of 22 ng/ml to 224 ng/ml of folate is concentrated in human milk. Milk concentrations vary between laboratories and between methods used, which have been substantially improved over the last decade. Laboratories that utilized the trienzyme extraction method developed for food folate (Tamura et al., 1997) have observed the highest concentration of milk folate (Lim et al., 1998;

Mackey and Picciano, 1999). Recently, several studies similarly estimated the average milk folate content in human milk to be 85  $\mu\text{L}$  (O'Conner et al., 1991; Lim et al., 1998). Further investigations are needed to develop methodologies that can be used as reference methods by all laboratories assessing milk folate.

*Plasma homocysteine.* Plasma total homocysteine has become a useful functional indicator of folate status during the last decade. Homocysteine (2-amino-4-mercaptobutanoic acid) is a nutritionally non-essential sulfur containing amino acid that serves as an intermediate in methionine metabolism or it can be converted to cystathionine by the trans-sulfuration pathway. When the availability of 5-mTHF is limiting due to impaired folate status, methylation of homocysteine to methionine does not occur. This results in the accumulation of intracellular homocysteine within the cell, which may leak into the plasma causing an increased plasma homocysteine concentration. Many studies have been published that show a negative correlation between plasma homocysteine and folate levels in serum and RBC's (see FNB, 1998 for references). Plasma homocysteine levels are maintained between 5-15  $\mu\text{mol/l}$  in normal individuals, but increase to 15 to 30  $\mu\text{mol/l}$  in cases of moderate hyperhomocysteinemia, 30 to 100  $\mu\text{mol/l}$  in cases of intermediate hyperhomocysteinemia, and  $> 100 \mu\text{mol/l}$  in those individuals with severe hyperhomocysteinemia (Selhub, 1999). Values of homocysteine in children appear to be half that of adults with a range of 4.9 to 7.4  $\mu\text{mol/l}$  (Selhub, 1999). Care must be taken when attributing hyperhomocysteinemia to folate, because it can also be influenced by vitamin B<sub>12</sub> status, vitamin B<sub>6</sub> status, gender, race, and some genetic abnormalities (FNB, 1998). Therefore, plasma total homocysteine alone should not be used to determine folate status of individuals.

*Histidine loading test.* The histidine loading test estimates the amount of FIGLU excreted in the urine after an oral loading dose of 2 to 5 g of histidine (Gibson, 1990). Normally, histidine is catabolyzed by a series of reaction with FIGLU as an intermediate. The FIGLU is converted to glutamic acid via the action of the enzyme formimino transferase and THF. In folate deficiency, THF is limiting to that reaction resulting in an increased FIGLU concentration, which subsequently is excreted in the urine. Therefore, an oral histidine load administered to individuals with folate deficiency will have FIGLU concentrations above 50  $\mu\text{g/ml}$  during an 8 h

collection period (Gibson, 1990). Although this test is sensitive to lowered folate status, it can also be influenced by disorders not associated by folate deficiency and is thus not routinely used for diagnostic purposes.

*Deoxyuridine suppression test.* The deoxyuridine (dU) suppression test is a test used on bone marrow cells to measure the methylation of dU monophosphate (dUMP) to thymidine monophosphate (dTMP), which is a folate-mediated step (Chanarin, 1986; Brody, 1991). Bone marrow cells are incubated with dU and tritium labeled thymidine to allow for competition between labeled and unlabeled dTMP incorporation into DNA. The extent of the competition is a measure of thymidylate synthetase activity, which depends on the availability of folate in the cells. After an incubation period, DNA is extracted, counted for its tritium content, and compared to control counts. In normal cells, the dU will be phosphorylated, converted to thymidine monophosphate and incorporated in DNA, whereby only about 10 % of the tritiated thymidine will be incorporated. In individuals with macrocytic anemia, folate depleted bone marrow cells are not able to utilize the dU and instead phosphorylate the tritiated thymidine and incorporate into DNA accounting for more than 10 %. This test can also be useful in distinguishing between folate and cobalamin deficiency. In the case with cobalamin deficiency, the addition of methylcobalamin or any folate other than 5-mTHF will increase suppression of labeled thymidine incorporation. Despite its usefulness in deciphering between a folate and cobalamin deficiency, this test is not commonly used due to the difficulty obtaining routine bone marrow samples from individuals.

*Hematological indexes.* Impaired cellular division as a result of a long-term deficiency in folate will be reflected in many hematological indexes in cells in the blood and other rapidly dividing tissues. Hematological indexes in severe folate deficiencies are easily analyzed with the help of automated machines in a clinical setting. One of the first signs of folate deficiency is macrocytosis, or an increase in the mean cell volume (MCV) of RBC's (Herbert and Das, 1994). The increase in MCV is also associated with an increased mean cell hemoglobin concentration (MCHC). Associated with an increased MCV and MCHC is a decline in hematocrit (HCT). The appearance of hypersegmented neutrophils, macrocytosis, and other abnormal hematological findings occurs late in the development of deficiency. It should be noted that macrocytic anemia

determined by hematological indexes can also be a result of cobalamin deficiency and differential diagnoses of the folate and cobalamin deficiency should be determined.

### *Methods and methodological issues*

The type of extraction procedures and methods selected to analyze folate in biological materials is a topic of much scientific research and discussion. Because 5-mTHF monoglutamate is the predominant folate derivative in plasma, plasma folate is by far one of the most simplest indicator to measure because it requires no extraction procedure. On the other hand, folate in body tissues (e.g. erythrocytes) and food exist as folate polyglutamates. Therefore, analysis of cellular and food folate require use of extraction procedures that deconjugate glutamate residues, release folate from binding proteins, and extract unwanted components from the sample. The most commonly used extraction procedure now employed is trienzyme treatment, which uses folate conjugase, protease, and  $\alpha$ -amylase (Tamura, 1997). A review of various extraction procedures has been published (Eitenmiller and Landen, 1998).

After extracting folate from biological samples, folate concentrations can be analyzed using methods including the microbial assay, radioimmunoassay (RIA), and HPLC. The microbial assay utilizes the microorganism, *Lactobacillus casei*, because its growth is dependant upon common monoglutamate and shorter polyglutamate forms of folate including 5-mTHF (Tamura, 1990; AOAC International, 1995). Other bacteria such as *S. faecalis* or *P. cerevisiae* respond to folate except 5-mTHF, and are therefore not used for plasma folate determination. However, the use of those bacteria are useful in differentiating between amounts of different folate derivatives in biological tissues. Growth of the organism in response to added known amounts of FA are used to compare growth of the organism to unknown plasma samples to determine folate concentration. The microbial assay method has been used to detect folate concentration in plasma, erythrocytes, body tissue, and food (Tamura, 1990).

The relative difficulty of maintaining the microbial assay organisms in a clinical setting and its interference with antibiotics in samples, led to the development of a RIA for folate. The assay involves the competitive binding of swine binding proteins to either  $I^{125}$  labeled FA or the folate in the sample. The use of the RIA offers advantages over the microbial assays because they are

commercially available, simple to use, require less time, and are less affected by antibiotics in blood samples. On the other hand, several conflicting reports exist that produce evidence for and against the use of RIA's compared to the microbial assay (Baril and Carmel, 1978; McGown et al., 1978; Molloy et al., 1998). In general, the performance of many commercial RIA's appears to be adequate for use on plasma folate and generally comparable to *L. casei* methods although there remains considerable variability in performance between commercial methods and between laboratories (Lindenbaum and Allen, 1995). However, the use of RIA's to assess folate in erythrocytes has received attention regarding its precision, accuracy, and general reliability of the assays (Wright et al., 1998).

High-pressure liquid chromatograph methods have been developed to determine folate levels in plasma (Shimoda, 1992), serum (Kohashi and Inoue, 1986), milk (Wigertz and Jagerstad, 1995), foods (Vahteristo et al., 1996; Konings, 1999; Ruggeri et al., 1999), citrus juice (White et al., 1991), bile (Shimoda et al., 1994), liver and brain (Bagley and Selhub, 2000). Many studies evaluating the folate content in various biological tissues used either HPLC with electrochemical detection or HPLC with fluorescence detection. The advantage of HPLC methods is differentiation between the various folate derivatives and gamma-glutamylfolate polymers. Several detailed reviews on the various analysis of folate in biological samples have been published (Eitenmiller and Landen, 1998; Mullin and Duch, 1992).

Many studies have been conducted to compare results of folate concentration in biological samples using the microbial assay, RIA, and HPLC methods (van den Berg et al., 1994; Wigertz and Jagerstad, 1995). In general, the microbial assay remains the "gold standard" for measuring folate status over the RIA and HPLC. However, HPLC, microbiological, and RIA assays of milk folate were in similar agreement (Wigertz and Jagerstad, 1995).

### *Folate Requirements*

With the exception of folate requirements established for humans, folate requirements established by the National Research Council (NRC) at best offer only offer mean minimum requirements. Mean minimum requirements are likely to fall below optimal ranges for the population and result in suboptimal folate nutrition in numerous individuals. In humans, the

Recommended Dietary Allowance (RDA) and Estimated Average Requirements (EAR) have been discussed and standardized based on Dietary Folate Equivalents (DFE) (FNB, 1998). Dietary folate equivalents are adjusted estimates based on the bioavailability of synthetic FA compared to natural folate in food.

Dietary folate requirements of selected species are summarized in Table 1. Dietary folate requirements differ within and between species, and most animal NRC requirements are based on a per kg feed basis, whereas those of humans are based on  $\mu\text{g}$  of a RDA. Many livestock species have either low or zero requirements for dietary folate due to the large contribution of folate from microbial synthesis in the gut. The exception to this is livestock animals that are young and have an immature gut with little microbial contribution of folate. Monogastric animals rely more heavily on the contribution of folate from dietary sources with a minor folate contribution from intestinal microbial synthesis. However, the horse is monogastric animal with a microbial population in the hindgut that is capable of fermentation of B-vitamins including folate (Carroll et al., 1949).

#### *Factors that influence folate requirements with specific reference to the horse*

Requirements for folate are influenced by numerous factors that can be of dietary and physiological origin. Some of those factors may include bioavailability of feed and forage folate, folate-drug interactions, microbial synthesis, and periods of increased physiological demand including pregnancy, lactation, growth, and exercise.

*Bioavailability.* In humans, the bioavailability of food folate and FA fortified foods has been studied extensively (Gregory, 2001). These studies led to the use of DFE (Table 1). The bioavailability of FA in fortified foods is highly available in human diets (Cuskelly et al., 1996; Pfeiffer et al., 1997), but folate inherent in foods was no more than 50 % of FA (Sauberlich et al., 1987). This is important in determining availability of folate in foods because many foods in the United States are now are fortified with FA (FNB, 1998). Folic acid fortification in foods appears to be an effective means of increasing the dietary intake of folate and improving folate status (FNB, 1998).

**Table 1.** Dietary folate requirement of selected species

<b>Animal</b>	<b>Class</b>	<b>Requirement<sup>a, b</sup></b>	<b>Source</b>	
Beef Cattle	Adult	Microbial synthesis	NRC, 1996	
	Calf	0.1 ppm milk replacer	NRC, 1996	
Cats	Adult	0.8 mg/kg	NRC, 1986	
Chicken	Leghorn, 0-6 wk	0.55 mg/kg	NRC, 1994	
	Leghorn, 6-18 wk	0.25 mg/kg	NRC, 1994	
	Laying	0.25 mg/kg	NRC, 1994	
	Broilers, 0-6 wk	0.5 mg/kg	NRC, 1994	
	Broilers, 6-8 wk	0.50 mg/kg	NRC, 1994	
Dairy cattle	Adult	Microbial synthesis	NRC, 1989	
Dog	Growing	0.2 mg/kg	NRC, 1985a	
Fox	Growing	0.2 mg/kg	NRC, 1982	
Goat	Adult	Microbial synthesis	NRC, 1981	
Horse	Adult	Microbial synthesis	NRC, 1989	
	Human	Infants, 0-12 mo	65-80 µg/d DFE <sup>c</sup>	FNB, 1998
		Children, 1-8 yr	120-160 µg/d DFE	FNB, 1998
		Teens, 9-18 yr	300-400 µg/d DFE	FNB, 1998
		Adults, 19+	400 µg/d DFE	FNB, 1998
		Pregnancy	600 µg/d DFE	FNB, 1998
Lactation		500 µg/d DFE	FNB, 1998	
Rat	All classes	1.0 mg/kg	NRC, 1995	
Swine	Growing/finishing	0.3 mg/kg	NRC, 1998	
	Gestating/lactating	1.30 mg/kg	NRC, 1998	
Sheep	Adult	Microbial synthesis	NRC, 1985b	
Turkey	Growing, 0-8 wk	1.0 mg/kg	NRC, 1994	
	Growing, 8-16 wk	0.8 mg/kg	NRC, 1994	

<sup>a</sup> Expressed as per unit of animal feed on either as-fed or dry matter basis

<sup>b</sup> Based on RDA or µg /d of dietary folate equivalents. Expressed as µg/kg of BW

<sup>c</sup> Dietary Folate Equivalent (DFE) adjusts for nearly 50 % lower bioavailability of food folate compared with that of folic acid: 1 µg of dietary folate equivalent = 0.6 µg of folic acid from fortified food or as a supplement taken with meals = 1 µg of food folate = 0.5 µg of a supplement taken on an empty stomach (FNB, 1998)

Bioavailability of food folate is in large part governed by the type of food, and the extent of digestion and absorption in the gut. Food composition may play a role in altering bioavailability of food folate. For instance, some dietary fiber may decrease the bioavailability of folate (Bailey, 1988), whereas many others dietary fibers appear to have no effect (Gregory, 1995). Additionally, dietary fiber had a positive influence on serum folate perhaps due to stimulation of microbial folate synthesis (Houghton et al., 1997). Therefore, the composition of the diet may affect folate bioavailability and may ultimately affect folate status in the individual. Also, secondary problems associated with malabsorption syndromes or drug administration may decrease the bioavailability of food folate and increase dietary requirements.

*Malabsorption.* In humans, intestinal malabsorption of folate can be caused by disorders including tropical sprue, celiac disease, regional enteritis (i.e. Crohn's disease), and congenital malabsorption in infants (Chanarin, 1986). Tropical sprue is caused by an unknown infective agent in tropical regions and is nearly always associated with megaloblastic anemia, but can be treated by antibiotics and folate supplementation (Chanarin, 1986). Celiac disease is due a life-long sensitivity to gluten of wheat that causes intestinal damage and results in the malabsorption of several nutrients including folate. Celiac disease will cause megaloblastic anemia, which is reversible by consumption of a gluten free meal and folate supplementation (Chanarin, 1986). Crohn's disease is a chronic inflammatory disorder of the bowel of unknown causation. Despite the malabsorption of folate, the lowered folate status that occurs in the individuals is not usually associated with frank megaloblastic changes (Chanarin, 1986). Lastly, a congenital intestinal malabsorption disorder in infants caused by an autosomal recessive inheritance has been described (Chanarin, 1986). In this disorder, infants suffer a severe megaloblastic anemia soon after birth caused by a lack of absorption of folate and/or problems associated with transport of folate in the body. Daily treatment with 5-fTHF is mandatory to prevent neurological problems including mental retardation.

In the horse, two cases of malabsorption were presented with one horse experiencing inflammatory bowel disease in the ileum and large intestine and the other horse experiencing minor pathological changes throughout the small intestine (Roberts, 1983). The first horse with the inflammatory bowel disease had adequate levels of serum folate (13.7 ng/ml), but had a

reduced RBC folate concentration (196 ng/ml) by human standards (Herbert and Das, 1984). The second horse had a low serum folate of 5 ng/ml, but an adequate RBC folate of 559 ng/ml. These findings suggest that malabsorption of folate in the intestine of the horse may play a minor role in affecting folate status of the horse.

*Pregnancy.* Pregnancy is a time of increased folate requirements due to rapid growth of the fetus, the placenta, breast tissue, and other maternal tissues. Requirements for folate during pregnancy and lactation may increase 5 to 10 fold over those of non-pregnant individual (Chanarin, 1986). Demand for folate is also elevated by an increased catabolism of folate during pregnancy (McNulty et al., 1993). Folate status declines during pregnancy, which can result in megaloblastic anemia (Babior, 1990), adverse pregnancy outcomes (Scholl and Johnson, 2000), low birth weights of infants (Chanarin, 1986), and congenital defects in infants (Butterworth and Bendich, 1996). In addition to the folate deficiency associated with single pregnancies, there is a higher risk of folate deficiency in pregnant women in a low socioeconomic class, carrying twins, of higher parity, and those that smoke (Hall et al., 1976). Folate supplementation during pregnancy has reduced the risk of megaloblastic anemia and neural tube defects in infants (FNB, 1998). Maintaining folate status during pregnancy is also important to reduce the risk of further depletion during lactation.

Folate status throughout pregnancy has not been assessed in the horse. Allen (1978) observed normal serum folate concentrations of 10.6 ng/ml (range: 6.4 to 15.8 ng/ml) in 30 Thoroughbred mares at stud. These concentrations were reportedly higher than in horses in training in the same study. Serum and RBC folate appeared to decline from early to mid gestation in mares, however no statistical analyses were performed on the data (Roberts, 1983). Pregnant mares treated for Equine Protozoal Myeloencephalitis (EPM) with pyrimethamine and sulfonamides, folic acid, and vitamin E had low serum folate concentrations and produced foals with congenital problems, suggesting an impaired folate status in both the mare and foal (Toribio et al., 1998).

*Lactation.* Lactation is a physiologically demanding period that can also lower folate status and in some cases has been estimated to be more demanding than pregnancy (Shapiro et al., 1965; Metz, 1970; O'Conner et al., 1997). In lactation, supply of folate to the infant via

mother's milk takes precedence over maintaining maternal stores (Metz, 1970). Low folate status during pregnancy may also predispose further folate depletion of the mother during lactation. In addition, low folate status during lactation may reduce reproductive efficiency in subsequent pregnancies. Folate supplementation of women in lactation is important to maintain maternal folate body stores and supply folate to milk to maintain infant folate body stores. Several reviews discuss the necessity and benefits of folate supplementation in women during lactation (Picciano, 1995; O'Conner et al., 1997; FNB, 1998).

Data is limited on the effects of lactation on folate status in the horse. Serum folate levels in recently foaled grazing mares was moderately low with a mean of  $7.4 \pm 2.2$  ng/ml (Seckington et al., 1967). Another study reported a range of serum and RBC folate of mares in the first mo of lactation to be between 10.1 to 15.4 and 243 to 656 ng/ml (Roberts, 1983). These findings suggest that grazing lactational mares may have moderate to normal folate status during lactation. It is important to note that assessment of folate status cannot be made on serum folate alone (see *Methods and methodological issues*).

*Age.* The effects of age on folate requirements have been noted in young growing humans and animals and in the elderly. In the young growing animal, rapid growth of tissues increases the requirement for folate. In humans, blood folate levels are higher in the infant than in the mother (Landon and Oxley, 1971; Ek, 1980). There is, however, a decline in serum and RBC folate levels associated with the first mo of rapid growth in the infant. Early growth in the newborn is supported by folate in the mother's milk, which has been shown to stabilize or increase throughout lactation (Ek, 1980; Smith et al., 1985). Infants that are breast fed are thought to be protected from declines in folate status because the supply of folate to milk in the mother takes precedence over her own folate stores (Tamura et al., 1980; O'Conner et al., 1997).

Studies assessing the effects of aging folate status in humans have been conflicting (see FNB, 1998 for references). Based on those studies, the RDA for individuals 51 yr or older was maintained at the same level of mature adults (FNB, 1998). However, the elderly are at a higher risk for folate deficiency for several reasons. First, the elderly are often times in a lower income bracket due to retirement and may not consume adequate amounts of folate through their diet.

Elderly are often institutionalized and receiving medications that may interfere with folate metabolism (Chanarin, 1986; Brody, 1991; Bailey, 1995). Elderly individuals with chronic disorders, receiving medications, or in a low-income class may require higher folate intakes.

*Genetics.* Enzymes, like the ones involved in one-carbon metabolism are regulated by the genetic make-up of the individual. Several genetic polymorphisms have been identified in key enzymes of one-carbon metabolism in humans (Bailey and Gregory, 1999b; Botto and Yang, 2000). One of the most important enzymes with a common gene variant is the 5,10-methylenetetrahydrofolate reductase (MTHFR) enzyme. The MTHFR enzyme catalyzes the conversion of 5,10-methylenetetrahydrofolate into 5-mTHF. The C677T allele is characterized by a point mutation at position 677 of the MTHFR gene that converts a cytosine into a thymine, resulting in the amino acid substitution (alanine to valine) in the enzyme. The other variant in the MTHFR enzyme is the A1298C allele that involves a point mutation due to an amino acid substitution of glutamate for alanine resulting in the conversion of adenine to cytosine. Individuals homozygous for the C677T mutation have a decline in MTHFR enzyme activity. The activity of the MTHFR enzyme is also decreased in individuals with the A1298C, but to a lesser extent (Botto and Yang, 2000). The lowered enzyme activity reduces the availability of 5-mTHF, which can impair the flux of carbons towards homocysteine remethylation and increase it towards nucleotide synthesis. Thus, individuals with the C677T gene variant in MTHFR have an increased requirement for folate. Individuals with gene variants in the MTHFR gene experience hyperhomocysteinemia and as such are at a higher risk of associated disorders including cardiovascular disease, neural tube defects in infants, presbyopia, Alzheimer's, and dementia (Bailey and Gregory, 1999b; Selhub, 1999; Kim, 2000).

*Folate antagonists.* Many drugs are routinely used to treat disorders in humans and animals that in some way interfere with folate metabolism. Examples of drugs that falls into that interfere with folate metabolism are DHF reductase (DHFR) inhibitors, sulfonamides, anticonvulsants, and alcohol. More commonly used DHF inhibitors include methotrexate, pyrimethamine, and trimethoprim. Dihydrofolate reductase inhibitors act by inhibiting the enzyme that catalyzes the conversion of DHF to THF, as well as the conversion of FA to DHF. Further, PYR and trimethoprim have been shown to induce folate malabsorption (Zimmerman et

al., 1987). The primary mode of action of PYR and trimethoprim is to interfere with DNA synthesis in addition to a number of secondary effects on cellular metabolism, such as inhibition of thymidylate synthetase and depletion of cellular folate. The fact that DHFR inhibitors have a far greater affinity for the DHFR enzyme in prokaryotic organisms compared to the enzyme in mammals (Burchall and Hitchings, 1965; Cocco et al., 1983) led to their use as agents in clinical practice. Despite this fact, hematological changes associated with drug administration have been reported (Waxman and Herbert, 1969). Therefore, it is important to assess folate status during treatment of disorders with DHF reductase inhibitors.

Pyrimethamine has been shown to be teratogenic in rats (Izumi, 1984; Kudo et al., 1988; Chung et al., 1993), hamsters (Sullivan and Takacs, 1971), pigs (Misawa, 1982; Yamamoto et al., 1985), and humans (Harpey et al., 1983). These findings and further understanding of the roles of the drugs in the interference with folate metabolism led to the routine supplementation of FA with PYR administration to prevent impaired folate status in the host. However, numerous investigators have observed an increased incidence of teratogenicity when FA is coadministered (Kudo et al., 1988; Hayama et al., 1991; Chung et al., 1993). The observed decline in plasma 5-mTHF during coadministration of FA and PYR (Tsunematsu et al., 1990) was also observed by Kudo et al. (1995) who delineated that the decline was likely due to a combination of the inhibition of reabsorption of bile reduced folate by FA and the inhibition of FA conversion to a reduced form in the liver by PYR. On the other hand, coadministration of PYR with folic acid supplementation increased plasma 5-mTHF and decreased fetal teratogenesis in rats compared to FA and PYR (Tsunematsu et al., 1990; Chung et al., 1993). In pigs, plasma THF and 5-mTHF decreased following orally administered single doses of oxidized, synthetic FA but not reduced forms -- folic acid, peptidoglycan or liver powder (Mizuno et al., 1997). Oral supplementation with peptidoglycan, an Ajinomoto product, has also been shown to increase litter size in sows and decrease incidence of infectious disease in their offspring. Therefore, folic acid may be a better supplement to ensure adequate folate status in individuals treated with PYR.

Sulfonamides include a wide variety of drugs that are commonly used to treat many disorders in humans and animals. Sulfonamides do not directly inhibit folate synthesis; rather they depress the biosynthesis of FA by microorganisms. Sulfonamides are stereochemically similar to para-

aminobenzoic acid (PABA), a major structural component of folate. The mechanism by which these drugs depress microbial folate synthesis is through competitive inhibition of PABA incorporation into folate (Wise, 1999). This may be of importance to animals that depend partially on microbial synthesis and coprophagy to meet folate requirements.

Specifically in horses, the combination of PYR and SDZ have been shown to be effective against *Sarcocystis neuuona* in vitro (Lindsay and Dubey, 1999) and are routinely used to treat the neurological syndrome EPM in the horse. The association between the mechanisms of action of the drugs led to suggestions that FA (approx. 40 mg/d) be supplemented along with PYR and sulfa drugs. However, pregnant mares treated for EPM with pyrimethamine, sulfonamides, FA, and vitamin E gave birth to foals with congenital defects (e.g. bone marrow aplasia and hypoplasia, renal nephrosis or hypoplasia, and skin lesions) that ultimately died (Toribio et al., 1998). Mares in that study had serum folate between 4.8 and 5.5 ng/ml whereas foals were between 0.4 and 5.2 ng/ml. Foals showed symptoms of lethargy and weakness, alopecia, skin lesions, and oral ulcers. Foals had low PCV, low WBC and platelet count, and high serum creatinine. Histopathological diagnoses were renal tubular nephrosis, lymphoid hypoplasia, and bone marrow aplasia, epidermal necrosis and renal hypoplasia and severe myeloid and mild erythroid hypoplasia, and myeloid atrophy and hypoplasia and renal hypoplasia.

Chronic treatment with anticonvulsants such as phenytoin, primidone, and pentobarbital has been shown to cause folate depletion via interaction with folate metabolism in humans (Mattson and Cramer, 1982; Pisciotto, 1982) and animals (Carl and Smith, 1984; Carl et al., 1987). The three potential mechanisms whereby anticonvulsants affect folate metabolism include interference with absorption of folate, changes in folate enzyme function, and impaired DNA synthesis (Chanarin, 1986). It has also been suggested that anticonvulsants may interfere with polyglutamate synthesis in tissues thereby decreasing the retention and increasing the loss of folate from those cells (Carl et al., 1987). In patients taking high doses of anticonvulsant medication, megaloblastic anemia may occur in addition to psychiatric manifestations including confusion, dementia, memory impairment and depression that can be reversed with 3 to 6 mo of folate supplementation (Chanarin, 1986).

*Alcohol.* Another factor that can contribute to a folate deficiency is chronic alcohol consumption. The direct relationship between alcohol consumption and folate status remains in question. One of the main theories describing the relationship is that alcohol directly affects enzymes involved in folate metabolism resulting in an impaired absorption of folate, impaired enterohepatic cycle, and increased urinary excretion (Chanarin, 1986). Another viewpoint considers the fact that individuals that are chronic alcohol consumers also generally have a poor diet. Therefore, the latter viewpoint associates the lowered folate status with a poor dietary intake of folate. In either case, megaloblastic anemia is reversible by withdrawal of alcohol and folate supplementation.

*Exercise.* Studies in the athletic horse associate intense strenuous exercise with lowered serum folate or RBC folate (Seckington et al., 1967; Allen, 1978; Allen and Powell, 1983; Roberts, 1983). One case report detailed the poor performance of a mature stabled gelding associated with a low serum folate (5.0 ng/ml), low hemoglobin (11.5 %), alterations in nuclear structure of bone marrow, and poor body condition that responded to folate supplementation (Seckington et al., 1967).

Several researchers observed low serum and RBC folate concentrations in young horses in race training (Allen, 1978; Allen and Powell, 1983; Roberts, 1983). Serum and RBC folate concentrations significantly declined following 20 wk of race training (Allen and Powell, 1983). Roberts (1983) observed low serum and RBC folate concentrations in non-supplemented working police horses, and in FA supplemented Thoroughbreds and Standardbreds in race training. Thoroughbred horses in training had lower serum folate concentrations than mares in various stages in reproduction and ponies kept on grass (Allen, 1978). The demand for increased erythropoiesis during prolonged exercise training in addition to high cell turnover in other tissues may be partly responsible for lowered blood folate concentrations in these horses. Also, limited access to pasture typically experienced by stabled racehorses may play a large factor in their increased risk for low folate status due to the lower folate content in hays.

### *Folate supplementation in the horse*

Horses are foraging hindgut fermenters capable of utilizing hindgut microbial synthesis of folate (Carroll et al., 1949). There are currently no dietary recommendations for folate in the horse (NRC, 1989b). Early findings of low serum folate levels in exercising horses (Seckington, 1967; Allen, 1978) led to the recommendation for routine supplementation of horses with either oral or i.m. synthetic FA. However, a preliminary study found the absorption of oral FA to be low in the horse (NRC, 1989b). The first report of folate supplementation was a case report that detailed poor performance of a mature stabled race-trained gelding, which had improvements in hemoglobin (14.6 %) and serum folate (12.0 ng/ml) associated with an increase in performance and body condition after 23 d of daily FA supplementation of 20 mg/d (method of administration not reported) (Seckington et al., 1967). Another study reported low serum and RBC concentrations in some Thoroughbreds and Standardbreds in race training that received both daily oral FA supplementation and either bi-monthly or weekly i.m. injections of FA (Roberts, 1983). In the same study, horses engaged in police work that had not been receiving folate supplementation had significantly lower serum and RBC folate compared to the supplemented horses in training.

The findings of low serum and RBC folate in strenuously exercising horses and that folate status may improve with folate supplementation raises some questions as to the efficacy of folate supplementation in the horse. Folic acid has been typically supplemented in horse feeds without knowledge of its absorption or kinetics in the horse. Roberts (1983) measured serum and RBC after i.m. injections of either 150 mg of FA or two 75 mg doses separated by 4 d. After the 150 mg injection, both serum and RBC folate increased significantly above baseline and returned to baseline within 24 h. Serum and RBC folate increased after the first 75 mg injection, but only RBC folate responded 4 d later after the second small injection. These findings indicate that FA is cleared slowly from the circulation. The finding that RBC folate increased after injection is novel because RBC folate is reportedly not influenced by recent dietary intake (Bailey, 1990) or in this case, i.m. folate injection.

More recently, folate deficiency was shown to occur in foals born to mares treated for EPM with PYR, sulfonamides, FA and vitamin E (Toribio et al., 1998). Upon admission, foals

showed signs of lethargy and weakness, alopecia, skin lesions, and oral ulcers. Clinical diagnosis revealed low hematocrit, low white blood cell and platelet count, and high serum creatinine associated with numerous congenital defects including bone marrow aplasia and hypoplasia, renal nephrosis or hypoplasia, and skin lesions. It was concluded that the treatment of FA potentiated the effects of PYR and sulfonamides, which resulted in a lowered folate status in the mare, and was further evident in the fetus. Clearly, the efficacy of FA supplementation needs to be evaluated further in the horse.

### *Causes of Folate Deficiency*

Folate deficiency in humans and other animals occurs over a length of time when the individual is not meeting their requirements for folate. Folate deficiency in humans primarily occurs due to inadequate dietary intake, impaired absorption or utilization, and increased folate requirements. Folate deficiency caused by inadequate dietary intake is more common in adults and infants of lower socioeconomic status and less affluent societies (Chanarin, 1986). Deficiencies of folate can also occur in individuals with impaired absorption and utilization due to the use of sulfonamides, malabsorption syndromes, and genetic defects. Lastly, folate deficiency may occur when conditions such as pregnancy, lactation, and growth cause an increased need for folate (Babior, 1990).

### *Indicators of Folate Deficiency*

*Humans.* Although there is considerable variability between individuals with folate deficiency, there is a general sequence of hematological events that occurs (Herbert and Das, 1994; Lindenbaum and Allen, 1995). One of the first clinical signs that occurs during folate deficiency is a lowered plasma folate concentration. The term “negative folate balance” has been used to describe the period associated with plasma folate below 6 ng/ml and a normal RBC folate concentration (> 200 ng/ml). As folate stores in the body are depleted, the RBC folate will typically fall below 160 ng/ml and plasma folate may also decline below 3 ng/ml (Herbert and Das, 1994). In addition, plasma homocysteine may increase above 15  $\mu\text{mol/L}$  (FNB, 1998). An increase in plasma homocysteine is known as hyperhomocysteinemia, which provides more of a function test of folate deficiency (FNB, 1998). When folate stores within the RBC become limited, stores within the body including the bone marrow also become limited. During this

period, changes in the peripheral blood become evident and the individual will have an abnormal dU suppression test. Changes in the peripheral blood include an increased neutrophil hypersegmentation (define as more than 5 % five-lobed or any six-lobed cells/100 granulocytes) and an increase in MCV and MCHC above the individual's normal baseline level (Lindenbaum and Allen, 1995). Macrocytosis occurs because the RBC that has grown larger and stored more hemoglobin in anticipation of cell division cannot divide due to the low availability of folate. Macrocytosis of the RBC impairs its function and megaloblastic anemia ensues associated with a decline in RBC counts, HCT, and Hb with anisocytosis and poikilocytosis occurring at varying degrees (Lindenbaum and Allen, 1995). In severe folate deficiency when HCT falls below 30 %, increases in serum lactate dehydrogenase and bilirubin, and a decline in white blood cell and platelet count may occur (Lindenbaum and Allen, 1995).

During the later stages of folate deficiency, symptoms of megaloblastic anemia occur. In humans, marginal folate deficiency produces general symptoms including weakness, lethargy, irritability, and decreased appetite (FNB, 1998). Symptoms associated with severe deficiency include abdominal pain, diarrhea, tachycardia, ulcers in the mouth and pharynx, skin changes, and hair loss (Lindenbaum and Allen, 1995; FNB, 1998). In humans with underlying coronary artery disease, angina pectoris may occur, leading to potentially fatal congestive heart failure and pulmonary edema (Lindenbaum and Allen, 1995). Also, neurological dysfunction may occur as a result of severe folate deficiency due to an impaired RNA synthesis in the brain including ataxia, depression, disorientation and confusion (Chanarin, 1986).

*Animals.* Symptoms of folate deficiency in various animals are presented in Table 2. Compared to humans, folate deficiency is less likely to occur in livestock animals due to the greater contribution of folate from microbial synthesis in the foregut of ruminants and hindgut of non-ruminants. However, folate deficiency has been experimentally induced in laboratory animals such as mice and rats (Bills et al., 1992; Walzem and Clifford, 1988). To experimentally produce folate deficiency in animals, folate-free diets have been fed in combination with sulfonamides and folate antagonists (McDowell, 2000). In animals, folate deficiency has been associated with megaloblastic anemia, leukocytopenia, poor growth, increased susceptibility to disease, dermatological lesions, and alopecia (Combs, 1992a; McDowell, 2000).

**Table 2. Symptoms of folate deficiency in selected species**

<b>Animal</b>	<b>Deficiency Syndrome</b>	<b>Source</b>
Cat	Anemia (macrocytic tendencies), leukopenia, weight loss	b
Chicken	Macrocytic anemia, reduced food intake, growth, erythrocyte and leukocyte counts, hemoglobin, bone and feather development, egg production and hatchability, increased lethargy, number of immature red blood cells, excretion of FIGLU, perosis, decolorization of the comb and mucous membranes, cervical paralysis, and mortality	a, b
Dog	Hypochromic anemia, erratic appetite, weight loss, glossitis, leukopenia, decreased antibody response, bone marrow hypoplasia	a, b
Fish	Macrocytic, normoblastic, and megaloblastic anemia, anorexia, reduced growth, and feed conversion, increased sensitivity to bacterial infection, pale gills, dark coloration	a, b
Guinea Pig	Anemia, reduced growth rate, reproductive capacity, increased mortality and rate of fetal resorption and abortion, anorexia fatty liver syndrome	a
Fox	Anorexia, loss of body weight, decreased hemoglobin, erythrocytes and white blood cells	b
Horse	Reduced serum folate, depressed hemoglobin, poor exercise performance; lethargy, recumbancy, skin lesions, alopecia, oral ulcers, lymphoid, myeloid and renal hypoplasia	a, b, c
Non-human primates	Megaloblastic, macrocytic anemia, leukopenia, weight loss, anorexia, listlessness, diarrhea, oral ulceration, increased susceptibility to infection	a, b
Mouse	Anemia, poor growth, reproductive and lactation performance, leukopenia, increased FIGLU excretion	a, b
Rat	Anemia, leukopenia, reduced serum and tissue folate, antibody response, reproductive performance, embryonic development and survival, protein metabolism, and increased FIGLU excretion	a, b
Swine	Normocytic and macrocytic anemia, leukopenia, poor growth, alopecia, mild thrombocytopenia, listlessness, erythroid hyperplasia of bone marrow, diarrhea	a, b
Sheep	Leukopenia, diarrhea, and increased mortality in young lambs	a
Turkey	Mild anemia, reduced growth, feather development, egg hatchability and embryonic development, increased mortality and cervical paralysis	a

<sup>a</sup> Harper, 1992; <sup>b</sup> McDowell, 2000; <sup>c</sup> Toribio et al., 1998

Carroll et al. (1949) fed horses a diet low in B-vitamins including folate (< 0.01 mg/kg) for up to 19 wk. After 4 mo, deficiency signs including anorexia, nervousness, incoordination in the hindquarters, loss of weight and general weakness were observed, which were associated with a thiamine deficiency. Assessment of fecal folate concentrations revealed a higher concentration than that of the diet indicating microbial synthesis of folate. Because folate status was directly assessed during this study, it is unknown to what extent the low dietary intake of folate contributed to the signs of vitamin deficiency. In one case report, a 7yr old stabled gelding consuming a diet lacking fresh grass was reported to have a low hemoglobin (11.5 %), low serum folate (5.0 ng/ml), poor body condition, and was suffering poor racing performance (Seckington et al., 1967). After 23 d of 20 mg/d FA supplementation (method of administration not reported), the horse had an improved hemoglobin and serum folate concentration, improved nuclear structure of bone marrow sample, and rise in performance and condition. More recently, folate deficiency was shown to occur in foals born to mares being treated for EPM with PYR, sulfonamides, FA and vitamin E (Toribio et al., 1998). Upon admission, foals showed signs lethargy and weakness, alopecia, skin lesions, and oral ulcers. Clinical diagnosis revealed low hematocrit, low white blood cell and platelet count, and high serum creatinine associated with numerous congenital defects including bone marrow aplasia and hypoplasia, renal nephrosis or hypoplasia, and skin lesions. Therefore, treatment of FA potentiated the effects of PYR and sulfonamides lowering folate status in the mare and foal. These findings suggest that folate deficiency in the horse is not common, but may occur in stabled horses during intense exercise or in pregnant mares supplemented with antifolate drugs and FA.

### *Toxicity*

There is generally not a high risk of toxicity associated with high oral supplemental intakes of folate because it is a water-soluble vitamin that can be excreted and not stored. Parental administration of pharmacological amounts nearly 1000 times the dietary requirement was associated with epileptic responses and renal hypertrophy in rats (Combs, 1992a), and individual cases of hypersensitivity to oral and parental FA have been reported in humans (FNB, 1998). Rats given high oral daily doses of 40 mg/kg of FA corresponding to 20 times their requirement for 3 wk showed no adverse affects (Achon et al., 2000). However, high folate intakes have been associated with limiting the absorption of zinc (Combs, 1992a; Tamura, 1995) and it can

also mask the effects of B<sub>12</sub> deficiency (Herbert and Das, 1994). In addition, FA supplementation along with folate antagonists may potentiate the effects of the drugs (Kudo et al., 1988; Hayama et al., 1991; Chung et al., 1993). Currently, the Tolerable Upper Intake Level (UL) for humans is set at 1,000 ug/d of folate from fortified food or as a supplemented exclusive of food folate (FNB, 1998). There has been no investigation into the tolerable intake limit of folate in the horse.

## CHAPTER III

### Manuscript 1. Folate status during lactation and growth in the Thoroughbred

**ABSTRACT:** Twenty Thoroughbred mares ( $609.9 \pm 16.2$  kg BW,  $12.9 \pm 1.1$  yr) and foals maintained on mixed grass/legume pasture were studied from foaling to 6 mo post-foaling to assess effects of lactation and growth on folate status during lactation and growth. Prior to foaling, mares were paired by age and foaling date and randomly assigned to either a corn and molasses based pasture supplement rich in starch and sugar (SS) or a fiber (beet pulp, soy hulls, oat straw) and fat (cereal by-product) based supplement (FF) that had the synthetic folic acid removed from the vitamin premix. Folate status was assessed by plasma folate, red blood cell folate, plasma homocysteine, and milk folate in monthly samples. During the experimental period, folate levels in pasture followed a similar pattern to that of growth of cool season grasses with peak values occurring in April with lowest levels occurring in June. All mares and foals in the present study had plasma folate and RBC folate concentrations well above those considered normal. Mare plasma folate declined moderately over 6 mo of lactation ( $P < 0.05$ ), whereas RBC concentrations initially increased after foaling up to 3 mo ( $P < 0.05$ ), but declined after that to concentrations similar to foaling. Plasma homocysteine was higher for SS mares compared to FF mares over the 6 mo period ( $6.9 \pm 0.4$  vs  $4.5 \pm 0.2$   $\mu\text{mol/L}$ ,  $P < 0.05$ ), which indicated a possible relationship between microbially derived B-vitamins and the regulation of homocysteine metabolism. Folate concentration in milk rapidly declined from foaling to 3 mo ( $P < 0.05$ ) followed by an increase up to 6 mo ( $P < 0.05$ ). Foal plasma folate initially declined from foaling ( $13.8 \pm 1.9$  ng/ml) to 1 mo ( $9.5 \pm 0.8$  ng/ml), but rapidly increased to  $22.3 \pm 1.6$  ng/ml at 6 mo ( $P < 0.05$ ). Stable concentrations of RBC folate was maintained in foals throughout the study. Plasma homocysteine in foals was unaffected by growth over the last 5 mo despite the high concentrations at foaling ( $13.4 \pm 1.9$  ng/ml;  $P < 0.05$ ). Therefore, the intake of dietary folate from feed and forage offered in this study was sufficient to maintain folate status in mares and foals during 6 mo of lactation and growth, respectively.

Key Words: Folate, homocysteine, mare, foal, lactation, milk

## Introduction

Lactation and growth are two nutritionally demanding periods due to the need for the body to maintain a high rate of cell turnover. Folate is an important B-vitamin during these periods due to its function as a one-carbon donor in DNA, RNA, and protein biosynthesis (Herbert and Das, 1994). In humans, folate deficiency during pregnancy and lactation can lead to megaloblastic anemia in mothers and congenital birth defects including neural tube defects (NTD) in their infants (Metz, 1970; Shapiro et al., 1965). These devastating disorders may be prevented by daily oral supplementation of synthetic folic acid (FA) by the mother throughout pregnancy and lactation (O'Conner et al., 1997; FNB, 1998).

Folate status during lactation and growth in the horse is less understood. Mares maintained on pasture and sampled during the first mo of lactation were reported to have moderately low concentrations of serum folate in one study (Seckington et al., 1967), but adequate concentrations of serum and red blood cell (RBC) folate in another (Roberts, 1983). Neither of these reports assessed folate status over the duration of lactation, nor did they assess the folate status of the foal.

The role of folate in normal DNA and protein synthesis can ultimately influence the growth of the young foal if folate is limited. Specifically, adequate milk folate concentrations are necessary to support early growth until the foal's gut matures and folate requirements can be met by consumption of feed, forage, and from utilization of microbially derived folate. In order to make informed decisions regarding diet formulation and folate supplementation in horses, the effects of lactation and growth on folate status over long periods should be investigated.

The objective of this study was to assess the effects of lactation and growth on folate status in Thoroughbred mares and foals in order to determine if additional dietary folate supplementation was warranted to support folate status during the two physiologically demanding periods.

## Materials and Methods

*Animals.* Twenty Thoroughbred mares ( $609.9 \pm 16.2$  kg BW,  $12.9 \pm 1.1$  yr) from the Virginia Tech Middleburg Agricultural Research and Extension Center were used for this study. Mares were bred during March and April of 1999 to one of four Thoroughbred stallions located at the Center or a nearby farm. Prior to foaling, mares were paired by age, breeding date, and covering sire and randomly assigned to 2 groups of ten. Mares were maintained on adjacent bluegrass/white clover mixed pasture and were offered orchardgrass/alfalfa hay ad libitum during the winter mo. Foaling occurred during April and May of 2000. Mares were placed in foaling stalls the day of foaling and were returned to pasture with foals within 1 wk of foaling. Weaning occurred during the mo of October and November by removing two to four mares from each treatment group/wk. The protocol was approved by the Institutional Animal Care and Use Committee.

*Diets.* Mares were fed their experimental diet twice daily beginning in late gestation to meet approximately one-third of their DE requirement (NRC, 1989) with the remaining requirement met by pasture and hay consumption. Each group of mares was fed either a corn and molasses supplement rich in starch and sugar (SS) or a fiber (beet pulp, soy hulls, oat straw) and fat (cereal byproduct) based supplement (FF). Supplements were developed to be isoenergetic and isonitrogenous so that they would differ only in the fiber and fat content. Diet compositions of the supplements are shown in Table 1. Synthetic folic acid was excluded from the vitamin premix so that the folate sources were only those contained inherently in the supplement and forage. During lactation, mares and foals were fed their experimental diets in quantities sufficient to meet one-half to one-third of the DE requirements (NRC, 1989). Mares were fed an average of 3.5 and 4.0 kg/d divided into 2 meals/d during late gestation and early lactation, respectively. Weanlings were fed 1.6 kg/d divided into 2 meals/d until their yearling year when they were fed 2.2 kg/d. Mares and foals were group fed in their respective pastures.

### *Sampling and analysis*

*Blood and milk sampling.* Samples of blood and milk were collected in order to assess folate status by red blood cell and plasma folate, milk folate, and plasma homocysteine. Sampling was

conducted from 0800 to 1100 every mo beginning at foaling and ending at weaning when the foals were approximately 6 mo of age. Whole blood (10 ml) was collected from mares and foals by venipuncture into EDTA tubes. Milk (13 ml) was collected manually into plastic Whirl-Pak™ bags, filtered through a 4 x 4 in gauze pad to remove foreign particles, and transferred to light protective polypropylene vial with sodium ascorbate (Sigma, St. Louis, MO) added to achieve a final concentration of 1.0 % sodium ascorbate (wt/vol). For processing of red blood cells, 100 µl of whole blood was removed from blood collection tubes, transferred to 2 ml polypropylene vials (Sarstedt, Newton, North Carolina) containing 1 ml of 0.3 % (wt/vol) sodium ascorbate dissolved in deionized water. The remaining whole blood sample was then centrifuged at 2000 x g for 5 min. Plasma was transferred into 2 ml polypropylene vials and sodium ascorbate was added to a final concentration of 0.3 % (wt/vol) sodium ascorbate. All biological samples were kept out of direct light to prevent photooxidation of folate and were stored at -80°C until analyzed.

*Supplement and forage sampling.* In order to assess dietary intake of folate, forage and feed samples were sampled. Pasture samples (1 to 2 kg wet weight) were obtained monthly by clipping forage using hand-held electric clippers with a 10.2 cm wide edge by collection at random stops in each pasture housing the horses. Hay samples (0.8 kg wet weight) were obtained by the core method (Blaser et al., 1986). Samples of each supplement (0.5 kg wet weight) were taken by random grab samples from five different supplement bags in storage. Samples of pasture, hay and supplement were weighed, dried in a forced-air oven, and then weighed again for DM determination. Forage and supplement samples were then ground through a 0.5 mm screen Wiley Mill (Model 4, Thomas Scientific, Swedesboro, NJ) prior to analysis of total folate concentration as determined by microbial assay (Ralston Analytical Laboratories, St. Louis, MO).

*Plasma, red blood cell, and milk folate.* After validation for the equine, samples of plasma, RBC, and milk were analyzed for total folate by radioimmunoassay (Folate Dualcount Solid Phase No Boil Assay, Diagnostic Products Corporation, Los Angeles, CA). Validation of the radioimmunoassay for horse plasma was conducted by assessing recovery of known amounts of added 5-methyltetrahydrofolate (Schirks Laboratories, Jona, Switzerland) to plasma and by

dilution parallelism (1:1, 1:2, 1:4, and 1:6) (Table 2). All plasma samples were diluted 1:4 prior to analysis and run in duplicate. For the determination of RBC folate, whole blood was diluted 1:11 in a 1 % (wt/vol) sodium ascorbate solution immediately following sampling as per kit directions. However, analysis of RBC folate at that dilution yielded concentrations of folate that were between 2 and 6 times lower than published concentrations in the horse (Roberts, 1983). Further validation of the commercial kit revealed the highest yield of folate could be obtained by diluting the samples to a dilution of 1:50 with distilled water prior to analysis. The amount of NaOH/KCN provided in the commercial assay was increased in the unknown samples only from 50  $\mu$ l/sample to 125  $\mu$ l/sample with the additional NaOH/KCN factored into the dilution for a final dilution of 1:51.25. The latter step was found to increase the yield of folate mostly likely by increasing the denaturation of intracellular folate binding proteins in the erythrocyte. Concentration of plasma folate was subtracted from the concentration of whole blood folate for each horse during each mo to estimate RBC folate.

Highest concentrations of milk folate were obtained using a 1:30 dilution of milk prior to analysis. Use of the trienzyme treatment using  $\alpha$ -amylase, protease, and rat serum folate conjugase shown to be successful at increasing extraction of folate from foods (Tamura et al., 1997) and human milk (Mackey and Picciano, 1999; Lim et al., 1998) and was therefore assessed in the present study. Alpha-amylase (3.2.1.1) and protease (3.4.24.31) was obtained from Sigma Chemical (St. Louis, MO). In order to remove endogenous folate from pooled rat serum, 5.0 g of activated acid washed charcoal (Sigma, St. Louis, MO) was mixed with 50 ml of pooled rat serum for 2 hr, centrifuged at 50,000 x g for 2 hours, and then the mixture was filtered through a 0.45  $\mu$ m filter (Millipore Corp., Bedford, MA) to remove remaining charcoal. Milk samples (1:30; 0.3 ml) were incubated with either 0.1 ml folate conjugase for 3 hr, 0.2 ml  $\alpha$ -amylase (10 mg/ml) for 1, 4, or 8 hr, or 0.1 ml protease (4 mg/ml) for 4, 8, or 12 hr. Total milk folate concentration was increased only after incubation of milk with 0.2 ml  $\alpha$ -amylase (10 mg/ml) for 1 and 4 hr (Figure 1). For analysis of experimental milk samples by radioimmunoassay, 0.5 ml of milk (1:30 dilution) was mixed with 0.3 ml  $\alpha$ -amylase and incubated for 37°C for 2 hr prior to analysis.

*Plasma homocysteine.* Plasma homocysteine was determined using a pre-column derivatization procedure followed by reverse-phase separation and fluorescence detection using high-pressure liquid chromatography. The pre-column derivatization and HPLC separation materials were purchased as a kit from Bio-Rad Laboratories (Homocysteine by HPLC, Bio-Rad Laboratories, Hercules, CA) and methods provided in the kit were followed. The HPLC system was an 1100 Series LC 3D ChemStation from Agilent Technologies (Wilmington, DE) equipped with a thermostatted autosampler, quaternary pump with degasser, fluorescence detector, and reverse-phase analytical column (70 mm x 3.2 mm ID) fitted with a Micro-Guard<sup>TM</sup> (Bio-Rad Laboratories, Hercules, CA). Briefly, 50 µl of plasma, 100 µl of internal standard, 50 µl of reduction reagent (NaBH<sub>4</sub>), and 100 µl of derivatization reagent (Ammonium 7-fluorobenzo-2-oxa1,3-diazole-4-sulphonate solution) were added together, mixed thoroughly, and incubated for 5 min at 50°C. After cooling the mixture at 4°C for 5 min, the proteins were precipitated by adding 100 µl of precipitation reagent (Trichloroacetic Acid), mixing thoroughly and then centrifuging the proteins into a pellet at 10,000 x g for 5 min. The supernatant was transferred to screw cap vials and placed in the autosampler. Twenty µl of plasma was injected on to the heated (45°C) column at a flow rate of 0.7 mL/min with the fluorescence detector at a wavelength of 385 nm for excitation and 515 nm for emission. The concentration of plasma homocysteine was quantified from its retention time relative to known standards, whereas peak areas were converted to concentration of plasma homocysteine by reference to the internal standard.

*Statistical analysis.* SAS for WINDOWS (Version 8, 1999; SAS Institute Inc, Cary, NC) was used for data tabulation and statistical analyses. Data were analyzed by analysis of variance (ANOVA) with repeated measures using the MIXED procedure with the REPEATED statement. Diet (FF or SS) and time (mo of lactation or growth) were treated as main effects, with diet by time interaction. A Tukey's test was used to determine differences between mo for the variables tested. Relationships among mare and foal indexes of folate status and folate intake were evaluated using regression analyses (SlideWrite for Windows, v. 9). A P-value of < 0.05 was chosen as the level of significance. Data are presented as means ± standard error (SE).

## Results

Mean BW of mares and BW and ADG of foals over the 6 mo experimental period are shown in Table 3. No differences in BW for mares and BW and ADG in foals were noted due to diet; however there was an effect due to mo of lactation in mares ( $P < 0.0001$ ) and growth in foals ( $P < 0.0001$ ). Mare BW was lowest at foaling ( $P < 0.0001$ ) and highest at 6 mo of lactation ( $P < 0.05$ ). As expected, the BW of foals increased significantly each mo ( $P < 0.05$ ). Highest ADG of foals was observed at 1 mo ( $P < 0.0005$ ) followed by a gradual decline to 6 mo of age.

Total folate concentration in SS, FF, and orchardgrass/alfalfa hay was  $1.6 \pm 0.4$ ,  $1.9 \pm 0.9$ ,  $1.4 \pm 0.2$  mg/kg DM, respectively. Total folate concentration in the bluegrass/white clover mixed pasture over the course of 1 yr, including the present study is presented in Figure 2. Folate levels in the pasture peaked in April ( $4.48 \pm 0.73$  mg/kg DM) and October ( $4.88 \pm 0.66$  mg/kg DM), with lowest values occurring in May ( $1.65 \pm 0.2$  mg/kg DM) and June ( $2.2 \pm 0.4$  mg/kg DM).

**Mare Folate Status.** Mare plasma folate concentrations are shown Figure 3. There was no main effect of diet although the SS mares had higher plasma folate concentrations compared to FF mares at 3 mo ( $P < 0.05$ ). There was a main effect of mo of lactation on plasma folate in mares ( $P < 0.002$ ). Plasma folate concentrations in SS mares during the last 3 mo were lower than the first 4 mo of lactation ( $P < 0.05$ ). Plasma folate concentrations in FF mares were highest at foaling ( $P < 0.05$ ) and lowest at 3 mo ( $P < 0.05$ ). There was no significant correlation between plasma folate concentration and pasture folate concentration.

Mare RBC folate concentrations are shown in Figure 4. There was a main effect due to diet observed indicating that mares fed SS had higher RBC folate concentrations compared to mares fed FF ( $P < 0.05$ ). In addition, mo of lactation influenced RBC folate concentrations in both groups. Red blood cell folate concentrations increased from foaling to 3 mo ( $P < 0.05$ ) in both groups of mares, followed by a decline resulting in lowest concentrations observed between 5 and 6 mo ( $P < 0.05$ ). There was no significant correlation between mare plasma folate and mare RBC folate, nor was there a significant correlation between mare RBC folate concentrations and pasture folate concentrations.

Plasma homocysteine concentrations were higher in mares fed FF compared to mares fed SS throughout lactation ( $P < 0.05$ ) (Figure 5). Plasma homocysteine was increased from  $6.2 \pm 0.56$   $\mu\text{mol/l}$  at foaling to  $7.4 \pm 0.60$   $\mu\text{mol/l}$  after 6 mo of lactation ( $P < 0.05$ ) in mares fed SS, whereas plasma homocysteine concentrations in mares fed FF remained relatively stable after foaling. There was no significant correlation found between mare plasma homocysteine concentrations and either mare plasma folate or mare RBC folate concentrations.

Folate concentration in mare's milk is shown in Figure 6. There was a trend for a higher milk folate concentration in SS mares compared to FF mares ( $P = 0.076$ ), and a main effect due to mo of lactation in SS and FF mares ( $P < 0.001$ ). Milk folate was highest at foaling in SS and FF mares than any other period ( $P < 0.05$ ). Mean milk folate concentration combined for both groups was  $217.8 \pm 12.4$  ng/ml at foaling. Milk folate concentration declined in both groups at 3 mo ( $P < 0.05$ ), followed by an increase at 4 mo ( $P < 0.05$ ), which again declined at 6 mo of lactation ( $P < 0.05$ ). There was no significant relationship observed between plasma and RBC folate concentration and milk folate concentration, or between milk folate concentration and pasture folate concentration.

*Foal Folate Status.* Plasma folate concentrations of foals are shown in Figure 7. There was no main effect due to diet; however plasma folate concentrations were higher in FF foals compared to SS foals from 4 to 6 mo of growth. Plasma folate concentrations rapidly declined from foaling to 1 mo of age in SS and FF foals ( $P < 0.05$ ) followed by a gradual increase with highest concentrations observed from 4 to 6 mo ( $P < 0.05$ ). Mean values for plasma folate in foals over the experimental period was  $16.2 \pm 1.6$  ng/ml. There was no significant correlation observed for foal plasma folate concentrations and pasture folate or mare's milk folate.

Red cell folate concentrations in foals is shown in Figure 8. Higher concentrations of RBC folate in FF foals were observed at 1 and 3 mo compared to SS foals ( $P < 0.05$ ). There were no differences among RBC folate concentrations in SS foals during the experimental period. However, FF foals experienced a decline in RBC folate at 6 mo ( $P < 0.05$ ). There were no significant correlations observed between foal RBC folate and plasma folate, pasture folate, or mare's milk folate concentrations.

Plasma homocysteine concentrations in foals are shown in Figure 9. There were no differences in plasma homocysteine concentrations between SS and FF foals during the 6 mo experimental period. Plasma folate concentrations for all foals were highest at foaling ( $13.35 \pm 1.9 \mu\text{mol/l}$ ) ( $P < 0.0001$ ). Plasma folate concentrations remained stable in all foals from 2 to 6 mo with a mean concentrations of  $4.7 \pm 0.09 \mu\text{mol/l}$ . There was no significant correlation observed between plasma homocysteine concentrations and foal plasma folate or RBC folate.

## Discussion

The objective of this study was to assess the effects of lactation and growth on folate status in Thoroughbred mares and foals up to 6 mo post-foaling. In the present study, folate status was influenced by lactation and growth in Thoroughbred mares and their foals fed pasture supplements and maintained on adequate mixed grass/legume pastures. However, folate status was not sufficiently lowered in either mares or foals to warrant folate supplementation.

Folate status assessment used in this study was based on the combination of RBC folate, plasma folate, plasma homocysteine, and milk folate. Unlike plasma folate, RBC folate concentrations do not fluctuate due to recent dietary intake, and RBC folate is reflective of tissue folate stores since the concentration of folate in the cells reflects the folate available to the cell at the time of erythropoiesis (Bailey, 1990). Plasma homocysteine was negatively correlated with dietary folate intake and plasma folate and has thus been used as a functional indicator of folate status (Lewis et al., 1992; O'Keefe et al., 1995; Mackey and Picciano, 1999).

Despite the moderate decline in plasma folate concentrations towards the end of lactation, all mares in the present study had plasma folate concentrations considered normal for humans ( $> 6 \text{ ng/ml}$ ) (Herbert and Das, 1994), which may be interpreted as normal for mares during lactation since neither mares or their foals suffered from any clinical abnormalities. Plasma folate concentrations at 1 mo of lactation were considerably higher than previous reports of serum folate sampled in recently foaled grazing mares (Seckington et al., 1967) and mares in the first mo of lactation (Roberts, 1983). Mares in the present study were maintained on quality

grass/legume pasture and fed supplements that inherently contained 1.6 to 1.9 mg/kg of folate, which is a relatively high amount of folate compared to other feedstuffs (NRC, 1989b).

All of the SS and FF mares had concentrations of RBC folate above those considered normal for humans ( $> 160$  n/gml) (Herbert and Das, 1994), which again can be interpreted as possible normal ranges for mares during lactation. Concentrations of RBC folate reported in this study were higher than RBC folate concentrations in mares at 1 mo of lactation (Roberts, 1983), and in horses engaged in submaximal routine exercise (Manuscript 2, Ordakowski, 2001). It is likely that the improved assay techniques utilized for RBC folate in this study were responsible for the higher folate concentrations observed in RBC compared to previous reports that also utilized radioimmunoassay (Roberts, 1983). The initial increase in RBC folate concentration following foaling suggests that the 11 mo gestation of pregnancy in the mare may have placed an increased folate demand on the mare resulting in the lowered RBC folate concentrations at foaling. In accordance with a decline in mare plasma folate, there was also a decline in RBC folate concentrations after 2 mo of lactation, which indicated that late lactation placed an increased folate demand on the mares. In humans, a decline in RBC folate concentrations during early lactation was shown to increase towards late lactation (Smith et al., 1983; Ek, 1983), which is inconsistent with our results. Since RBC folate concentrations are considered a better indicator of folate status, and no significant correlation between RBC folate concentrations and plasma folate concentrations were found indicates that plasma folate concentrations is a poor indicator of folate status in the horse.

The assessment of milk folate concentration is an essential component of folate status because it can be used to determine maternal folate needs due to mobilization of folate in milk and dietary folate intake in nursing infants. The decline in folate content in mares' milk during the initial 3 mo of lactation may indicate that less folate is mobilized into the milk in order to maintain body stores of folate in the mare. Possible reasons why this may occur in the mare is due to the preservation of folate status in the mare for the benefit of the foal since mare's can become pregnant within 7 to 9 d after foaling. Therefore, the increase in maternal folate stores ensures a good reproductive efficiency for future foals. In humans, mobilization of folate into milk for the purpose of maintaining folate status in the infant has precedence over maintaining

folate stores (Metz, 1970; O'Conner et al., 1997). Milk folate concentrations during lactation in the human have been shown to either increase (Cooperman et al., 1982; Ek, 1983; Smith et al., 1983) or remain stable (Tamura et al., 1980). There was no significant association between the decline in milk folate and the decline in pasture folate that occurred during the same period. Also, milk folate had no correlation with RBC folate in mares indicating that milk folate is mobilized in mare milk regardless of folate status in the mare. Studies in humans also found no association between maternal folate stores and milk folate concentrations (Smith et al., 1983).

Concentrations of milk folate obtained in the present study are much higher than a previous estimate of 1.4 ng/ml of milk folate in horse milk 10 d post foaling (Collins et al., 1951). The higher milk folate concentrations in our study was most likely due to the 2 hour incubation of milk samples with  $\alpha$ -amylase prior to analysis using the RIA, which increased folate concentrations by 54 %. Milk folate concentrations in horse milk obtained in our study were similar to reports in human studies that analyze folate concentrations by microbial assay (Lim et al., 1998; Mackey and Picciano, 1999) after utilization of the trienzyme extraction method developed for food folate (Tamura et al., 1997). We were unable to find increased folate concentrations due to the addition of protease or rat serum conjugase addition. Therefore, the 2 h incubation of milk samples with  $\alpha$ -amylase prior to analysis by RIA was sufficient to liberate folate concentrations in horse milk.

The similar milk folate concentrations between SS and FF mares indicated that the added dietary fat and fiber had no harmful effects on mobilization of folate into milk. The lowered folate concentration observed during the warm summer mo and cold winter mo was similar to growth patterns typical of cool season grasses with growth periods occurring in the fall and spring and dormancy occurring during the summer and fall. The lower folate content observed in the orchardgrass/alfalfa hay fed during the winter mo compared to the folate concentrations in pasture suggest that the 7 to 9 mo of storages likely resulted in a decline in folate concentrations in the hay.

Homocysteine is a non-essential sulfur containing amino acid whose metabolism is catalyzed by folate, B<sub>12</sub>, and B<sub>6</sub> dependant enzymes (Selhub, 1999). Homocysteine is converted to

methionine via methionine synthase, an enzyme that requires 5-methyltetrahydrofolate as a methyl donor and vitamin B<sub>12</sub> as a co-factor. When 5-mTHF is limited, homocysteine becomes concentrated and leaks out of the cell into the plasma. Mean ranges of plasma homocysteine observed in this study were between 3.7 to 7.8  $\mu\text{mol/l}$ . These are the first reported values of plasma homocysteine concentration in normal horses. By comparison, plasma homocysteine levels maintained between 5-15  $\mu\text{mol/l}$  are considered normal in humans (Selhub, 1999). Plasma homocysteine has been used as a functional indicator of folate status in humans because it is negatively correlated to dietary folate intake and plasma folate (Lewis et al., 1992; O'Keefe et al., 1995; Mackey and Picciano, 1999). No correlation was observed between RBC folate and plasma homocysteine concentrations most likely due to the fact that all mares in the present study had an adequate folate status.

Higher concentrations of plasma homocysteine in SS mares compared to FF mares was not likely to be related to folate status since SS mares had either similar or higher concentrations of RBC folate throughout lactation. The B-vitamins B6 and B12 are also important to homocysteine metabolism and when in limited amounts in the body, can cause increased plasma homocysteine concentrations (Selhub, 1999). Therefore, the lower concentration of plasma homocysteine concentrations in FF mares may have been due to a greater utilization of microbially derived B vitamins in the mares consuming SS. The FF supplement was designed to have a variety of fiber sources and length of fibers such that it would stimulate and promote a healthy and diverse microbial population in the gut of the horse.

Concentrations of plasma folate and RBC folate in foals over the 6 mo experimental period were also well above concentrations considered normal in humans (Herbert and Das, 1994). The rapid decline in plasma folate concentrations in foals occurred during a time of rapid growth indicated by a high ADG (Table 3). However, after 2 mo, the plasma folate concentrations increased over concentrations obtained at foaling. Despite a similar increase in pasture folate concentration, no significant correlation between pasture folate and plasma folate concentration was found. Unlike the plasma folate, concentration of RBC folate remained relatively stable in foals over the 6 mo observational period indicating a stable folate status. As in the mares, no significant correlation was observed between plasma folate concentrations and RBC folate

concentrations in the foals again showing that plasma folate status is not a good indicator of folate status. In addition, no significant correlation was observed between milk folate concentration and either plasma folate concentration and RBC folate concentrations indicating that folate status in foals occurred regardless of milk folate concentrations in mare's milk.

The drastic decline in plasma homocysteine concentrations at foaling is difficult to interpret since plasma folate concentrations were adequate in foals at foaling. The collection and isolation of plasma on foals at birth often leads to hemolysis due to the fragility of the foal erythrocytes. Visual examination of some of the plasma samples revealed hemolysis did in fact occur causing some of the data for those horses to be removed from analysis. Therefore, the increased plasma homocysteine may be due to leakage of intracellular homocysteine into the plasma.

Alternatively, the plasma homocysteine may have been due to a low B<sub>12</sub> or B<sub>6</sub> status, which was offset after foaling by milk consumption and B-vitamin microbial synthesis by the foal. Despite the fluctuations in plasma folate and RBC folate, plasma homocysteine concentration was not affected by the 6 mo growth period. The mean concentration of plasma homocysteine observed during the 5 mo following foaling was  $4.7 \pm 0.09 \mu\text{mol/l}$ .

Comparison of mares and foals revealed that foals generally had a higher RBC folate and lower plasma homocysteine concentrations indicative of a higher folate status. This same phenomenon also occurred in humans (Ek, 1980; Tamura, 1990; Smith et al., 1983). Although milk folate declined during early lactation and folate status in the mare was lowered after 4 mo of lactation, no adverse effects on folate status were observed in the foal. Therefore, the dietary intake of folate from feed and forage offered in this study was effective in maintaining folate status in mares and foals during 6 mo of lactation and growth, respectively.

### **Implications**

Mares and foals maintained on quality grass/legume pasture and fed pasture supplements similar to those used in this experiment do not require additional folate supplementation to maintain folate status during lactation and growth.

## Tables

**Table 1. Ingredient and nutrient composition of Starch/sugar (SS) and Fiber/fat (FF) feed<sup>a</sup>**

Item	SS (n=6)	FF (n=6)
Ingredient (%)		
Dent yellow grain corn	60.0	1.5
Soybean meal 48%	15.5	2.0
Oat Straw	7.0	7.0
Alfalfa	-	13.5
Soybean hulls	4.0	4.0
Beet pulp	-	10.0
Cereal by-product	-	56.0
Cane molasses	10.0	5.0
Dicalcium phosphate	1.5	-
Limestone	1.0	-
Mineral premix <sup>b</sup>	0.5	0.5
Vitamin premix <sup>c</sup>	0.5	0.5
Nutrient composition		
DM, %	95.8 ± 0.4	97.9 ± 0.69
DE, Mcal/kg	3.7 <sup>f</sup> ± 0.05	3.0 <sup>g</sup> ± 0.00
CP, %	15.8 ± 1.1	13.9 ± 0.22
ADF, %	7.4 <sup>d</sup> ± 0.95	22.3 <sup>e</sup> ± 0.90
NDF, %	15.8 <sup>d</sup> ± 1.4	31.4 <sup>e</sup> ± 0.68
Fat, %	2.8 <sup>f</sup> ± 0.18	10.2 <sup>g</sup> ± 1.0
NSC, %	59.4 <sup>f</sup> ± 3.1	34.1 <sup>g</sup> ± 1.9
Ash, %	6.2 <sup>d</sup> ± 0.75	11.7 <sup>e</sup> ± 0.14
Ca, %	0.86 ± 0.14	2.3 ± 0.03
P, %	0.63 ± 0.08	0.93 ± 0.08
Mg, %	0.20 <sup>f</sup> ± 0.02	0.57 <sup>g</sup> ± 0.05
K, %	1.0 ± 0.08	1.2 ± 0.01
Na, %	0.21 ± 0.03	0.27 ± 0.02
S, %	0.20 ± 0.01	0.19 ± 0.01
Cl, %	0.86 ± 0.40	0.50 ± 0.03

<sup>a</sup> Analyzed by DHI Forage Testing Laboratory (Ithaca, NY). Data are summarized on a DM basis as means SE's.

<sup>b</sup> The mineral premix provided the following per kilogram of supplement: NaCl, 3,774 g; Zn, 422 g; Fe, 208 g; Cu, 89.5 g; Mn, 50.3 g; Se, 1.095 g; and KI, .415 g

<sup>c</sup> The vitamin premix provided the following per kg of a supplement: vitamin A, 1,380,080 IU; vitamin D3, 258,000 IU; vitamin E, 26,455 IU; riboflavin, 701mg; niacin, 3009 mg; folic acid, 66 mg; thiamin, 1400 mg; biotin, 42 mg; and -carotene, 3527 mg;

<sup>d,e</sup> Within a row, means without a common superscript differ ( $P < 0.01$ )

<sup>f,g</sup> Within a row, means without a common superscript differ ( $P < 0.0001$ )

**Table 2.** Recovery of plasma folate after spiking and dilution parallelism of radioimmunoassay<sup>a</sup>

Procedure	Observed (O)	n	Expected (E)	O/E (%)
Dilution Factor				
1:1	8.93	5	-	-
1:2	5.03	5	4.47	112
1:4	2.55	5	2.23	114
1:6	1.60	5	1.49	107
5-mTHF Added (ng/ml)				
0	8.93	5	-	-
1.5	5.85	5	5.97	98
3	7.29	5	7.47	98
6	9.58	5	10.47	91
12	13.02	5	16.47	79

<sup>a</sup> Folate Dualcount Solid Phase No Boil Assay (Diagnostic Products Corporation, Los Angeles, CA)

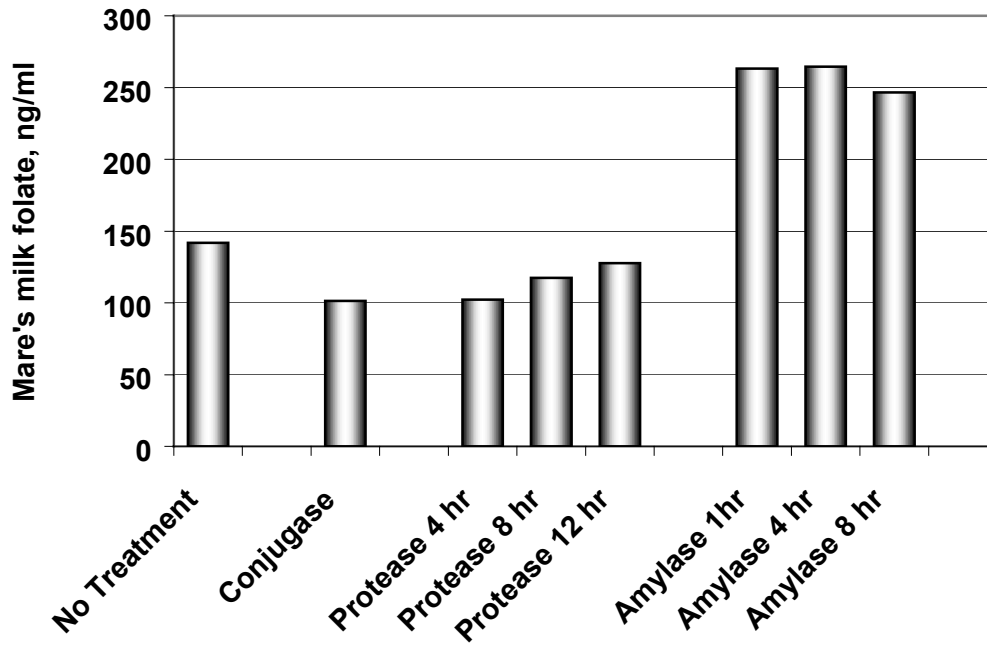
<sup>b</sup> Abbreviations: 5-mTHF: 5-methyltetrahydrofolate

**Table 3.** Mean body weight of mares and foals and average daily gain of foals fed either starch/sugar (SS) or fiber/fat (FF) supplements

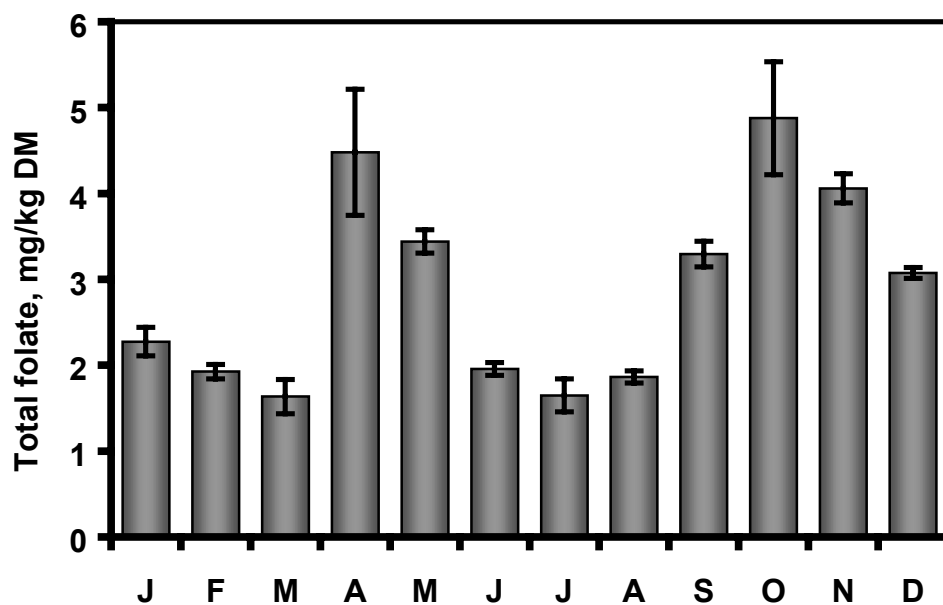
Group	Month							SE
	Foaling	1	2	3	4	5	6	
Mare								
SS	598.5 <sup>a</sup>	616.9 <sup>b</sup>	624.1 <sup>b</sup>	617.7 <sup>b</sup>	620.7 <sup>b</sup>	624.2 <sup>b</sup>	633.1 <sup>c</sup>	16.2
FF	580.9 <sup>a</sup>	592.0 <sup>b</sup>	597.8 <sup>b</sup>	602.9 <sup>b</sup>	604.3 <sup>b</sup>	611.2 <sup>b</sup>	621.9 <sup>c</sup>	16.2
Foal								
SS	58.6 <sup>a</sup>	100.6 <sup>b</sup>	142.2 <sup>c</sup>	177.3 <sup>d</sup>	217.0 <sup>e</sup>	249.6 <sup>f</sup>	279.0 <sup>g</sup>	3.9
FF	60.8 <sup>a</sup>	99.2 <sup>b</sup>	140.7 <sup>c</sup>	182.4 <sup>d</sup>	219.5 <sup>e</sup>	252.3 <sup>f</sup>	275.6 <sup>g</sup>	3.9
ADG								
SS	-	1.8 <sup>a</sup>	1.3 <sup>b</sup>	1.2 <sup>b,c</sup>	1.2 <sup>b,c</sup>	1.1 <sup>c</sup>	0.9 <sup>d</sup>	0.04
FF	-	1.6 <sup>a</sup>	1.4 <sup>b</sup>	1.3 <sup>b,c</sup>	1.2 <sup>b,c</sup>	1.0 <sup>c</sup>	0.7 <sup>d</sup>	0.04

a,b,c,d,e,f,g Within a row, means with unlike superscript differ ( $P < 0.05$ )

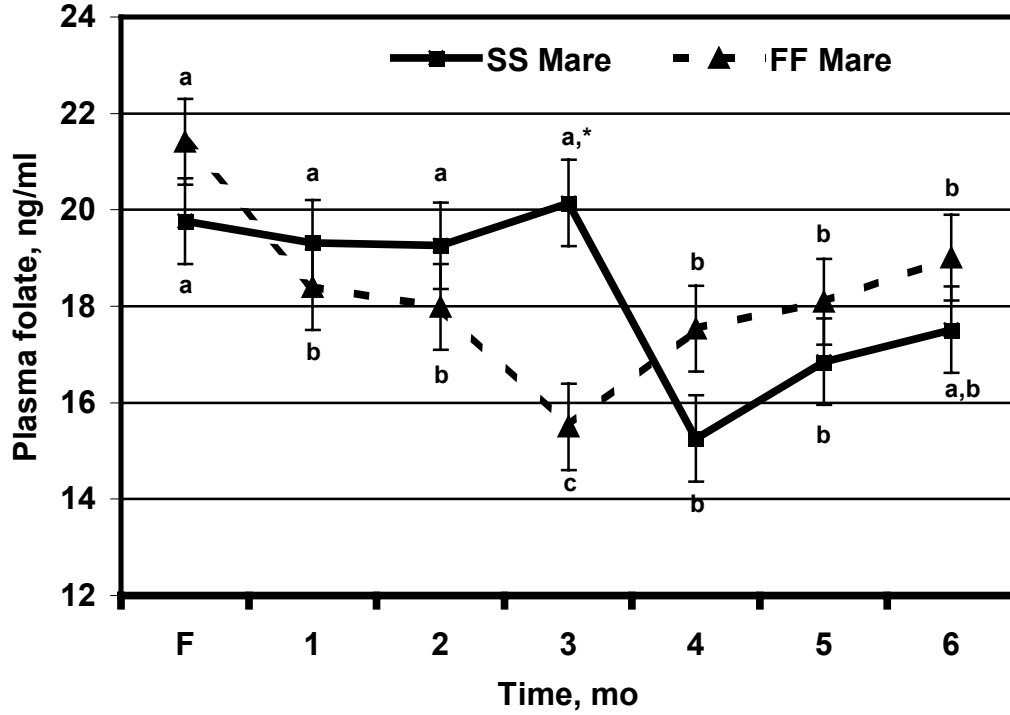
## Figures



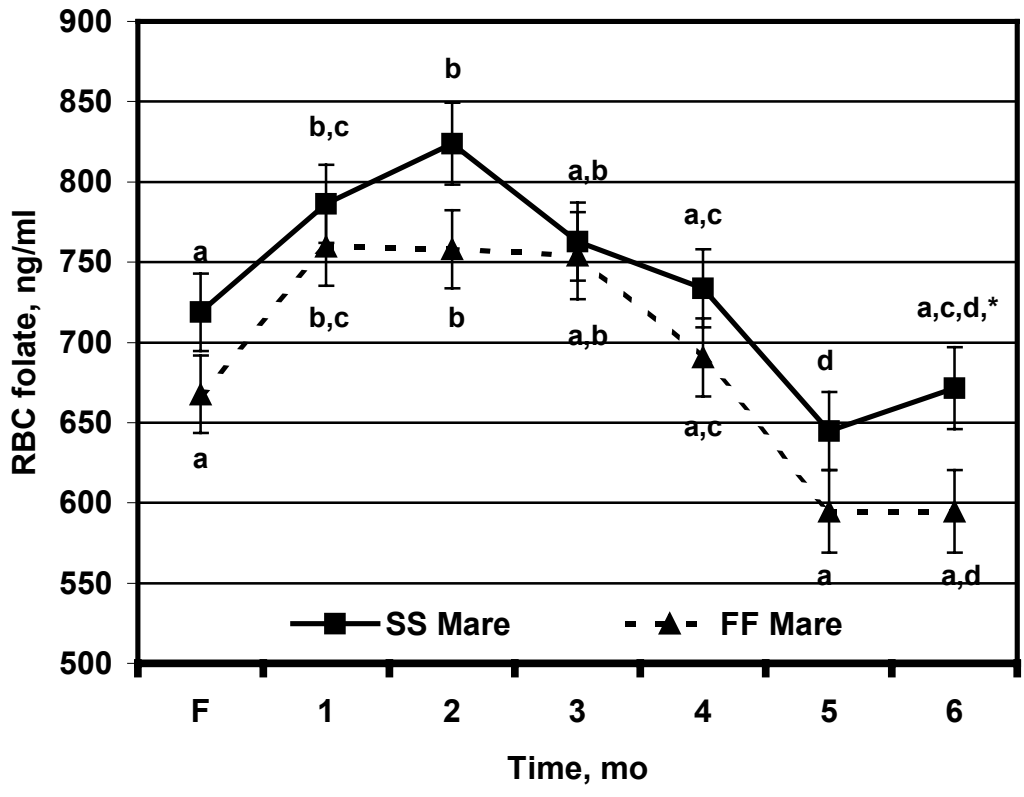
**Figure 1.** Total folate in mare's milk after either no treatment, or after incubation with rat serum conjugase for 3 hr, protease (4 mg/ml) for 4, 8, or 12 hr, or  $\alpha$ -amylase (10 mg/ml) for 1, 4, and 8 hr.



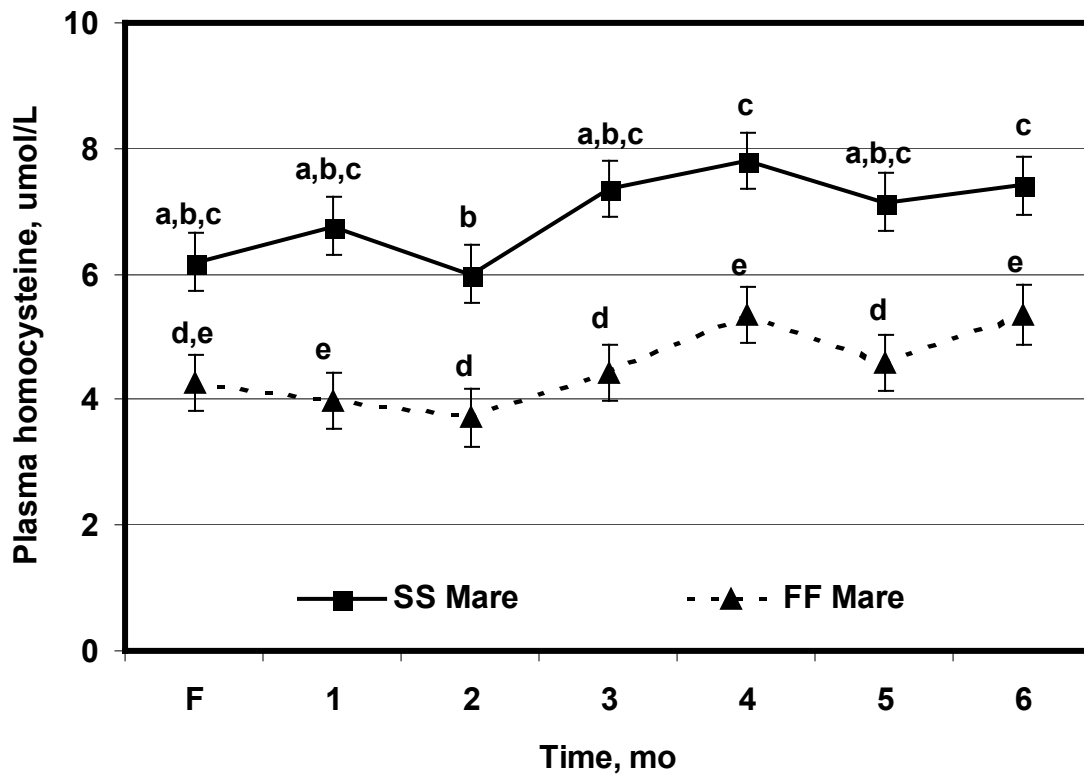
**Figure 2.** Monthly total folate concentrations (mg/kg DM) in bluegrass/white clover mixed pasture as determined by microbial assay



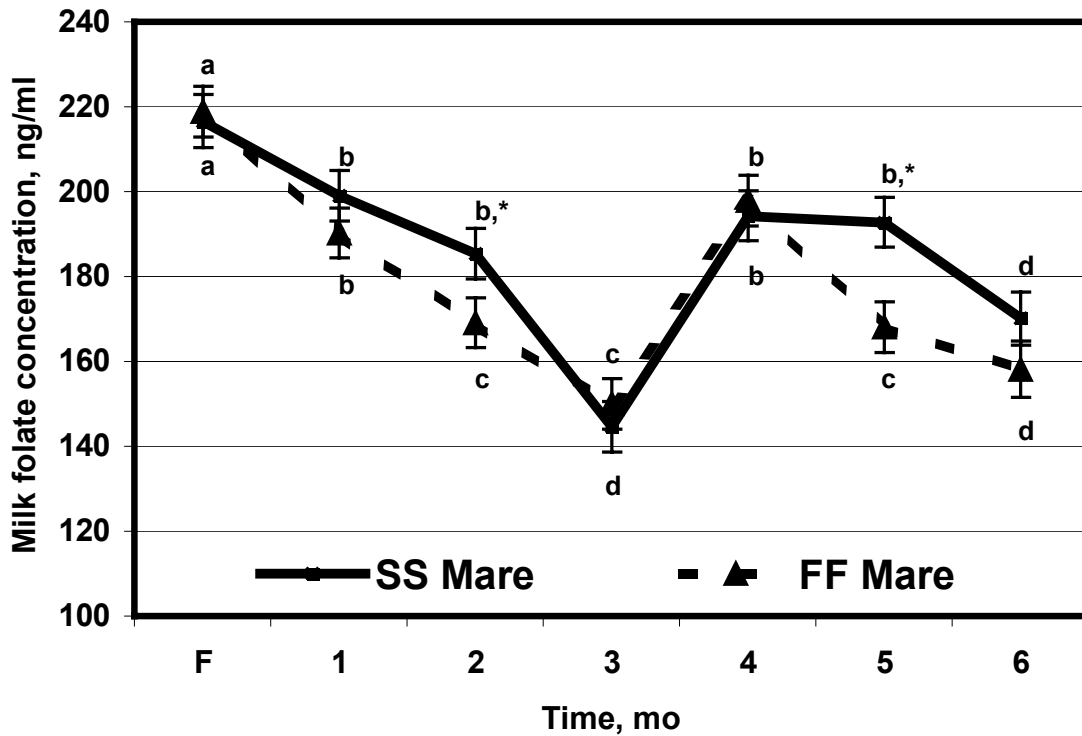
**Figure 3.** Plasma folate concentrations in mares fed sugar/starch (SS) or fiber/fat (FF) from foaling (F) to 6 mo of lactation. <sup>a,b,c</sup> Means with unlike superscripts differ within treatment ( $P < 0.05$ ). \* Higher plasma folate concentrations in SS mares compared to FF mares ( $P < 0.05$ ).



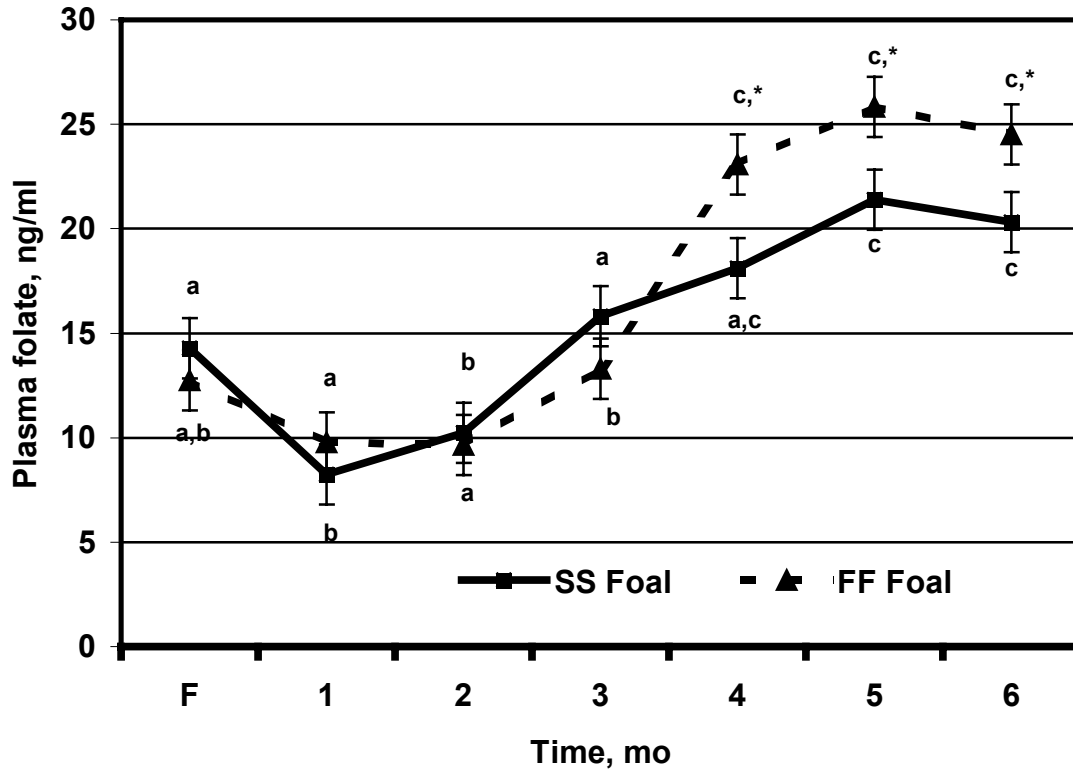
**Figure 4.** Red cell folate concentrations in mares fed sugar/starch (SS) or fiber/fat (FF) from foaling (F) to 6 mo of lactation. <sup>a,b,c,d</sup> Means with unlike superscripts differ within treatment ( $P < 0.05$ ). \* Higher RBC folate concentration in SS mare compared to FF mares ( $P < 0.05$ ).



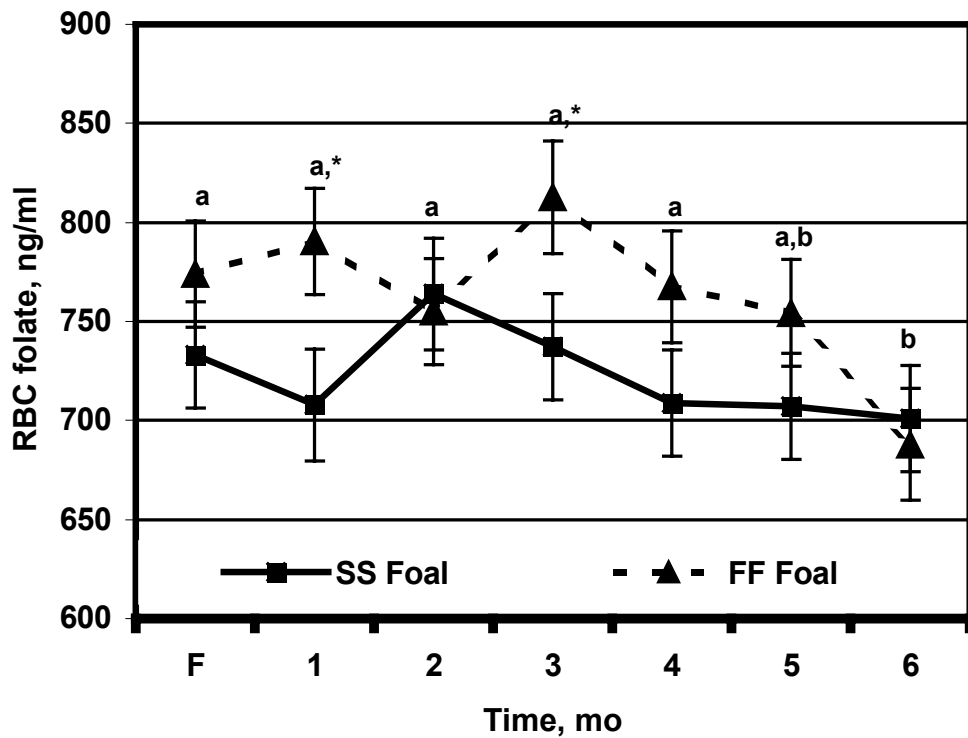
**Figure 5.** Plasma homocysteine concentrations in mares fed starch/sugar (SS) or fiber/fat (FF) from foaling (F) to 6 mo of lactation. Mares fed SS had higher concentrations of plasma homocysteine compared to mares fed FF ( $P < 0.05$ ). <sup>a,b,c</sup> Means with unlike superscripts differ within and between treatment ( $P < 0.05$ ).



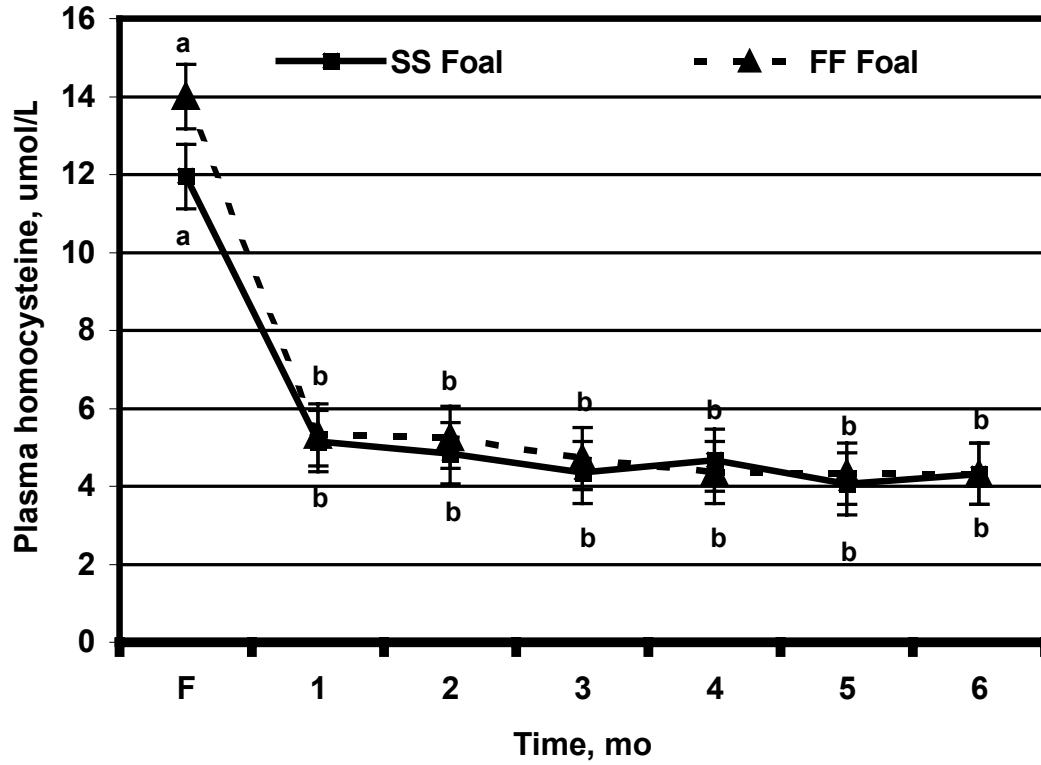
**Figure 6.** Total milk folate concentrations in mares fed starch/sugar (SS) or fiber/fat (FF) from foaling (F) to 6 mo of lactation. There was a trend for higher milk folate concentrations in SS compared to FF mares ( $P = 0.76$ ). <sup>a,b,c,d</sup> Means with unlike superscripts differ within treatment ( $P < 0.05$ ). \* Milk folate concentrations higher in SS mares compared to FF mares ( $P < 0.05$ )



**Figure 7.** Plasma folate concentration in foals fed starch/sugar (SS) or fiber/fat (FF) from foaling (F) to 6 mo of age. <sup>a,b,c</sup> Means with unlike superscripts differ within treatment ( $P < 0.05$ ). \* Plasma folate concentrations higher in FF compared to SS foals ( $P < 0.05$ ).



**Figure 8.** Red blood cell folate concentrations in foals fed starch/sugar (SS) or fiber/fat (FF) from foaling (F) to 6 mo of age. <sup>a,b</sup> Means with unlike superscripts differ within treatment ( $P < 0.05$ ). \* Plasma homocysteine concentration higher in FF compared to SS foals ( $P < 0.05$ ).



**Figure 9.** Plasma homocysteine concentrations in foals fed starch/sugar (SS) or fiber/fat (FF) from foaling (F) to 6 mo of age. No differences in plasma homocysteine concentration were noted between dietary groups. <sup>a,b</sup> Means with unlike superscripts differ within treatment ( $P < 0.05$ ).

## CHAPTER IV

### **Manuscript 2. Oral folic acid supplementation does not influence folate status or oxidative stress in mature horses engaged in routine submaximal exercise**

**ABSTRACT:** A study was conducted to determine the effects of oral folic acid (FA) supplementation on folate status, oxidative stress, and athletic performance during submaximal exercise tests in 22 horses ( $12.1 \pm 0.9$  yr) participating in a routine submaximal exercise program. Horses were paired by age and level of riding lesson activity and randomly assigned to either a control group (CON) or a treatment group that was orally administered 25 mg of FA mixed into their morning concentrate 5 d/wk for 12 wk. Horses were housed on mixed grass/legume pastures and fed a commercial concentrate and orchardgrass/alfalfa hay in stalls 4 d/wk and offered hay only during rest periods. Horses participated in  $4.0 \pm 0.6$  equitation and/or jumping lessons/wk over 4 d work period followed by a 3 d rest period. At the beginning and end of the study, horses performed a submaximal 48 min standard exercise test (SET) to assess exercise performance before and after FA supplementation. Folate status was assessed by means of plasma and red blood cell (RBC) folate and plasma homocysteine. Oxidative stress was assessed by means of bi-monthly analysis of plasma vitamin E and white blood cell glutathione peroxidase (WBC GPx) activity. There were no differences observed in folate status, oxidative stress, or submaximal SET performance due to oral supplementation of 25 mg of FA 5 d/wk. The 12 wk submaximal exercise regimen moderately reduced folate status ( $P < 0.05$ ), which did not result in an increased plasma homocysteine concentration. Changes in some hematological indexes occurred as a result of the submaximal exercise regimen, but not from oral FA supplementation. The 12 wk submaximal exercise period was associated with an increased oxidative stress as indicated by a decline in plasma vitamin E concentrations during the last half of the study ( $P < 0.05$ ) and an increase in the activity of WBC GPx throughout the study ( $P < 0.05$ ). The common practice of orally supplementing FA to horses in low to moderate exercise regimens appears to be a futile practice. Alternative sources of folate that are effective in improving folate status in the horse are necessary. In addition, supplementation of antioxidants may be warranted to combat the increased oxidant load in submaximally exercised horses.

Key Words: Folate, submaximal exercise, horse, homocysteine, oxidative stress

## Introduction

Supplementation of synthetic folic acid (FA) in equine concentrates and vitamin supplements became common practice after reports of low serum folate (Seckington et al., 1967; Allen, 1978; McMeniman et al., 1995) and low serum and red cell folate (Allen and Powell, 1983; Roberts, 1983) in stabled horses that were intensely exercised. Seckington et al. (1967) reported improvement in serum folate, hematocrit, body condition, and racing performance after 23 d of 20mg/d of FA supplementation (method of administration not included) in one horse. Despite this common practice, the bioavailability of FA is low in the horse (Roberts, 1983; NRC, 1989) and no controlled studies have been conducted evaluating the potential benefits of FA supplementation on folate status in the horse.

In addition to causing ineffective DNA synthesis, impaired folate status can lead to an inability of 5-methyltetrahydrofolate to methylate homocysteine to form methionine, which can lead to a disorder called hyperhomocysteinemia (Brody, 1991). Hyperhomocysteinemia has been associated with numerous disorders in the human including oxidative stress (Huang et al., 2001; Moat et al., 1999). Horses in moderate to heavy exercise have an increased risk of oxidative stress and subsequently reduced performance due to the production of reactive oxygen species (ROS) during energy metabolism needed for continued muscle contraction (Hinchcliff et al., 1998; Witt et al., 1992; Ji, 1999). Low dose FA supplementation in humans reduces plasma homocysteine concentrations (Brouwer et al., 1999; Jacques et al., 1999) and has been shown to have free radical scavenging activity *in vitro* (Joshi et al., 2001). Thus, FA supplementation may be beneficial as a preventive measure towards minimizing oxidative stress during exercise training and performance.

The objective of this study was to evaluate the effects of long-term routine oral supplementation of FA on folate status, oxidative stress, and athletic performance during submaximal exercise tests.

## Materials and Methods

*Animals.* Twenty-two horses ( $12.1 \pm 0.9$  yrs old) of mixed breeding (15 Thoroughbreds, 2 Quarter Horses, and 5 Warmbloods) and sex (18 geldings and 4 mares) that participated in the Fall 2000 Virginia Tech Equitation Program were used in this study. The feeding, health, and exercise management practices used in this study complied with procedures already established for the Virginia Tech Equitation Program. All horses began participating in the lesson program at least 3 wk prior to the start of the study. Geldings were housed in two adjacent 5-acre paddocks separated into a dry lot with a covered shed and a mixed grass/legume pasture. Mares were housed nearby in a 10-acre pasture consisting of the same mixed grass/legume pasture species. All but 3 horses (1 gelding and 2 mares) were brought into box stalls 4 d/wk, while the remaining horses were brought into box stalls during the afternoon. While in stalls, horses were fed and removed for daily lessons. All horses were returned to their paddocks in the evening. The lesson program consisted of beginning and intermediate equitation and beginning, intermediate, and advanced jumping lessons lasting 1 h each. During the study, horses averaged  $4.0 \pm 0.6$  lessons/wk. Horses were usually rested 3 d/wk unless they participated in additional schooling events and shows on the weekends. The lesson, show, and additional schooling activity of each horse were recorded. The protocol was approved by the Institutional Animal Care and Use Committee.

*Diets.* Horses were allowed free access to mixed grass/legume pasture during the evening and morning hours; however, availability of pasture was limited. Therefore, horses were fed hay and concentrate once or twice daily to maintain a body condition score between 4.5 and 6 (Henneke, 1983). Horses were fed approximately 1.8 kg of orchardgrass/alfalfa hay and between zero and 1.4 kg of a commercial concentrate in the morning and/or afternoon. The same orchardgrass/alfalfa hay was distributed twice daily (0800 and 1600 h) to the three fields housing the horses. Twenty of the horses were fed Reliance T 12 % (Southern States, Richmond, VA), whereas two horses were fed Equine Senior (Southern States, Richmond, VA). Each concentrate had been fortified with 0.88 mg/kg of synthetic FA during processing. Concentrates were fed 4 d/wk unless the horse participated in a weekend activity in which case they received their normal concentrate ration.

In order to estimate folate intake, samples of pasture, hay, and concentrate were taken. Pasture samples (0.8 kg wet weight) were obtained at the beginning of the study by clipping forage using electric hand-held electric clippers by stopping at random in each of the three pastures. Samples of pasture could not be taken at any other interval due to a lack of forage availability. Orchardgrass/alfalfa hay samples (0.3 kg wet weight) were obtained by the core method (Blaser et al., 1986). Samples of each supplement (0.8 kg wet weight) were obtained at random in several different bags of concentrates. Samples of pasture, hay and supplement were weighed, dried in a forced-air oven, and then weighed again for DM determination. Forage and supplement samples were then ground through a .5mm screen Wiley Mill (Model 4, Thomas Scientific, Swedesboro, NJ) prior to analysis of total folate concentration as determined by microbial assay (Ralston Analytical Laboratories, St. Louis, MO). Nutrient composition of the pastures, hay, and feeds are shown in Table 1. In addition to their normal dietary constituents, some of the horses were receiving supplements. Seven horses received chondroitin sulfate (Max Flex™, Equicare® Products, Phoenix, AZ), three horses received biotin (H.B.-15™, Farnam Companies, Inc., Phoenix, AZ), one horse received aspirin (AspirEase™, BioNutrition Labs, Santa Fe, NM), and MSM (Vita-Flex Nutrition®), and one horse received aspirin, MSM and chondroitin sulfate. Supplements did not contain supplemental folate.

*Treatments.* Horses were paired by age and lesson activity level and randomly assigned to either the control (CON) or oral FA supplementation group that was administered 25 mg of synthetic FA (Rovimix® Folic 80 SD, Roche Vitamins Inc., Parsippany, NJ) 5 d/wk for 12 wk. The FA was dissolved in distilled water and 5 cc of the solution was mixed into the horses daily morning concentrate feeding. One d/wk, the oral FA solution was mixed into concentrate and hand fed to FA supplemented horses in the field.

*Standard Exercise Test.* Nine horses from each group performed a standard exercise test (SET) at the beginning (SET 1) and at the end (SET 2) of the experimental period. Exercise tests were carried out on an outdoor sand arena. Data for three horses from each group had to be omitted because they could not participate in one or both of the SET's due to lameness or illness. In order to have all horses tested on the same day, three identical consecutive SET's were conducted consisting of five to seven horses each. Horses were ridden by members of the

Virginia Tech Equestrian Team. All attempts were made to have the same person ride the same horse for each SET. Equipment used on each horse during the SET had been previously assigned for use with that particular horse. Average weight of the riders and equipment was  $61.9 \pm 2.1$  and  $10.0 \pm 0.22$  kg, respectively.

Each SET consisted of 10 min walk (1.5 m/s), 20 min trot (3.5 m/s), 2 min walk, 8 min medium canter (7.5 m/s), 2 min walk, and 6 min hand gallop (9 m/s). A pace horse was used to establish the speed for the other horses, and two individuals were placed in the center of the sand riding ring to call out commands to riders to make sure all horses were at the proper speed. Horses were asked to change directions midway through each walk, trot, canter, and hand gallop. Blood samples and heart rates were taken before (PRE), immediately after (POST), 30 min post-SET (REC1) and 60 min post-SET (REC2). Heart rates were obtained by using a stethoscope by volunteers at the same time blood was being obtained by another volunteer. Plasma was analyzed for creatine kinase (CK), aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and ascorbate.

*Sampling and analysis.* During the 12 wk supplementation period, whole blood samples were obtained bi-monthly by venipuncture into EDTA coated blood collection tubes. At the beginning, middle, and end of the study, whole blood was submitted for the determination of a complete blood count (Virginia Maryland Regional College of Veterinary Medicine, Blacksburg, VA) including red blood cell (RBC) count, hemoglobin (HGB), hematocrit (HCT), mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), white blood cell (WBC) count, platelets, and fibrinogen. For the determination of red blood cell (RBC) folate, 100  $\mu$ l of whole blood was added to 1 ml of a 1.0 % (wt/vol) sodium ascorbate solution, mixed thoroughly to lyse cells, and stored. For the determination of white blood cell glutathione peroxidase (WBC GPx), the buffy coat was removed after centrifugation of whole blood at 2500 x g for 10 min. White blood cells were washed once in a lysis buffer (0.15 M  $\text{NH}_4\text{Cl}$ , 0.01 M  $\text{NaHCO}_3$ , 0.03 M EDTA free acid) to lyse RBC's, washed twice with in Hank's Balanced Salt Solution (HBSS, Life Technologies, Carlsbad, CA), resuspended in 1 ml HBSS, and stored. For the determination of plasma folate, whole blood was centrifuged at 2000 x g for 5 min to separate plasma. Plasma was transferred into polypropylene vials with added sodium

ascorbate for a final concentration of 0.3 % (wt/vol). Remaining plasma was stored for the determination of plasma vitamin E and homocysteine. All samples were kept out of direct light to prevent photooxidation of folate and were stored at  $-80^{\circ}\text{C}$  until analyzed.

Plasma and RBC were analyzed for total folate by radioimmunoassay (Folate Dualcount Solid Phase No Boil Assay, Diagnostic Products Corporation, Los Angeles, CA) as previously described in Manuscript 1. Plasma was diluted 1:4 and RBC was diluted 1:30 and deconjugated with chicken pancreas prior to analysis as in Manuscript 1. Plasma homocysteine was determined by reverse-phase HPLC with fluorescence detection using a commercially available kit (Homocysteine by HPLC, Bio-Rad Laboratories, Hercules, CA) as previously described in Manuscript 1. Vitamin E was analyzed following the method of Bieri et al. (1979) and Miller and Yang (1985).

Samples taken before, during and after the SET were analyzed for plasma CK, AST, and lactate by an automated clinical chemistry analyzer (Beckman Synchron Clinical System CX5, Beckman Instruments Inc., Fullerton, CA). Plasma ascorbate was analyzed by normal phase separation with ultraviolet detection using high-pressure liquid chromatography. The HPLC system was an 1100 Series LC 3D ChemStation from Agilent Technologies (Wilmington, DE) equipped with a temperature controlled autosampler, quaternary pump with degasser, ultraviolet/visible detector, and normal phase Lichrosorb Si 60, 5  $\mu\text{m}$  column (4.6 mm x 30 cm). In brief, 1.0 ml of plasma was added to 4.0 ml of 5.0 % metaphosphoric acid (vol/vol) and the mixture was vortexed and centrifuged at 2200 x g for 10 min. Two ml of clear upper layer was transferred to a 5.0 ml polypropylene tube, and 0.125 ml 0.5 N iodine solution, 0.125 ml 5.0 % thiourea solution (wt/vol), 0.250 ml 0.02 % 2,4-dinitrophenylhydrazine (wt/vol) and mixed well after each addition. The mixture was then stored overnight in the dark at room temperature. Formed derivatives were extracted by adding 1.0 ml of ethylacetate containing 2 % acetic acid and mixed well on a mechanical horizontal shaker for 2 min. The mixture was centrifuged at 2200 x g for 10 min at  $10^{\circ}\text{C}$ . The clear orange-colored organic phase was transferred to screw cap vials and placed in the autosampler. One hundred  $\mu\text{l}$  of the sample was injected at a flow rate of 1.0 mL/min with the UV detector set at an absorbance of 520 nm. The mobile phase contained 29.4 % ethyl acetate, 68.6 % methyl t-butyl ether and 2 % acetic acid. External

standardization was performed using five known concentrations of ascorbate between 0.8 and 50 µg/ml. The concentration of plasma ascorbate was quantified from its retention time and peak areas relative to known standards.

*Statistical analysis.* SAS for WINDOWS (Version 8, 1999; SAS Institute Inc, Cary, NC) was used for data tabulation and statistical analyses. Data were analyzed by analysis of variance (ANOVA) with repeated measures using the MIXED procedure with the REPEATED statement. An autoregressive covariate was used and the horse was entered as the subject. A Tukey-Kramer comparison was used to determine differences between wk for the variables tested. A paired T-test was used to determine differences between variables tested in SET 1 and SET 2 for each horse. A P-value of < 0.05 was chosen as the level of significance. Data are presented as means ± standard errors (SE).

## Results

Plasma folate, RBC folate, and plasma homocysteine are presented in Table 2. Oral supplementation of FA had no effect on any of the indices assessed to determine folate status. However, the 12 wk submaximal exercise regimen had a main effect on plasma folate, RBC folate, and plasma homocysteine ( $P < 0.05$ ). There was a 19 % decline in plasma folate concentrations at wk 2 compared to initial concentrations ( $P < 0.05$ ), which remained lower than initial values for the remainder of the study ( $P < 0.05$ ). Concentrations of RBC folate were unchanged until a decline occurred at 3 mo ( $P < 0.05$ ). Mean concentration of plasma homocysteine during the 12 wk experimental period was  $4.0 \pm 0.16$  µmol/l. Plasma homocysteine concentrations were stable from wk 0 and 8. There was a 34 % decline in plasma homocysteine concentrations from wk 10 to 12 ( $P < 0.05$ ).

Plasma vitamin E concentration and WBC GPx activity are shown in Table 2. There was no effect of oral supplementation of FA on either plasma vitamin E or WBC GPx activity. However, there was a main effect due to the 12 wk submaximal exercise regimen ( $P < 0.05$ ). Plasma vitamin E concentrations declined during the last 4 wk of the study compared to baseline concentrations ( $P < 0.05$ ). White blood cell GPx activity steadily increased from wk 0 to wk 10, with highest levels observed at 8 and 10 wk ( $P < 0.05$ ). After wk 10, activity of WBC GPx

declined by 62 % ( $P < 0.05$ ), such that levels at wk 10 were similar to initial values obtained at wk 0.

Complete blood count data for horses during the 12 wk experimental period are shown in Table 3. There were no differences observed for any of the variables due to the oral supplementation of FA. However, there was a trend for lower MCV ( $P = 0.097$ ), lower MCH ( $P = 0.14$ ), and higher platelet count ( $P = 0.07$ ) in horses supplemented with FA compared to CON horses. In response to the submaximal exercise regimen, RBC, HGB, and HCT steadily increased during wk 6 and 12 compared to values obtained at the beginning of the study ( $P < 0.05$ ). Decreases in MCH, MCV, and fibrinogen occurred at wk 12 compared to initial values ( $P < 0.05$ ). There were no changes in MCHC, WBC, or platelets during the experiment.

Variables assessed before and after horses completed SET's 1 and 2 are shown in Table 4. As expected, there were no treatment effects observed during SET 1 since the horses had not been assigned to the treatment. There were no differences detected between the CON and FA horses for any of the variables assessed in SET 2. Increases in HR and plasma lactate over resting concentrations were observed in SET 1 and 2 ( $P < 0.05$ ), which returned to resting levels within 30 min. Comparison of plasma lactate concentrations revealed that lower lactate concentrations occurred in all horses after completing SET 2 compared to SET 1 ( $P < 0.05$ ). Concentrations of plasma AST were elevated in horses after performing both SET 1 and 2 ( $P < 0.05$ ), which returned to resting values within 30 min. Plasma CK concentrations were unaffected in horses performing SET 1. However, CON horses had higher plasma CK concentrations at rest in SET 2 compared to their resting concentrations in SET 1 ( $P < 0.05$ ). The submaximal exercise performed in SET 1 and 2 did not influence plasma ascorbate concentrations. However, plasma ascorbate concentrations at several sampling times were higher for horses in SET 2 than when they performed the same exercise test in SET 1 ( $P < 0.05$ ).

## Discussion

The main objective of the present study was to determine if long-term routine oral supplementation of 25 mg of FA to horse engaged in routine submaximal exercise would have any benefits on folate status and on exercise performance during a submaximal SET. A secondary aim of the study was to assess whether oral FA decreased plasma homocysteine concentrations and whether the decline would be associated with a decline in oxidative stress. In the first long-term oral FA supplementation study in horses, we found that oral supplementation of 25 mg of FA 5 d/wk for 12 wk had no beneficial effects on folate status, antioxidant status, or performance during a submaximal exercise test.

One possible explanation why oral FA supplementation did not influence folate status in the present study may be that synthetic FA has a low bioavailability in the horse, contrary to what is observed in humans (Gregory, 2001). In the human, orally administered FA is absorbed and utilized (Bhandari and Gregory, 1992; Gregory, 2001), resulting in an increased folate status (Mackey and Picciano, 1999), lowered plasma homocysteine (Jaques et al., 1999) and a decreased incidence of folate deficiency related disorders (Butterworth and Bendich, 1996). A preliminary report in the horse indicated that absorption of oral FA was low (NRC, 1989) and preliminary work administering intramuscular FA to exercise trained horses observed a slow clearance of FA from the plasma (Roberts, 1983). The only study observing a beneficial effect of FA supplementation reported that one mature stabled gelding experiencing poor race performance associated with a low serum folate, low hemoglobin, alterations in nuclear structure of bone marrow, and poor body condition improved after 23 d of 20 mg of daily FA supplementation (no mode of administration reported) (Seckington et al., 1967). Despite the lack of information regarding the bioavailability of FA in the horse, early reports of low serum folate (Seckington et al., 1967; Allen, 1978) and low serum and red cell folate concentrations (Allen and Powell, 1983; Roberts, 1983) in stabled horses that were intensely exercised led to the routine oral supplementation of FA in most equine concentrates and vitamin supplements.

Studies regarding folate status in the exercising horse have focused mainly on horses in race training (Seckington et al., 1967; Allen, 1983; Roberts, 1983). We chose to investigate the role

of oral FA supplementation in horses in routine submaximal exercise since a large portion of the equine population is engaged in similar exercise activity. The horses in the present study participated in an average of  $4.0 \pm 0.6$  lessons/wk. It would have been ideal to record individual feed intakes of the horses to estimate folate intake, but one constraint of the study was that the experimental procedures had to complement the current management practices of the horses in the equitation program. However, the management practices in the present study are typical of current industry practices including offering hay and concentrate to horses that are stalled for a portion of the day followed by turn out in a fenced lot with limited pasture availability for grazing. Although oral FA supplementation had no effect on any variables assessed in the present study, the feeding and exercise management used in this study was associated with changes in folate status, antioxidant status, and performance during the SET.

The decline in plasma folate concentration after 2 wk and the moderate decline in RBC folate concentration at wk 12 indicated that folate status was lowered by the 3 mo submaximal exercise regimen. The decline in folate status was most likely caused by an increased folate requirement that was not met by consumption of folate in the diet or by utilization of microbially derived folate in the hindgut. An increased erythropoiesis during training has been suggested to increase the requirements of folate to support the cell turnover (Allen and Powell, 1983), which would be in agreement with our study since we observed increases in RBC and HCT levels during the 12 wk period.

There may be several possibilities why the diet failed to support folate status towards the end of the study. First, the high stocking density of the pasture and the low availability of pasture for grazing resulted in an increased proportion of hay in the diet. The concentration of folate in the hay was lower than the concentration of folate determined in the pasture at the start of the study. Previous reports indicate that stabled horses fed hay had a lower serum folate concentration than horses maintained on pasture (Seckington et al., 1967; Allen, 1978; Roberts, 1983). Also, the decline in RBC folate concentrations was observed in November coinciding with the beginning of the colder winter mo, which has been associated with lower folate status in horses in a previous report (Roberts, 1983).

Oral supplementation of FA did not influence plasma homocysteine concentrations in the present study. Homocysteine is a non-essential sulfur containing amino acid whose metabolism is catalyzed by folate, B<sub>12</sub>, and B<sub>6</sub> dependant enzymes (Selhub, 1999). Homocysteine is converted to methionine via methionine synthase, an enzyme that requires 5-mTHF as a methyl donor and vitamin B<sub>12</sub> as a co-factor. When the concentration of 5-mTHF is limited to the body, homocysteine accumulates within the cell and may leak out into the plasma. As a result, high levels of plasma homocysteine occur which is also known as hyperhomocysteinemia. Plasma homocysteine has been used as a functional indicator of folate status in humans because it is negatively correlated to dietary folate intake and plasma folate (Lewis et al., 1992; O'Keefe et al., 1995; Mackey and Picciano, 1999).

Low dose FA supplementation has been shown to reduce plasma homocysteine concentrations in humans (Brouwer et al., 1999; Jacques et al., 1999). In the present study, oral supplementation of FA did not have any effect on lowering plasma homocysteine concentrations due to the fact that homocysteine concentration in the plasma was already low ( $4.0 \pm 0.16$   $\mu\text{mol/l}$ ). The mean concentration of plasma homocysteine observed in this study was within the range of concentrations observed in lactating mares and foals (Manuscript 1, Ordakowski, 2001). By comparison, normal plasma homocysteine concentrations in the human are maintained between 5-15  $\mu\text{mol/l}$  (Selhub, 1999). Although folate status declined during the 12 wk submaximal exercise program, plasma homocysteine concentration did not increase. The decline in plasma homocysteine concentrations observed during the last wk of the study was likely due to the horses having an additional 1 wk rest period due to a University holiday.

Hyperhomocysteinemia has been associated with numerous folate deficiency related disorders including oxidative stress (Moat et al., 1999; Huang et al., 2001; McDowell and Lang, 2000). The exact mechanism by which hyperhomocysteinemia causes an increased oxidative stress is unknown, however, autoxidation resulting in an increased production of ROS (McDowell and Lang, 2000) and inhibition of the activity of GPx, and important antioxidant enzyme (Moat et al., 1999; Loscalzo, 1996) have been suggested. More recently, FA has been shown to have free radical scavenging activity *in vitro* (Joshi et al., 2001), which indicates a potential antioxidant role of FA in addition to promoting folate status.

Horses in moderate to heavy exercise have an increased risk of oxidative stress and subsequently performance due to the production of reactive oxygen species (ROS) during energy metabolism needed for continued muscle contraction (Hinchcliff et al., 1998; Witt et al., 1992; Ji, 1999). In the present study, we examined the concentration of the cellular antioxidant, vitamin E, and the WBC activity of the antioxidant enzyme, GPx, as indicators of oxidative stress. Vitamin E plays an important role in decreasing oxidative stress by scavenging free radicals produced during normal metabolism (Combs, 1992b), whereas GPx catalyzes a reaction that converts the pro-oxidant, hydrogen peroxide, to water (Hinchcliff et al., 1998).

In the present study, there was no effect of oral FA supplementation on either plasma vitamin E or WBC GPx activity during the 12 wk experimental period. However, we observed a decline in vitamin E concentrations during the last half of the study and an increase in WBC GPx activity throughout the study indicating an increased oxidant load was placed on the horse during the 12 wk submaximal exercise program. Similar to our reports, vitamin E concentrations declined (McMeniman and Hintz, 1992; McMeniman et al., 1995) and WBC GPx activity increased (Avellini et al., 1999) in horses engaged in prolonged race training. The mean vitamin E concentration observed for all horses in this study was  $2.1 \pm 0.07$   $\mu\text{g/ml}$  (range 1.0 to 3.8  $\mu\text{g/ml}$ ), which is similar to previous reports in clinically normal horses (Butler and Blackmore, 1983; Steis et al., 1994). The submaximal exercise used in this study may have increased the oxidant load on the horse by increasing production of reactive oxygen species (ROS) caused by an increased aerobic metabolism to support muscle contraction (Ji, 1999).

All of the hematological indexes assessed in the present study were within normal ranges reported in horses (Rose and Hodgson, 1994a). Although FA supplementation had no effect on any of the hematological variables tested, the submaximal exercise program resulted in a number of changes. The significant increase in RBC count, hemoglobin, and hematocrit over the 12 wk submaximal exercise period are normal hematological responses to exercise that have also been observed to occur in race-trained horses (Allen and Powell, 1983; Tyler-McGowan et al., 1999). The apparent increase in erythropoiesis may have contributed to the decline in folate status observed in the present study, which was similarly documented in race-trained horses (Allen and

Powell, 1983). The reason for the decline in fibrinogen concentration was unknown, but was not related to any clinical illness in the horses.

The SET's were conducted at the beginning and at the end of the experimental period in order to evaluate any potential benefits of FA supplementation on some indicators of submaximal exercise performance. The testing procedure was the same for all horses. Although there were no differences contributed to the 12 wk of oral FA supplementation, there were moderate increases in HR, lactate and AST after completion of the SET's indicating that the exercise was only submaximal at best. The lower lactate concentrations and higher plasma ascorbate changes observed in SET 2 compared to SET 1 indicated that there were changes associated with exercise conditioning over the 12 wk period. The lower lactate concentrations during SET 2 may be associated with an improvement in aerobic capacity due to the exercise-conditioning period (Miller and Lawrence, 1983; Thornton et al., 1983; Rose and Hodgson, 1994b).

The submaximal exercise during the SET's did not alter concentrations of plasma ascorbate, which is consistent with other reports when horses experienced more intense exercise tests that simulated a race (McMeniman et al., 1995). Ascorbate is an extracellular antioxidant that quenches free radicals and regenerates vitamin E (Ji, 1999). The increase in plasma ascorbate concentrations at various sampling periods in SET 2 compared to SET 1 may be due to an increased synthesis by the horses in response to the increased oxidative load during the course of the study.

An increase in the plasma concentrations of muscle enzymes such as CK and AST is often used as an indicator of muscle damage since levels of these enzymes are usually low in horses not experiencing clinical symptoms of muscle damage (e.g., exertional rhabdomyolysis). Similar responses of CK and AST to submaximal exercise have been reported in horses (Judson et al., 1983). A large response of the muscle specific enzymes CK and AST were not expected since the SET's were not designed to result in a maximal effort for the horses.

This is the first controlled study that investigated the effects of long-term effects of FA supplementation on folate status in horses performing daily submaximal exercise. This study

failed to demonstrate any significant effect of routine oral supplementation of 25 mg of FA on folate status, oxidative stress, and exercise performance during a submaximal SET. However, we did identify numerous alterations in the horse due to the 12 wk exercise-conditioning period including a decline in the antioxidant status of the horse.

### **Implications**

The common practice of supplementing FA to horses in low to moderate exercise regimens is a futile practice. The lack of a response of folate status to daily administration of 25 mg of FA indicates that FA has a low bioavailability in the horse. Future studies concerned with the folate nutriture in the horse should focus on evaluating alternative sources of folate that are effective in maintaining or increasing folate status in the exercising horse. Supplementation of antioxidants may be effective in combating the increased oxidative stress experienced by horses engaged in routine submaximally exercise.

## Tables

**Table 1.** Nutrient composition (DM basis) of pasture, hay, and concentrate offered to horses <sup>a,b</sup>

Item	Pasture	Hay	Reliance	Senior
CP, %	22.3	17.1	13.9	16.5
ADF, %	32.7	42.5	15.4	24.0
NDF, %	51.5	57.7	30.0	39.0
NSC, %	8.5	13.4	43.7	25.9
Fat, %	3.5	2.6	4.5	8.1
Ash, %	14.1	9.2	7.9	10.5
TDN, %	57.7	57	73	73
Ca, %	0.58	0.80	1.14	1.28
P, %	0.42	0.34	0.64	0.73
Mg, %	0.30	0.26	0.34	0.44
K, %	2.99	2.77	1.52	2.09
Na, %	0.01	0.02	0.46	0.58
Fe, ppm	208.0	201.0	241.0	447.0
Zn, ppm	28.0	19.0	111.0	218.0
Cu, ppm	11.7	8.0	33.0	51.0
S, %	0.28	0.19	0.31	0.35
Cl, %	1.08	0.71	0.80	1.03
Folate, ppm	3.1	2.2	3.80	3.90
DE, Mcal/lb	1.16	0.96	1.46	1.25

<sup>a</sup> Analysis by Dairy One, Ithaca, NY

<sup>b</sup> Pasture: mixed grass/legume, mean of samples from three field housing horses; Hay, orchardgrass/alfalfa mixed hay; Reliance, Reliance Textured 12 %, Southern States, Richmond, VA; Senior, Equine Senior, Southern States, Richmond, VA

**Table 2.** Plasma and RBC folate, plasma homocysteine, and white blood cell glutathione peroxidase in control horses (CON) and horses supplemented with folic acid (FA) during 12 wk of moderate exercise

Item	Week							SE
	0	2	4	6	8	10	12	
Plasma folate, ng/ml								
CON	22.1 <sup>a</sup>	16.6 <sup>a,b</sup>	19.6 <sup>a,b</sup>	16.5 <sup>a,b</sup>	16.9 <sup>a,b</sup>	15.1 <sup>b</sup>	16.7 <sup>a,b</sup>	1.33
FA	21.2	18.4	18.9	16.0	17.8	15.4	17.9	1.33
RBC folate, ng/ml								
CON	568.3		559.52		544.7		472.1**	29.1
FA	562.0		540.6		593.0		464.0**	29.1
Plasma homocysteine, $\mu$ mol/L								
CON	5.3 <sup>a</sup>	-	5.0 <sup>a</sup>	-	4.4 <sup>a</sup>	-	2.9 <sup>b</sup>	0.42
FA	4.2 <sup>a</sup>	-	4.1 <sup>a</sup>	-	3.8 <sup>a</sup>	-	2.5 <sup>b</sup>	0.42
Plasma Vitamin E, $\mu$ g/ml								
CON	2.6 <sup>a</sup>	2.4 <sup>a</sup>	2.4 <sup>a</sup>	2.3 <sup>a</sup>	2.0 <sup>b</sup>	2.1 <sup>a,b</sup>	2.0 <sup>a,b</sup>	0.17
FA	2.1 <sup>a,b</sup>	2.1 <sup>a</sup>	2.1 <sup>a</sup>	1.9 <sup>a,b</sup>	1.7 <sup>b</sup>	1.7 <sup>a,b</sup>	1.6 <sup>a,b</sup>	0.17
White blood cell glutathione peroxidase, U/g protein								
CON	3.1 <sup>a</sup>	4.3 <sup>a</sup>	6.7 <sup>a,b</sup>	5.5 <sup>a</sup>	9.3 <sup>b,c</sup>	10.6 <sup>c</sup>	3.9 <sup>a</sup>	0.76
FA	2.0 <sup>a</sup>	4.8 <sup>a,b</sup>	6.9 <sup>b</sup>	6.0 <sup>b</sup>	6.0 <sup>b</sup>	10.7 <sup>c</sup>	4.3 <sup>a,b</sup>	0.76

<sup>a,b,c</sup> Within a row, means with unlike superscripts differ ( $P < 0.05$ )

\*\* Main effect of wk ( $P < 0.05$ )

**Table 3.** Complete blood count data for control (CON) horses and horses supplemented with folic acid (FA) during 12 wk of moderate exercise

Item	Week			SE
	0	6	12	
Red blood cell, 10 <sup>12</sup> /L				
CON	7.0 <sup>a</sup>	7.2 <sup>a,b</sup>	7.7 <sup>b</sup>	0.28
FA	7.3 <sup>a</sup>	7.6 <sup>a,b</sup>	8.0 <sup>b</sup>	0.28
Hemoglobin, g/dl				
CON	12.4 <sup>a</sup>	12.9 <sup>a,b</sup>	13.4 <sup>b</sup>	0.40
FA	12.6 <sup>a</sup>	13.2 <sup>a,b</sup>	13.7 <sup>b</sup>	0.40
Hematocrit, %				
CON	34.3 <sup>a</sup>	36.2 <sup>a,b</sup>	37.1 <sup>b</sup>	1.15
FA	34.8 <sup>a</sup>	36.5 <sup>a,b</sup>	38.3 <sup>b</sup>	1.15
Mean cell volume, fl				
CON	49.2 <sup>a,b</sup>	50.1 <sup>a</sup>	48.5 <sup>b</sup>	0.77
FA	47.9	48.3	47.8	0.77
Mean cell hemoglobin, pg				
CON	17.8 <sup>a,b</sup>	17.9 <sup>a</sup>	17.5 <sup>b</sup>	0.29
FA	17.3 <sup>a,b</sup>	17.5 <sup>a</sup>	17.1 <sup>b</sup>	0.29
Mean cell hemoglobin conc., %				
CON	36.1	35.7	36.1	0.25
FA	36.1	36.2	35.8	0.25
White blood cell, x10 <sup>9</sup> /L				
CON	6.9	6.1	6.5	0.64
FA	7.3	6.9	7.1	0.64
Platelets, x10 <sup>9</sup> /L				
CON	155.3	161.1	120.1	13.8
FA	174.2	169.9	172.6	13.8
Fibrinogen, mg/dl				
CON	236.4 <sup>a,b</sup>	300.0 <sup>a</sup>	172.7 <sup>b</sup>	28.9
FA	236.4	236.4	181.8	28.9

<sup>a,b</sup> Within a row, means with unlike superscripts differ ( $P < 0.05$ )

**Table 4.** Heart rate (HR) and plasma lactate, aspartate aminotransferase (AST), creatine kinase (CK), and vitamin C in control (CON) and folic acid supplemented (FA) horses before (PRE), immediately after (POST), and at 30 min after (REC1) and 60 min after (REC2) performing standard exercise tests (SET) 1 and 2

Item	SET 1					SET 2				
	PRE	POST	REC1	REC2	SE	PRE	POST	REC1	REC2	SE
Heart rate, beats/min										
CON	37.2 <sup>a</sup>	80.7 <sup>b</sup>	44.1 <sup>a</sup>	38.1 <sup>a</sup>	2.5	32.8 <sup>a</sup>	83.1 <sup>b</sup>	45.2 <sup>c</sup>	41.0 <sup>a,c</sup>	2.8
FA	33.2 <sup>a</sup>	75.8 <sup>b</sup>	44.0 <sup>a</sup>	37.7 <sup>a</sup>	2.5	34.8 <sup>a</sup>	92.0 <sup>b,**</sup>	42.7 <sup>a</sup>	38.0 <sup>a</sup>	2.8
Plasma lactate, mmol/L										
CON	0.75 <sup>a</sup>	2.0 <sup>b</sup>	0.88 <sup>a</sup>	0.72 <sup>a</sup>	0.17	0.39 <sup>a,**</sup>	0.90 <sup>b,**</sup>	0.37 <sup>a,**</sup>	0.45 <sup>a,*</sup>	0.12
FA	0.75 <sup>a</sup>	1.6 <sup>b</sup>	0.77 <sup>a</sup>	0.68 <sup>a</sup>	0.17	0.46 <sup>a,b,**</sup>	0.93 <sup>a,b</sup>	0.44 <sup>a</sup>	0.43 <sup>a,**</sup>	0.12
Plasma CK, IU/L										
CON	294.6	398.0	382.4	321.3	49.5	433.8 <sup>**</sup>	464.4	460.6	359.8	75.2
FA	405.7	328.9	335.7	373.6	49.5	508.4	384.2	459.3	478.5	75.2
Plasma AST, IU/L										
CON	273.9 <sup>a</sup>	299.5 <sup>b</sup>	290.0 <sup>a,b</sup>	271.9 <sup>a,b</sup>	21.8	250.4 <sup>a</sup>	266.6 <sup>b</sup>	248.3 <sup>a</sup>	250.1 <sup>a,b</sup>	11.0
FA	254.2	268.6	258.2	261.1	21.8	283.9	290.0	280.8	285.4	11.0
Plasma ascorbate, ug/ml										
CON	0.79	0.86	0.86	1.1	0.11	1.2 <sup>a,*</sup>	1.4 <sup>b,**</sup>	1.4 <sup>b,**</sup>	1.3 <sup>a,b</sup>	0.20
FA	0.80	0.76	0.77	0.88	0.11	1.18 <sup>*</sup>	1.3	1.3 <sup>**</sup>	1.2	0.20

<sup>a,b,c</sup> Within a row, means with unlike superscripts differ ( $P < 0.05$ )

<sup>\*\*</sup> Means differ between SET 1 and 2 ( $P < 0.05$ )

<sup>\*</sup> Means differ between SET 1 and 2 ( $P < 0.10$ )

## CHAPTER V

### **Manuscript 3. Pyrimethamine and sulfadiazine lowers plasma folate and increases plasma homocysteine in horses**

**ABSTRACT:** A study was conducted to evaluate effects of oral administration of pyrimethamine (PYR) and sulfadiazine (SDZ) for 9 wk followed by 6 wk of coadministration of either Peptidoglycan (PG) or folic acid (FA) on folate status and hematological indexes in eight healthy mature Thoroughbred geldings ( $583.3 \pm 20.1$  kg initial BW) at maintenance. Geldings were maintained on a 0.5-acre dry lot with an adjacent shed, and were fed 1 to 2 yr old orchardgrass/alfalfa hay to maintain their initial BW. During Period 1, each horse was orally administered 1mg/kg BW of PYR and 20mg/kg BW SKZ once daily for 9 wk. For the remaining 6 wk, horses were maintained on the same drug regimen and were paired by age and BW and randomly assigned to either once daily oral administration of 20 mg FA or 35 g of PG as a source of formylated folate derivatives. Body wt, rectal temperature, and blood were obtained weekly. Hematological indexes assessed were variable, but were within normal limits for horses during both periods. After 1 wk of PYR/SDZ administration, plasma folate concentrations decreased 59 % compared to baseline levels ( $P < 0.05$ ). Folate status was impaired during 9 wk of PYR/SDZ administration as determined by a decreased plasma folate concentration ( $P < 0.05$ ) and an increased plasma homocysteine concentration ( $P < 0.05$ ). During Period 2, coadministration of either PG or FA were not effective in preventing further decline of plasma folate and increases in plasma homocysteine. Despite a lowered folate status in the horses, no abnormal hematological indexes were reported indicating clinical anemia did not occur in these horses as a result of PYR and SDZ administration. A moderate case of hyperhomocysteinemia occurred as a result from an impaired folate status, but not from lowered B<sub>12</sub> status in our horses during the experimental period. The stable concentrations of plasma B<sub>12</sub> indicate that administration of PYR and SDZ did not sufficiently impair microbial synthesis of that B-vitamin. These data indicate that 15 wk of oral daily administration of 0.1 mg/kg BW PYR and 20 mg/kg BW SDZ impaired folate status in the horse resulting in moderate hyperhomocysteinemia. In addition, 6 wk oral coadministration of either 35 g of PG or 20 mg of FA was not effective in preventing a further decline in folate status.

**Key Words:** Folate, horse, pyrimethamine, sulfadiazine, homocysteine

## Introduction

Pyrimethamine (PYR) is a dihydrofolate reductase (DHFR) inhibitor shown to be teratogenic in many species including rats (Izumi et al., 1984; Kudo et al., 1988; Chung et al., 1993), hamsters (Sullivan and Takacs, 1971), pigs (Misawa et al., 1982; Yamamoto et al., 1985), dogs (Castles et al., 1971), and humans (Harpey et al., 1983). The failure of dihydrofolate to be further reduced by DHFR to tetrahydrofolate results in decreased circulatory levels of 5-methyltetrahydrofolate (Tsunematsu et al., 1990) and limited availability of folate derivatives for one-carbon metabolism imperative for normal DNA and protein synthesis.

Although coadministration of folic acid (FA) was once considered to decrease toxic effects of PYR, FA potentiates the effects of the drug, further impairing folate homeostasis and increasing the incidence of teratogenicity (Kudo et al., 1988; Hayama et al., 1991; Chung et al., 1993). Recently, pregnant mares treated for Equine Protozoal Myeloencephalitis (EPM) with daily oral dosing of PYR and sulfadiazine (SDZ) or sulfamethoxazole, FA, and vitamin E produced foals with congenital defects, most likely caused by impaired maternal folate status (Toribio et al., 1998). Since then, the general practice of coadministration of PYR, sulfonamides and FA in horses has been strongly discouraged.

Intraperitoneal administration of the reduced formylated folate derivative, 5-formyltetrahydrofolate (5-fTHF), was effective in preventing a decline in folate status, thus lowering the incidence of PYR induced teratogenic effects compared to animal coadministered PYR and FA (Kudo et al., 1988; Tsunematsu et al., 1990; Chung et al., 1993). Oral administration of Peptidoglycan, an alternative folate source containing formylated derivatives, was found to increase plasma folate concentrations to a higher degree than oral administration of FA pigs (Mizuno et al., 1997).

Therefore, the objective of this study was to assess folate status and hematological indexes in horses after long-term administration of PYR and SDZ alone or with coadministration of either Peptidoglycan or FA.

## Materials and Methods

*Animals and diet.* Eight mature Thoroughbred geldings ( $586.3 \pm 20.1$  kg initial BW) from 6 to 14 yr old were maintained on a mixed grass/white clover pasture for at least 4 mo prior to the start of the study and were had been offered orchardgrass/alfalfa hay during winter mo. At the start of the study, horses were removed from pasture and placed on a 0.5-acre dry lot with an adjacent run in shed and an automatic water source. Horses were fed 1 to 2-yr old orchardgrass/alfalfa hay harvested from the Virginia Tech M.A.R.E. Center and the hay ration was adjusted weekly to maintain initial BW. Hay was offered in flakes on the ground to all eight horses at 0900 and 1700 h. Estimates of daily hay intake range from 20 to 25 lb/d. Samples of hay were taken three times during the study by using a hay core. Wet samples (approx. 1.0 kg) were weighed, dried in a forced air oven, reweighed for determination of DM, and then ground through a 0.5 mm screen Wiley Mill (Model 4, Thomas Scientific, Swedesboro, NJ). Dried hay samples were composited and subsamples were analyzed to determine concentration of total folate by microbial assay (Ralston Analytical Laboratories, Saint Louis, MO). Hay nutrient composition and folate concentration is shown in Table 1. The protocol was approved by the Institutional Animal Care and Use Committee.

*Experimental procedures.* Period 1 consisted of a 9 wk drug administration period during which each horse received once daily oral doses of PYR (1.0 mg/kg BW q 24h) and SDZ (20 mg/kg BW q 24 h) purchased from Wedgewood Pharmacy (Sewell, NJ). Drugs were mixed in approximately 0.2 kg of a corn/soybean meal/oat straw based horse feed as the carrier with molasses as the binder and orally administered once daily at 0800 h. To test the efficacy of two different folate sources on repleting folate stores, horses were paired by age and BW and randomly assigned to 6 wk of once daily oral administration of 35 g of Peptidoglycan® (PG, Ajinomoto Co., Inc., Tokyo, Japan) or 20 mg of synthetic FA (Rovimix® Folic 80 SD, Roche Vitamins Inc., Parsippany, NJ) during Period 2. The amount of PG was calculated to provide 20 mg of formylated folate derivatives. Each folate source was mixed into the same horse feed used for the drugs and orally administered at 1700 h. Concentrated apple juice was added to mask the salty taste of the PG and prevent refusals. Body wt was recorded weekly to adjust hay intake and maintain a constant BW throughout the trial. Weekly rectal temperatures were monitored as a

potential early indicator of poor health. In addition, horses were observed twice daily for any behavioral changes resulting from drug administration. Weekly samples of whole blood (30 ml) were collected by venipuncture into EDTA coated tubes (Fisher Scientific, Pittsburgh, PA). Twenty ml of whole blood was centrifuged at 2000 x g for 5 min to separate plasma. Plasma was transferred into polypropylene vials and sodium ascorbate was added to a final concentration of 0.3 % (wt/vol). Every 2 wk, 10 ml of whole blood was submitted for a complete blood count with differential (Marion Dupont Scott Equine Medical Clinic, Leesburg, VA). All biological samples were kept out of direct light to prevent photooxidation of folate and were stored at – 80°C until analyzed.

*Blood analysis.* Plasma was analyzed for total folate and B<sub>12</sub> by radioimmunoassay (Folate Dualcount Solid Phase No Boil Assay, Diagnostic Products Corporation, Los Angeles, CA) following methods previously described in Manuscript 1. Plasma homocysteine was determined using a pre-column derivatization procedure followed by reverse-phase separation and fluorescence detection using high-pressure liquid chromatography as described previously in Manuscript 1.

*Statistical analysis.* SAS for WINDOWS (Version 8, 1999; SAS Institute Inc, Cary, NC) was used for data tabulation and statistical analyses. Data were analyzed by analysis of variance (ANOVA) with repeated measures using the MIXED procedure with the REPEATED statement and horse as the subject. Time was used in the model statement to test for differences between variables during wk 0 to 9 during Period 1. Time, treatment and their interaction were used in the model statement to test for differences in variables due to treatment during wk 9 to 15. The covariance structure chosen was either unstructured, compound symmetry, or autoregressive and was selected based on the largest Akaike's Information Criterion determined by SAS. A Tukey's test was used to test for differences between weeks for variables tested. During wk 7 and 8, data for BW were not collected due to mechanical problems with the scale. Therefore, BW data collected during wk 0 to 6 and also wk 9 to 15 were analyzed using model statements discussed previously. A P-value of 0.05 was chosen as the level of significance. Data are presented as mean ± standard error (SE) unless otherwise stated.

## Results

### *Period 1*

Weekly BW and rectal temperatures obtained during Period 1 are shown in Table 2. Mean initial BW for the eight horses was  $586.3 \pm 20.1$  kg. Body wt of horses remained constant during the first 5 wk after which horses lost an average of 56.9 kg from wk 5 to 6 ( $P < 0.05$ ). There were no effect of drug administration on rectal temperature during Period 1.

Plasma folate and B<sub>12</sub> concentrations during Period 1 are shown in Figure 1. There was a significant effect of time on plasma folate levels during 9 wk of PYR and SDZ administration ( $P < 0.05$ ). One wk of oral PYR/SDZ administration resulted in a 59 % reduction in plasma folate concentrations ( $P < 0.05$ ), which remained lowered than baseline levels during wk 2 and 3 ( $P < 0.05$ ). However, there was an increase in plasma folate concentrations at wk 3 and wk 4 ( $P < 0.05$ ). Plasma folate concentrations once again declined after wk 4 ( $P < 0.05$ ) to levels similar to those obtained during wk 2 and 3. There was no effect of PYR/SDZ administration on plasma B<sub>12</sub> concentrations during Period 1. Plasma homocysteine concentration obtained during Period 1 is shown in Figure 2. Plasma homocysteine was stable from wk 0 and 5, but increased above initial concentrations during wk 5 and 9 ( $P < 0.05$ ). One horse (#133) had abnormally high values of plasma homocysteine noted in Figure 2, which was removed from the statistical analysis after confirmation of a Z test.

Hematological indexes assessed during Period 1 are shown in Table 3. Administration of PYR/SDZ over the 9 wk period had a main effect on all of the indexes tested ( $P < 0.05$ ) with the exception of fibrinogen. Values for WBC, RBC, and hemoglobin were highest at wk 2 ( $P < 0.05$ ), whereas platelet count was highest at 6 wk ( $P < 0.05$ ). The MCV declined at wk 6 ( $P < 0.05$ ) followed by an increase over baseline levels at 8 and 9 wk in response to PYR/SDZ administration ( $P < 0.05$ ). Decreases occurred in hematocrit at wk 6 and 9, whereas decreases in MCHC declined slowly from baseline to 9 wk ( $P < 0.05$ ).

### *Period 2*

There were no differences due to either PG or FA treatment between any of the variables assessed during Period 2, thus all data were combined and presented for all eight horses. Horses

maintained BW throughout Period 2 (Table 3). Rectal temperatures were affected by the PYR/SDZ administration during Period 2 ( $P < 0.05$ ) with the highest rectal temperatures occurring at wk 13 ( $P < 0.05$ ) (Table 3).

Plasma folate concentrations declined further during Period 2 despite coadministration with either PG or FA ( $P < 0.05$ ) (Figure 1). There was no effect of either PG or FA on plasma folate concentrations during Period 2. No effect of folate and PYR/SDZ coadministration on plasma B<sub>12</sub> concentrations was observed during Period 2. Plasma homocysteine concentrations remained stable between wk 9 and 12 wk, followed by an increase at wk 15 ( $P < 0.05$ ) (Figure 2). There was no effect of either PG or FA on plasma homocysteine concentrations during Period 2. Administration of PYR/SDZ resulted in a decline of plasma B<sub>12</sub> only at wk 14 ( $P < 0.05$ ).

Hematological indexes assessed during Period 2 are presented in Table 3. There was no main effect due to treatment of either PG or FA throughout the 6 wk coadministration period. The 6 wk of PYR/SDZ and folate coadministration did not affect red blood cell, hemoglobin, hematocrit, mean cell volume, mean cell hemoglobin, or MCHC. The levels of WBC were highest at 12 wk compared to all other wk ( $P < 0.05$ ). Platelet counts were variable during Period 2 with lowest values at wk 10 ( $P < 0.05$ ) and highest values at wk 15 ( $P < 0.05$ ).

## Discussion

The objective of this study was to determine effects of the antifolate drugs, PYR and SDZ, on folate status in the horse, and whether coadministration of either PG or FA would ameliorate or potentiate the antifolate effects of the drugs. Folate status was impaired in the horse after daily oral administration of 0.1 mg/kg of PYR and 20 mg/kg SDZ during a 9 wk period indicated by a lowered plasma folate concentration and increased plasma homocysteine concentration. In addition, neither 35 g of PG nor 20 mg of FA were effective in countering the decline in folate status due to PYR and SDZ administration during the 6 wk coadministration period.

Over the entire 15 wk, mean dose of PYR and SDZ administered was 586 mg and 12 g of SDZ, respectively. The PYR and SDZ dose and length of administration used in this study were

similar to those commonly used for treatment of EPM in horses (Dubey et al., 2001; Toribio et al., 1998). Toribio et al. (1998) reported three cases of pregnant mares that had been treated for EPM with daily coadministration of PYR, sulfonamides, vitamin E, and 40 mg of FA that resulted in congenital birth defects in their foals. Low serum folate between 0.4 and 5.2 ng/ml, abnormal clinical blood chemistries, and histopathological diagnoses clearly indicated that a folate deficiency had developed in the foal caused by an apparent low folate status in the dam. Teratogenic effects of PYR have been shown to occur at doses between 1.2 and 2.7 mg/kg/d (Chung et al., 1993) in rats and 1.8 to 3.6 mg/kg/d in pigs (Misawa et al., 1982). Thus, it appears that teratogenicity of PYR occurs at lower doses in the horse.

Coadministration of PYR and SDZ alone during Period 1 resulted in an impaired folate status indicated by the 51 % decline in plasma folate concentration and 53 % increase in plasma homocysteine. Despite the impaired folate status, all of the hematological variables assessed were within normal limits for horses. There was a significant increase in mean cell volume during the wk 8 and 9. Macrocytic anemia due to folate deficiency is associated with numerous hematological changes including increased mean cell volume, mean cell hemoglobin concentration, and neutrophil segmentation, and lowered hemoglobin (Herbert and Das, 1984). Although drug administration lowered folate status during Period 1, it was not sufficient to cause a clinical disorder.

Plasma homocysteine is negatively correlated with plasma folate in humans (Selhub, 1999) and is thus used as a functional indicator of folate status in that species. Homocysteine (2-amino-4-mercaptobutanoic acid) is a nutritionally non-essential sulfur-containing amino acid that serves as an intermediate in methionine metabolism or it can be converted to cystathionine by the trans-sulfuration pathway. When the availability of 5-mTHF is limiting due to impaired folate status, methylation of homocysteine to methionine does not occur. This results in the accumulation of homocysteine within the cell, which will subsequently leak into plasma increasing plasma homocysteine concentrations.

In humans, normal plasma homocysteine levels are maintained between 5-15  $\mu\text{mol/l}$ , but increase to 15 to 30  $\mu\text{mol/l}$  in cases of moderate hyperhomocysteinemia, 30 to 100  $\mu\text{mol/l}$  in

cases of intermediate hyperhomocysteinemia, and  $> 100 \mu\text{mol/l}$  in those individuals with severe hyperhomocysteinemia (Selhub, 1999). In the present study, plasma homocysteine increased from baseline levels of  $8.9 \mu\text{mol/L}$  to  $18.0 \mu\text{mol/L}$  after 9 wk of PYR/SDZ administration followed by a further increase to  $26.1 \mu\text{mol/L}$  after 6 wk of coadministration of PYR/SDZ and either PG or FA. Moderate declines in plasma folate were not associated with increases in plasma homocysteine in lactating mares during early and late lactation (Manuscript 1, Ordakowski, 2001) and in moderately exercising horses (Manuscript 2, Ordakowski, 2001). Therefore, plasma homocysteine may only respond to more severe declines in folate as observed in the present study due to administration with anti-folate drugs.

Care must be taken when attributing hyperhomocysteinemia to folate deficiency, because it can also be influenced by vitamin B<sub>12</sub> and B<sub>6</sub> status (Selhub, 1999). The stable concentrations of plasma B<sub>12</sub> throughout both periods in this study indicate that microbial synthesis of B<sub>12</sub> was adequate to maintain B<sub>12</sub> status and that it did not influence plasma homocysteine. Although B<sub>6</sub> status was not assessed in this study, it is likely that microbial synthesis of B<sub>6</sub> was also adequate such that it did not influence homocysteine metabolism.

Plasma homocysteine concentrations in one horse were considerably higher than the mean value of the eight horses (Figure 2). Despite plasma homocysteinemia concentrations peaking at  $58.1 \mu\text{mol/L}$  at wk 15, the gelding had plasma folate or B<sub>12</sub> concentrations within the range of the other horses. It may be possible that the hyperhomocysteinemia observed in the one gelding was caused by a genetic defect in an enzyme in folate and/or homocysteine metabolism similar to those reported in humans (Selhub, 1999). In a previous study (Manuscript 1), a lactating mare had abnormally high levels of plasma homocysteine although her folate status was normal compared to other grazing lactating mares. These rare incidents give rise to questions whether a possible genetic defect in genes that encode for enzymes in the homocysteine metabolism may exist.

Numerous studies have observed that oral FA potentiated the effects of PYR by impairing folate status even further, but that coadministration of intraperitoneal 5-fTHF ameliorated the antifolate effects of the drugs in rats (Sullivan and Takacs, 1971; Hayama et al., 1991; Chung et

al., 1993) and dogs (Castles et al., 1971). In our study, coadministration of PYR/SDZ with either PG or FA during Period 2 was associated with a further decline in folate status. Despite plasma folate concentrations reaching 6.0 ng/ml at wk 14 and 15 with four horses having levels < 6.0 ng/ml during both wk, none of the hematological indexes were out of ranges considered normal for horses. However, intermittent diarrhea occurred during wk 13 to 15 although specific horse(s) could not be ascertained, which may have been attributable to impaired folate status, since intestinal cells require folate to support rapid turnover. Several horses became more irritable and difficult to handle during sampling as the study progressed. One individual horse displayed signs of discomfort, lethargy, and ataxia from wk 13 to the end of the study.

In order for FA to become utilized in the body, DHFR must reduce the compound to dihydrofolate and then further to tetrahydrofolate (Brody, 1991). Pyrimethamine inhibits the reduction of dihydrofolate to tetrahydrofolate by DHFR resulting in a decreased availability of folate for one-carbon metabolism, and the reduction of FA to dihydrofolate by the same enzyme. Therefore, FA is made unavailable to the animal. In addition, FA disturbs normal folate homeostasis by competing with bile-derived folate from enterohepatic circulation thus limiting their reabsorption (Kudo et al., 1995; Bhandari and Gregory, 1992).

The coadministration of 5-fTHF with PYR instead of FA in the horse has potential benefits because 5-fTHF is readily absorbed and converted to 5-mTHF for utilization in man without the need for DHFR (Nixon and Bertino, 1972; Bhandari and Gregory, 1992). For instance, humans orally supplemented with 15 mg of 5-fTHF had increases in plasma 5-mTHF concentrations over 6 h with peaks at 2 h indicating effective absorption and conversion of the formylated form (Mehta et al., 1978). We chose to explore the effectiveness of PG in the horse due to its high concentration of formylated folate as well as its potential to provide an easy carrier and additional stability of the folate. Peptidoglycan, derived from the cell wall of non-pathogenic bacteria has been shown to increase plasma folate concentrations in swine after oral administration (Mizuno et al., 1997).

Despite the potential benefits of PG coadministration, 35 g of PG containing approximately 20 mg of 5-fTHF did not counter the antifolate effects of PYR and SDZ administration. Reasons

why the PG could not be utilized are unknown. It is possible that PYR or SDZ interfered with PG absorption or utilization. The observation of intermittent diarrhea experienced by one or more horses throughout the study may indicate PYR/SDZ administration impaired intestinal function and absorption.

### **Implications**

Horses administered PYR and SDZ at the doses used in this study may be at risk of an impaired folate status that will not be improved by FA or PG supplementation. Additional sources of folate along with their mode of administration need to be further explored as effective means of maintaining folate status in horses administered PYR and SDZ.

## Tables

**Table 1.** Nutrient composition (DM basis) of orchardgrass/alfalfa hay fed to geldings <sup>a,b</sup>

Item	Hay (n=2)
CP, %	16.1 ± 0.41
ADF, %	35.1 ± 1.6
NDF, %	48.6 ± 0.78
NSC, %	25.3 ± 0.29
Fat, %	2.0 ± 0.29
Ash, %	7.7 ± 0.09
Ca, %	1.1 ± 0.04
P, %	0.29 ± 0.05
Mg, %	0.21 ± 0.01
K, %	2.2 ± 0.17
Na, %	0.009 ± 0.002
Fe, ppm	368.0 ± 187.0
Zn, ppm	31.0 ± 8.2
Cu, ppm	8.0 ± 0.82
Mn, ppm	41.5 ± 7.8
S, %	0.21 ± 0.03
Folate, mg/kg	1.4 ± 0.07
DE, Mcal/kg	2.3 ± 0.07

<sup>a</sup> Analyzed by DHI Forage Testing Laboratory (Ithaca, NY).

<sup>b</sup> Data are summarized on a DM basis as means SE's

**Table 2.** Body weight, rectal temperature, and hematological indexes in geldings administered Pyrimethamine and Sulfadiazine during Period 1 <sup>a,b</sup>

Item	Week										SE
	0	1	2	3	4	5	6	7	8	9	
Body wt <sup>b</sup> , kg	586.3 <sup>c</sup>	597.1 <sup>c</sup>	598.6 <sup>c</sup>	593.1 <sup>c</sup>	592.3 <sup>c</sup>	594.6 <sup>c</sup>	537.7 <sup>d</sup>	-	-	-	19.7
Rectal temperature, °F	98.7	98.2	98.2	98.1	98.5	98.0	98.3	97.8	98.0	98.0	0.23
White blood cell, 10 <sup>9</sup> /l	7.9 <sup>c</sup>	-	9.0 <sup>d</sup>	-	7.6 <sup>c</sup>	-	7.6 <sup>c</sup>	-	7.0 <sup>c</sup>	7.1 <sup>c</sup>	0.56
Red blood cell, 10 <sup>9</sup> /l	7.2 <sup>c</sup>	-	8.1 <sup>d</sup>	-	7.3 <sup>c,d</sup>	-	7.2 <sup>c</sup>	-	6.7 <sup>c</sup>	7.2 <sup>c</sup>	0.30
Hemoglobin, g/dl	12.4 <sup>c,d</sup>	-	13.7 <sup>d</sup>	-	12.4 <sup>c,d</sup>	-	12.1 <sup>c,e</sup>	-	11.5 <sup>c</sup>	12.4 <sup>d,e</sup>	0.56
Hematocrit, %	35.4 <sup>c</sup>	-	40.1 <sup>c</sup>	-	35.9 <sup>c</sup>	-	34.9 <sup>d</sup>	-	33.6 <sup>d</sup>	36.4 <sup>c</sup>	1.30
Mean cell volume, fl	49.4 <sup>c</sup>	-	49.4 <sup>c</sup>	-	49.2 <sup>c</sup>	-	48.9 <sup>d</sup>	-	50.2 <sup>e</sup>	50.4 <sup>e</sup>	0.67
Mean cell hemoglobin, pg	17.4 <sup>c</sup>	-	17.0 <sup>c,d</sup>	-	17.0 <sup>d,e</sup>	-	16.9 <sup>c</sup>	-	17.2 <sup>c,e</sup>	17.2 <sup>c,d</sup>	0.29
Mean cell hemoglobin concentration, g/dl	35.2 <sup>c</sup>	-	34.3 <sup>c,d,e</sup>	-	34.5 <sup>c,d,e</sup>	-	34.5 <sup>d</sup>	-	34.2 <sup>d,e</sup>	34.0 <sup>e</sup>	0.20
Platelets, 10 <sup>9</sup> /l	165.6 <sup>c</sup>	-	151.4 <sup>c</sup>	-	168.1 <sup>c</sup>	-	186.0 <sup>d</sup>	-	170.4 <sup>c,d</sup>	145.9 <sup>c,d</sup>	11.90
Fibrinogen, g/l	312.5	-	287.5	-	287.5	-	237.5	-	237.5	287.5	41.30

<sup>a</sup> Pyrimethamine (1.0 mg/kg BW q 24h) and sulfadiazine (20 mg/kg BW q 24 h)

<sup>b</sup> Body wt not recorded during wk 7 and 8 due to mechanical problem with scale.

<sup>c,d,e</sup> Within a row, means with unlike superscripts differ ( $P < 0.05$ ).

**Table 3.** Body weight, rectal temperature, and hematological indexes in geldings coadministered Pyrimethamine and Sulfadiazine and either Peptidoglycan® or folic acid during Period 2 <sup>a,b</sup>

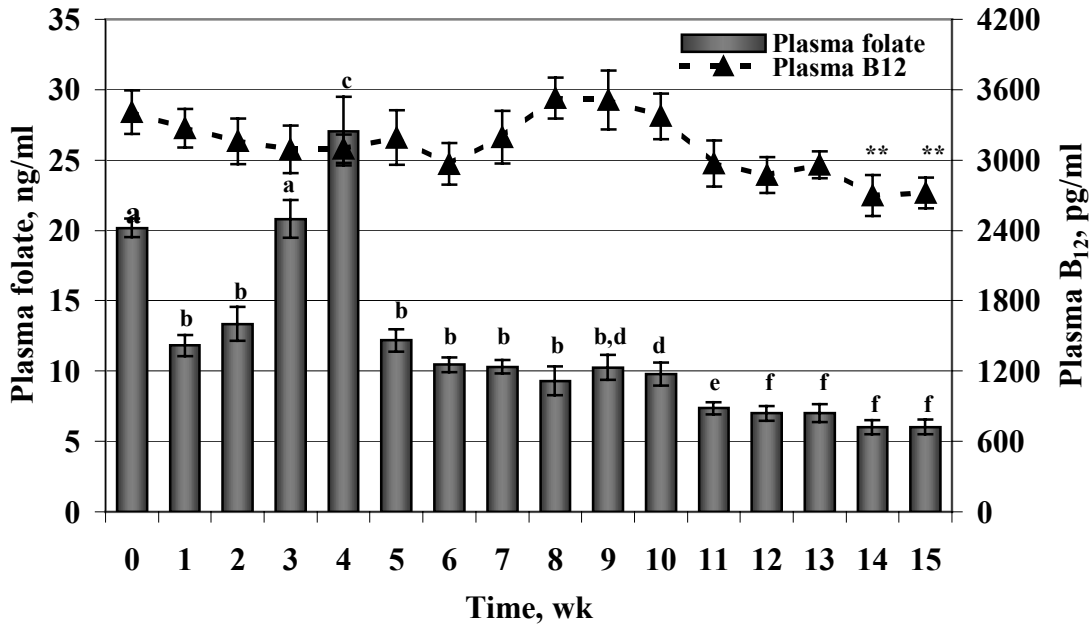
Item	Week							SE
	9	10	11	12	13	14	15	
Body wt <sup>b</sup> , kg	590.0	581.5	584.8	584.5	591.6	583.4	581.1	20.7
Rectal temperature, °F	98.0 <sup>c,d</sup>	97.4 <sup>c</sup>	98.0 <sup>c,e</sup>	97.3 <sup>c</sup>	98.7 <sup>d,e</sup>	98.1 <sup>c,d</sup>	98.1 <sup>c,d</sup>	0.25
White blood cell, 10 <sup>9</sup> /l	7.1 <sup>c,d</sup>	7.2 <sup>c,d</sup>	7.2 <sup>c,d</sup>	8.6 <sup>d</sup>	7.6 <sup>d</sup>	7.7 <sup>d</sup>	7.0 <sup>d</sup>	0.62
Red blood cell, 10 <sup>9</sup> /l	7.2	7.6	7.1	8.2	7.7	7.4	6.9	0.37
Hemoglobin, g/dl	12.4	12.8	12.2	13.9	13.5	12.8	12.1	0.60
Hematocrit, %	36.4	38.5	35.9	41.5	39.3	38.2	35.8	1.90
Mean cell volume, fl	50.4	50.7	50.8	51.1	51.0	51.4	51.8	0.70
Mean cell hemoglobin, pg	17.2	16.9	17.3	17.1	17.2	17.3	17.5	0.26
Mean cell hemoglobin concentration, g/dl	34.0	33.4	34.0	33.5	33.7	33.7	30.1	1.40
Platelets, 10 <sup>9</sup> /l	145.9 <sup>c,d,e</sup>	165.4 <sup>c,d,e</sup>	136.1 <sup>d</sup>	178.1 <sup>e</sup>	176.8 <sup>c,d,e</sup>	151.8 <sup>d</sup>	190.5 <sup>e</sup>	11.30
Fibrinogen, g/l	287.5 <sup>c,e</sup>	175.0 <sup>d</sup>	237.5 <sup>e</sup>	250.0 <sup>e</sup>	262.5 <sup>e</sup>	212.5 <sup>e</sup>	250.0 <sup>e</sup>	42.70

<sup>a</sup> Pyrimethamine (1.0 mg/kg BW q 24h) and sulfadiazine (20 mg/kg BW q 24 h) coadministered with either Peptidoglycan® (PG, 0.1 g/kg BW) or folic acid (FA, 0.05 mg/kg BW)

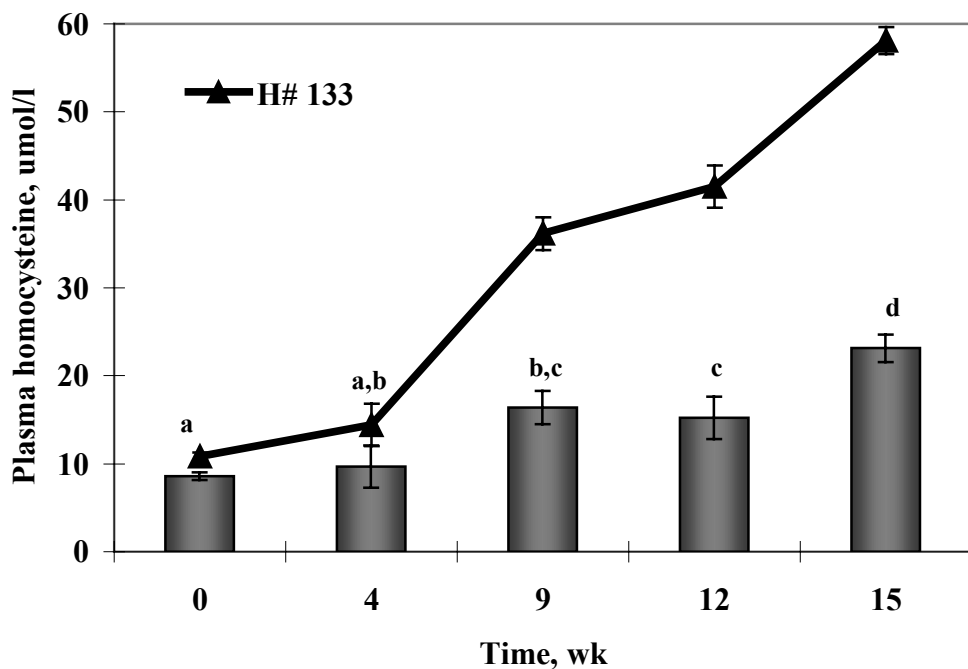
<sup>b</sup> Data were combined for PG and FA horses because no treatment effects were observed.

<sup>c,d</sup> Within a row, means with unlike superscripts differ ( $P < 0.05$ )

## Figures



**Figure 1.** Plasma folate (ng/ml) and B<sub>12</sub> (pg/ml) in geldings administered Pyrimethamine (1.0 mg/kg BW q 24h) and sulfadiazine (20.0 mg/kg BW q 24h) for 15 wk and orally dosed with either Peptidoglycan ® (PG) or folic acid (FA) during the last 6 wk. <sup>a,b,c</sup> Means with unlike superscripts differ ( $P < 0.05$ ). <sup>d,e,f</sup> Means with unlike superscripts differ ( $P < 0.05$ ). \*\* Denotes differences in plasma B<sub>12</sub> levels compared to concentrations at wk 9 through 13 in Period 2 ( $P < 0.05$ ).



**Figure 2.** Plasma homocysteine in geldings administered Pyrimethamine (1.0 mg/kg BW q 24h) and Sulfadiazine (20.0 mg/kg BW q 24h) during Period 1 (wk 0 to 9) and then coadministered drugs with either Peptidoglycan® (PG) or folic acid (FA) during Period 2 (wk 9 to 15). Bars represent mean of seven horses. Solid line represents data from one horse that was withdrawn from analysis after a Z-test. <sup>a,b</sup> Means with unlike superscripts differ in Period 1 ( $P < 0.05$ ). <sup>c,d</sup> Means with unlike superscripts differ in Period 2 ( $P < 0.05$ ).

## Chapter VI

### Manuscript 4. Bioavailability of oxidized and reduced folate sources in Thoroughbred horses

**ABSTRACT:** Two studies were conducted to test the bioavailability of food folate and natural and synthetic supplemental forms of folate administered orally or intravenously in the horse. Study 1 was a 6 x 6 Latin Square using six Thoroughbred mares at maintenance assigned to six treatments of either oral or intravenous sources of 5-methyltetrahydrofolate (5-mTHF), 5-formyltetrahydrofolate (5-fTHF), or folic acid (FA). Horses were catheterized, administered the treatment, and blood samples taken at 0, 0.5, 1, 3, 6, 9, and 12 h after treatment administration. In the second study, four feeding tests were conducted using either 10 Thoroughbred mares at maintenance fed either a sugar/starch pasture supplement (M/SS) or a fiber/fat pasture supplement (M/FF), or 12 Thoroughbred mares in early lactation fed either SS (EL/SS) or FF (EL/FF). Horses were fasted overnight prior to jugular catheterization the morning of the study. Mares were fed 1.82 kg of feed, and samples at 0.5, 1, 3, 6, 9, and 12 h after feed was given. Plasma was analyzed for total plasma folate by radioimmunoassay in both studies. In study 1, i.v. FA yielded a plasma folate response that decreased exponentially over time. The rate of FA removal from the plasma was 0.29 mg/h indicating a slow uptake by cells or removal by the kidney. Efficiency of FA absorption was calculated as the quotient of the oral/i.v. area subtended by the plasma folate/time curves. The minimum estimate was 11 % in 12 h. Plasma folate responses after feeding SS and FF concentrates to horses were small, variable, and often below baseline values. Significant increases in plasma folate over baseline levels were only observed in M/SS mares. These data indicate that the bioavailability of oral sources of FA, as well as other oral sources and food sources of folate are low in the horse. A discussion of the problems and limitations in methodology encountered are also discussed.

Key Words: folate, horse, bioavailability, leucovorin, peptidoglycan

## Introduction

Reports of low serum or RBC folate levels in stabled and exercised horses fed hay (Seckington et al., 1967; Allen, 1978; Roberts, 1983) prompted widespread use of synthetic folic acid (FA) in commercial concentrates and vitamin supplements for horses. Despite this common practice, little is known regarding bioavailability of folate and its potential as a supplement to maintain or increase folate status in horse. In addition, no data exist on the bioavailability of folate in feed and forages fed to horses. However, reports of adequate folate status in horses of various physiological states maintained on pasture (Seckington et al., 1967; Roberts, 1983) indicate that folate in forages are available to the horse. There is also evidence indicating that horses are capable of utilizing microbially derived folate in the hindgut of the horse (Carroll et al., 1949).

The greater stability of FA compared to other natural reduced folate derivatives including 5-formyltetrahydrofolate (5-fTHF) and the main circulating form, 5-methyltetrahydrofolate (5-mTHF), led to its use as an oral and intravenous supplement. From experimental data in humans, the bioavailability of FA fortified in foods is 85 % compared to natural food folate that were no more than 50 % of folic acid (FNB, 1998; Gregory, 2001). In the horse, oral FA has been reported to be poorly absorbed (abstract cited by NRC, 1989). In addition, FA administered intramuscularly in horses resulted in rapid increases of serum and RBC folate concentrations that were cleared slowly from the plasma returning to baseline levels within 24 h (Roberts, 1983). A novel product containing predominately formylated sources of folate known as Peptidoglycan (PG®) was shown to have potential as a folate supplement in swine (Mizuno et al., 1997).

The objective of our first study was to evaluate the bioavailability and kinetics of intravenous or oral administration of two reduced and one oxidized form of folate in order to assess the potential of the sources for their use as dietary supplements in the horse. The second study was conducted to assess the bioavailability of folate from two different types of pasture supplements fed to maintenance or lactating mares to see if bioavailability is altered by the type of diet and/or by physiological state.

## Material and Methods

### *Study 1*

*Animals.* Six Thoroughbred mares ( $560 \pm 14.2$  kg initial BW) at maintenance between the ages of 4 and 8 yr were used for this study. Mares were maintained on a 10-acre bluegrass/white clover mixed pasture with access to trace mineral salt. The protocol was approved by the Institutional Animal Care and Use Committee.

*Experimental procedures.* The study design was a 6 X 6 Latin Square. Mares were randomly assigned to receive one of six treatments once /wk over a 6 wk period (Table 1). The treatments were separated into three intravenous (i.v.) sources of folate and three oral complementary sources of folate. The i.v. treatments were synthetic folic acid (FA, 0.05 mg/kg BW), 5-formyltetrahydrofolate (5-fTHF, 0.05 mg/kg BW) and 5-methyltetrahydrofolate (5-mTHF, 0.02mg/kg). The oral treatments were FA (0.05 mg/kg BW), 5-mTHF (0.05 mg/kg BW), and peptidoglycan® containing 0.58 mg/g of total folate and 0.43 mg/g of tetrahydrofolate derivatives (Ajinomoto PG®, 0.1 g/kg BW).

Synthetic folic acid (Rovimix® Folic 80 SD) for oral administration was generously provided by Roche Vitamins Inc. (Parsippany, NJ). The i.v. source of FA (Folvite®) was obtained from Lederle Parenterals (Carolina, Puerto Rico). The 5-mTHF (6R,S-5-Methyl-5,6,7,8-tetrahydrofolate, calcium salt) used for oral and i.v. administration was purchased from Schircks Laboratories (Jona, Switzerland). The i.v. source of 5-fTHF (Leucovorin Calcium for Injection) was obtained from Gensia Sicor Pharmaceuticals Inc. (Irvine, CA). Peptidoglycan® was donated by Ajinomoto Co., Inc. (Tokyo, Japan). The i.v. and oral doses of 5-mTHF were prepared by reconstituting the 5-mTHF in saline with added sodium ascorbate (1.0 % wt/vol). The oral FA and PG® dose was prepared by reconstitution in distilled water. Oral doses were administered via a 35 or 60 cc syringe and i.v. doses were administered via the jugular vein using either a 1 or 3 cc syringe with a 22 gauge needle.

The morning of the tests, horses were brought in from the pasture and placed individually in 14 m<sup>2</sup> individual box stalls. Feed was withheld from mares on the day of the study, but they had free access to water. A jugular venous catheter was placed in each horse at approximately 0630

followed by a 15 min adjustment period after the placement of the last catheter. Shortly thereafter, the treatment was administered and a series of blood samples was collected at 0, 0.5, 1, 3, 6, 9, and 12 h. During blood collection, an initial 10 cc of blood was removed from the catheter and discarded as waste and then 20 ml of blood was collected into a plastic syringe and placed into two 10 ml EDTA coated Vacutainer tubes (Fisher Scientific, Pittsburgh, PA). Additionally, 15 ml of heparinized (0.01 % vol/vol) saline was administered following the sample removal to prevent clotting in the catheter. Blood was immediately centrifuged at 2,000 x g for 5 min to separate plasma. Plasma was transferred to 2 ml polypropylene vials (Fisher Scientific, Pittsburgh, PA) and sodium ascorbate (Sigma-Aldrich Co., St. Louis, MO) was added for a final concentration of 0.3 % (wt/vol). Samples were immediately stored at -80 °C. Plasma was analyzed for total folate by radioimmunoassay (Folate Dualcount Solid Phase No Boil Assay, Diagnostic Products Corporation, Los Angeles, CA) as previously described (Manuscript 1).

*Statistical and kinetic analysis.* Data are presented as means ± standard error (SE) unless otherwise stated. Data were analyzed by analysis of variance (ANOVA) using the MIXED procedure of SAS for WINDOWS (Version 8, 1999; SAS Institute Inc, Cary, NC) with the REPEATED statement. The model included period, treatment, and their interaction with horse as the subject. A P-value of < 0.05 was chosen as the level of significance.

The kinetics and absorption of FA were evaluated by using the magnitudes of the responses of plasma folate (areas under concentration/time curves, AUC). The i.v. AUC were determined by fitting exponential equations using graphical software (SlideWrite for Windows, v. 8, 1999). The monoexponential equation,  $y = ae^{-kt}$  was used where  $a$  was the initial concentration of FA at time 0,  $t$  was time, and  $k$  was the slope or rate constant.

The data following oral doses failed to represent the expected rising and falling curves of plasma folate plotted against time. Consequently, the AUC for a period of 12 h were obtained by trapezoidal approximations. The i.v. AUC exceeded the oral AUC only for FA, so the efficiency of absorption, calculated as the quotient of oral AUC and i.v. AUC, was obtained only for FA.

## Study 2

*Animals and diets.* Ten Thoroughbred mares ( $595.4 \pm 7.6$  kg BW) at maintenance (M) and twelve Thoroughbred mares ( $656.3 \pm 12.0$  kg BW) in early lactation (EL) between 5 and 18 yr were used for the feeding experiment. Prior to the start of the study, mares at maintenance were paired by age and weight, and pregnant mares were paired by weight and foaling date, and within each group, horses were randomly assigned to one of two feeds. Half of the mares in each group were fed either a corn and molasses feed rich in starch and sugar (SS) or a fiber (beet pulp, soy hulls, oat straw) and fat (cereal byproduct) based supplement (FF). Therefore, the treatment groups were denoted M/SS, M/FF, EL/SS, and EL/FF. Diet compositions of the feeds are shown in Table 2. Folate analysis of the supplements by microbial assay (Ralston Analytical Laboratories, St. Louis, MO) found the SS and the FF had 1.9 and 2.5 mg/kg DM, respectively. Mares were maintained on mixed bluegrass/white clover and fed approximately 2.0 kg twice daily in order to meet approximately one-third to one-half of their DE requirement (NRC, 1989). The protocol was approved by the Institutional Animal Care and Use Committee and performed at the Virginia Tech Middleburg Agricultural Research and Experiment Center.

*Experimental procedures.* Four feeding tests were conducted between mid-May to early June. The EL/SS and EL/FF mares tested first followed by the M/SS and M/FF mares 1 wk later. A period of 1 d separated the tests for SS and FF mares within each group. The evening prior to the tests, horses were weighed (Tyrel platform, TC-105, Alweights Hamilton Scale Corp., Richmond, VA) and placed in 14 m<sup>2</sup> individual box stalls. Mares were allowed free access to water, but feed was withheld overnight (approx. 16 h). The morning of the feeding test, a jugular venous catheter was placed in each horse at approximately 0630 followed by a 15 min adjustment period after the placement of the last catheter. A series of blood samples was collected before and at 0.5, 1, 3, 6, 9, and 12 h after feed was given. The amount of SS and FF fed was 1.82 kg and the time for the mares to consume the feed was recorded. An initial 10 cc of blood was removed from the catheter and discarded as wasted followed by the sample blood collection, which was placed into two 10 ml EDTA coated Vacutainer tubes (Fisher Scientific, Pittsburgh, PA). Additionally, 15 cc of heparinized saline was administered following the sample removal to prevent clotting in the catheter. Blood was processed to obtain plasma and analyzed for folate by methods detailed above.

*Statistical and kinetic analysis.* Data were analyzed by analysis of variance (ANOVA) using the MIXED procedure with the REPEATED statement in SAS for WINDOWS (Version 8, 1999; SAS Institute Inc, Cary, NC). For comparison of plasma folate responses within physiological stage, data were analyzed using diet, time, and their interaction in the model with horse as the subject. For comparison of plasma folate responses within diet, data were analyzed using physiological stage, time and their interaction in the model with horse as the subject. A P-value of < 0.05 was chosen as the level of significance. Data are presented as means  $\pm$  standard error (SE) unless otherwise specified.

## Results

### *Study 1*

Mean baseline concentrations of plasma folate for horses during all 6 wk was  $25.9 \pm 1.7$  ng/ml. Mean baseline concentrations of plasma folate were similar between each week indicating that 1 wk between treatments was a sufficient wash-out period. Mean plasma folate levels before and up to 12 h after i.v. and oral administration of folate sources are shown in Figures 1 and 2, respectively. Best fitting exponential equations are presented in Table 3. These equations were used to calculate i.v. AUC, hence plasma clearance rates (Table 3). The i.v. AUC adjusted for doses were 107.0, 2.86, and 5.34  $\text{h}\cdot\text{ng}\cdot\text{ml}^{-1}\cdot 10^{-6}$  for FA, 5mTHF and 5fTHF, respectively. Corresponding oral AUC adjusted for doses were 12.13, 12.39, and 11.20  $\text{h}\cdot\text{ng}\cdot\text{ml}^{-1}\cdot 10^{-6}$ . Thus an efficiency of absorption could be calculated only for FA, which was 11.3%.

### *Study 2*

The mean time taken by the M and EL mares to consume the SS and FF feeds was  $18.1 \pm 0.06$  min and  $21.3 \pm 0.05$  min ( $P < 0.04$ ), respectively. Baseline levels of plasma folate were for  $36.2 \pm 2.8$  and  $36.4 \pm 2.1$  ng/ml for the M and EL mares, respectively. For the M mares (Figure 3), there was a significant effect of diet ( $P < 0.01$ ), time ( $P < 0.01$ ) and their interaction ( $P < 0.01$ ) on plasma folate levels. Plasma folate concentrations were similar at all time points within the M/SS mares indicating no response of plasma folate to consumption of FF. The largest plasma folate response in the M/SS mares was at 30 min after feed consumption, which returned

to baseline within 1 h. The concentrations of plasma folate were consistently lower in the M/SS mares compared to the M/FF mares with the exception of the 30 min sampling period ( $P < 0.05$ ).

In the EL mares, there was a main effect of time ( $P < 0.05$ ) and a trend for a main effect of diet ( $P < 0.10$ ) on plasma folate levels. Plasma folate responses were similar between treatment groups at all time periods except at 6 h when EL/SS mares had higher plasma folate concentrations than the EL/FF mares ( $P < 0.05$ ). There was a moderate decline in plasma folate from 1 to 2 h in EL/SS mares ( $P < 0.05$ ), but levels were not significantly different from baseline. The EL/FF mares had a moderate increase in plasma folate at 3 h, but this increase was not significant.

Comparison of physiological state within the group of mares fed SS revealed main effects of stage, time and their interaction ( $P < 0.05$ ). The M/SS mares had lower concentrations of plasma folate than EL/SS mares at 0, 1 and 6 h after food consumption. Comparison of M/FF and EL/FF mares revealed a main effect of stage and time ( $P < 0.05$ ), but not their interaction. The M/FF mares had higher concentrations of plasma folate at 0, 0.5, and 6 h after consumption of FF.

## **Discussion**

### *Study 1*

These studies were conducted to evaluate the kinetics and bioavailability of reduced or oxidized folate sources either supplemented to the horse or available as food folate. Information regarding the bioavailability of FA is especially important in order to support or refute routine supplementation of FA to horses through fortification of feedstuffs or as vitamin supplements. In our first study, the design was such that we could evaluate the bioavailability of three sources, FA, 5-mTHF, and 5-fTHF by providing the supplements both orally and intravenously.

Folic acid was chosen as a supplement in our study because it is the most commonly used folate source due to its greater stability compared to reduced forms of folate (Gregory, 1989). However, the oxidized FA is found in low amounts in feeds and forages and is therefore foreign

to the gastrointestinal tract of a horse. We also chose to investigate 5-fTHF based on its availability as an injectable folate supplement and for its potential as an alternative oral folate supplement. Studies in humans found that 5-fTHF is effectively absorbed, converted to 5-mTHF, and utilized (Nixon and Bertino, 1972; Mehta et al., 1978; Bhandari and Gregory, 1992). The other oral folate source was a loose powder comprised of digested non-pathogenic bacterial cell wall known as Peptidoglycan. Peptidoglycan is a good source of 5-fTHF and has potential as an oral folate supplement in that it could provide additional stability to the formylated derivative if used to fortify feeds or be included in vitamin supplements.

Data analysis for the bioavailability of the three sources proved difficult. The only feasible result for a minimum efficiency of absorption for FA was 11.3 % in 12 h. Similar efficiencies are likely for 5-mTHF and 5-fTHF, because the oral AUC adjusted for doses were similar. This estimate of 11.3 % is regarded as a minimum, because the oral plasma folate/time curves showed no consistent trend for a decrease during the observational period. Thus, the strongest statement that can be based on these data is that the efficiency of folate absorption exceeds 11.3 % following the administration of about 30 mg of FA and probably also 5-mTHF and 5-fTHF.

Low levels of plasma folate observed from 3 to 12 h following administration of 5-mTHF and 5-fTHF suggest that absorbed folate was rapidly removed from plasma. In other words, the plasma folate response was "L-shaped" and most of the early curve was missed because the first blood sample was taken at 30 minutes after folate administration. Thus, the AUC for the i.v. doses were greatly underestimated. If this is the case, then the slower initial rate of removal of folate from plasma following administration of the synthetic form, FA, suggests that this form of plasma folate, which was estimated generically, represents a different form, perhaps still oxidized FA, from the natural forms present in plasma following administration of the THF's.

Studies of FA supplementation in the horse are limited. One horse was reported to have an improvement in racing performance associated with an increased serum folate, hemoglobin, and body condition after 23 d of 20 mg of folic acid supplementation (mode of administration not documented) (Seckington et al., 1967). Roberts (1983) observed low serum and RBC folate concentrations in Thoroughbreds and Standardbreds in race training that received daily oral folic

acid supplementation and/or bi-monthly or weekly i.m. injections of folic acid. Roberts (1983) observed significant increases in serum and RBC folate after i.m. injections of either 150 mg of folic acid or two 75 mg doses separated by 4 d. Slow clearance of FA from the plasma observed in the present study is supported by the Roberts (1983) study in that plasma folate returned to baseline levels within 12 h after i.m. administration of FA.

More recently, folate deficiency was observed in foals born to mares being treated for Equine Protozoal Myelencephalitis with PYR, sulfonamides, folic acid and vitamin E (Toribio et al., 1998). Upon admission, foals showed signs lethargy and weakness, alopecia, skin lesions, and oral ulcers. Clinical diagnosis revealed low hematocrit, low white blood cell and platelet count, and high serum creatinine associated with numerous congenital defects including bone marrow aplasia and hypoplasia, renal nephrosis or hypoplasia, and skin lesions. It was concluded that the oral coadministration of FA potentiated the effects of PYR and sulfonamides, which resulted in a lowered folate status in the mare that was further evident in the foal.

One of the objectives of this study was to evaluate the potential use of either FA or 5-fTHF as folate sources for routine supplementation. The data obtained in the present study are inconclusive and fail to support or refute the routine use of either folate source. The slow removal of FA and the presumably quick removal of 5-fTHF from the plasma would support the use of 5-fTHF (as Leucovorin®) as an i.v. source. The use of i.v. sources of folate is more practical under clinical settings suggesting that 5-fTHF could be used under such conditions, but would be impractical under field conditions. The lack of a plasma folate response in horses supplemented with both oral FA and 5-fTHF indicate that these sources may not be effective as folate supplements at the amounts given. Further studies need to be conducted to evaluate the effects of long-term i.v. or oral supplementation of 5-fTHF on folate status in the horse.

Several methodological problems and limitations were encountered in the first study. First, the sampling periods were not adequate to accurately assess the responses of plasma folate. In the case of the i.v. doses, sampling periods needed to be more frequent soon after treatment administration up to 30 min. Also, sampling periods should have been extended beyond 12 h for the oral doses in order to determine when and if plasma folate concentrations returned to baseline

concentrations. It is clear that the design of the study did not provide an adequate observational period and further studies should separate investigations of oral and intravenous treatments into separate studies. In addition to altering the sampling times, the physiologically relevant amounts of FA, 5-mTHF, and 5-fTHF used in this study may have been too small in order to detect a measurable plasma folate response. Also the large variability within and between animals given the small doses of folate increased the difficulty of the assessment of bioavailability. Increasing the amount of folate administered orally may be beneficial in future studies in the attempt to observe a measurable plasma folate response.

### *Study 2*

The objective of the second study was to assess the bioavailability of food folate in two pasture supplements containing different feed components and to see if a demanding physiological period such as early lactation would alter the bioavailability of food folate. The SS feed was designed to resemble a sweet feed typical of many commercial products, which contain a large amount of starch and sugar. The FF supplement was designed to include a larger variety of fiber sources in order to promote a healthy and diverse population of microbes in the hindgut and to contain a higher concentration of fat as an alternative energy source to carbohydrate. It was also anticipated that the larger amount of fiber sources would result in slower time for consumption of the feed by horses, which was found to be correct. The slower time for consumption may have advantages in that it can increase the time for digestion and absorption and decrease the incidence of digestive upsets.

After consumption of SS or FF, only the M/SS mares had a significant response in plasma folate. The lack of a response of plasma folate in the other three groups may be contributed to many factors. First, the rate of entry and removal of folate from the plasma after consumption of a meal is similar resulting in a stable plasma folate concentration. Also, the experimental period may need to be extended in order to determine if fermentation in the hindgut and microbial synthesis contribute a greater amount of plasma folate after 6 h meal consumption. The early onset of an increase in plasma folate in the M/SS mares may indicate that digestion and subsequent absorption of folate occurred in the large intestine, whereas the FF supplement may contribute a greater amount of absorbable folate in the cecum after fermentation.

Another possible contributing factor to the lack of an increase in plasma folate after consumption of a feed is the fact that the horses were fasted overnight. The fasted conditions were necessary for a companion study measuring the glucose and insulin response to meal feeding. In the human, increases in plasma folate have occurred after fasting (Cahill et al., 1998; Pietrzik et al., 1990), but it is not known whether the same occurs in the horse.

The use of stable isotopes to assess whole body folate metabolism has proven useful in humans (Gregory, 2001). Advantages of stable isotopes are that they are more sensitive and can trace body pools of folate in addition to plasma. The main limitation to using stable isotopes in the horse is the greater contribution of the microbially derived folate compared to the humans which is an additional folate pool to consider, which alters greatly with dietary changes. Other limitations of using stable isotopes are the difficulty in synthesis and the expense of the sophisticated equipment needed for analysis.

In summary, the results from these studies indicate that the bioavailability of i.v. and oral sources of FA, as well as other oral sources and food sources of folate are low in the horse. However, several methodological problems and limitations were encountered in the present study including time and frequency of sampling, amount of treatment, and methods used to detect bioavailability in the horse.

### **Implications**

The low bioavailability of FA indicates that the routine daily oral supplementation of FA in the horse industry may be safe but futile. Studies investigating the potential benefits of 5-fTHF supplementation to maintain or increase folate status are warranted in the horse.

## Tables

**Table 1.** Latin square design for six treatments (A to F)<sup>a</sup> administered to six horses (H1 to H6) over a 6 wk experimental period

Week	Horse					
	1	2	3	4	5	6
1	A	B	C	D	E	F
2	B	D	A	F	C	E
3	C	F	E	B	A	D
4	D	A	B	E	F	C
5	E	C	F	A	D	B
6	F	E	D	C	B	A

<sup>a</sup> Treatment assignments were: A, i.v. folic acid (Folvite®), 0.05 mg/kg BW); B, i.v. 5-methyltetrahydrolate (5-mTHF, 0.02mg/kg); C, i.v. 5-formyltetrahydrofolate (5-fTHF,0.05 mg/kg BW); D, oral folic acid (Rovimix® Folic 80 SD, 0.05 mg/kg BW); E, oral 5-mTHF (0.05 mg/kg BW); and peptidoglycan containing 0.58 mg/g of total folate (Ajinomoto PG®, 0.1 g/kg BW)

**Table 2.** Ingredient and nutrient composition of Starch/sugar (SS) and Fiber/fat (FF) fed in Experiment 2<sup>a</sup>

Item	SS (n=6)	FF (n=6)
Ingredient (%)		
Dent yellow grain corn	60.0	1.5
Soybean meal 48%	15.5	2.0
Oat Straw	7.0	7.0
Alfalfa	-	13.5
Soybean hulls	4.0	4.0
Beet pulp	-	10.0
Cereal by-product	-	56.0
Cane molasses	10.0	5.0
Dicalcium phosphate	1.5	-
Limestone	1.0	-
Mineral premix <sup>b</sup>	0.5	0.5
Vitamin premix <sup>c</sup>	0.5	0.5
Nutrient composition		
DM, %	95.8 ± 0.4	97.9 ± 0.69
DE, Mcal/kg	3.7 <sup>f</sup> ± 0.05	3.0 <sup>g</sup> ± 0.00
CP, %	15.8 ± 1.1	13.9 ± 0.22
ADF, %	7.4 <sup>d</sup> ± 0.95	22.3 <sup>e</sup> ± 0.90
NDF, %	15.8 <sup>d</sup> ± 1.4	31.4 <sup>e</sup> ± 0.68
Fat, %	2.8 <sup>f</sup> ± 0.18	10.2 <sup>g</sup> ± 1.0
NSC, %	59.4 <sup>f</sup> ± 3.1	34.1 <sup>g</sup> ± 1.9
Ash, %	6.2 <sup>d</sup> ± 0.75	11.7 <sup>e</sup> ± 0.14
Ca, %	0.86 ± 0.14	2.3 ± 0.03
P, %	0.63 ± 0.08	0.93 ± 0.08
Mg, %	0.20 <sup>f</sup> ± 0.02	0.57 <sup>g</sup> ± 0.05
K, %	1.0 ± 0.08	1.2 ± 0.01
Na, %	0.21 ± 0.03	0.27 ± 0.02
S, %	0.20 ± 0.01	0.19 ± 0.01
Cl, %	0.86 ± 0.40	0.50 ± 0.03

<sup>a</sup> Analyzed by DHI Forage Testing Laboratory (Ithaca, NY). Data are summarized on a DM basis as means SE's.

<sup>b</sup> The mineral premix provided the following per kilogram of supplement: NaCl, 3,774 g; Zn, 422 g; Fe, 208 g; Cu, 89.5 g; Mn, 50.3 g; Se, 1.095 g; and KI, .415 g

<sup>c</sup> The vitamin premix provided the following per kg of a supplement: vitamin A, 1,380,080 IU; vitamin D<sub>3</sub>, 258,000 IU; vitamin E, 26,455 IU; riboflavin, 701 mg; niacin, 3009 mg; folic acid, 66 mg; thiamin, 1400 mg; biotin, 42 mg; and -carotene, 3527 mg;

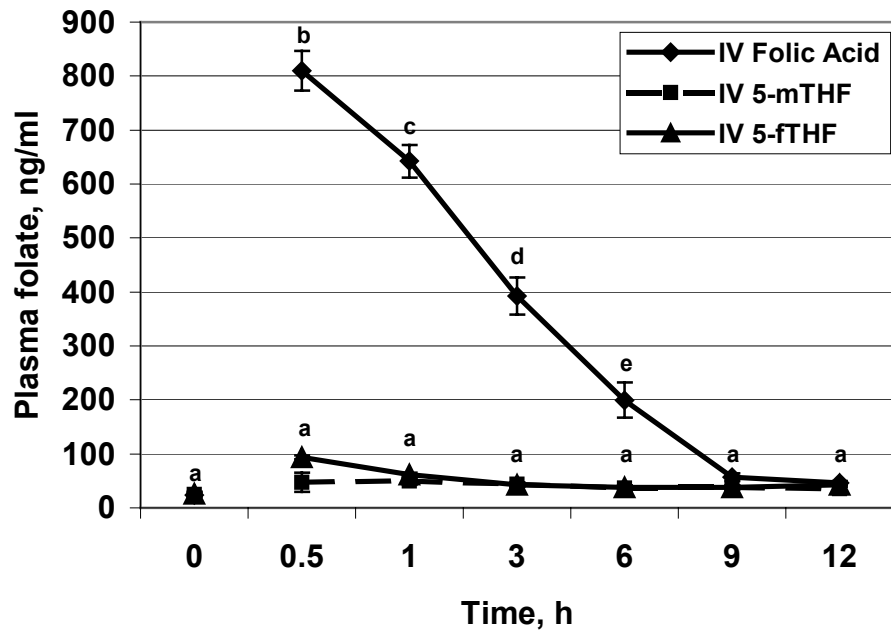
<sup>d,e</sup> Within a row, means without a common superscript differ ( $P < 0.01$ )

<sup>f,g</sup> Within a row, means without a common superscript differ ( $P < 0.0001$ )

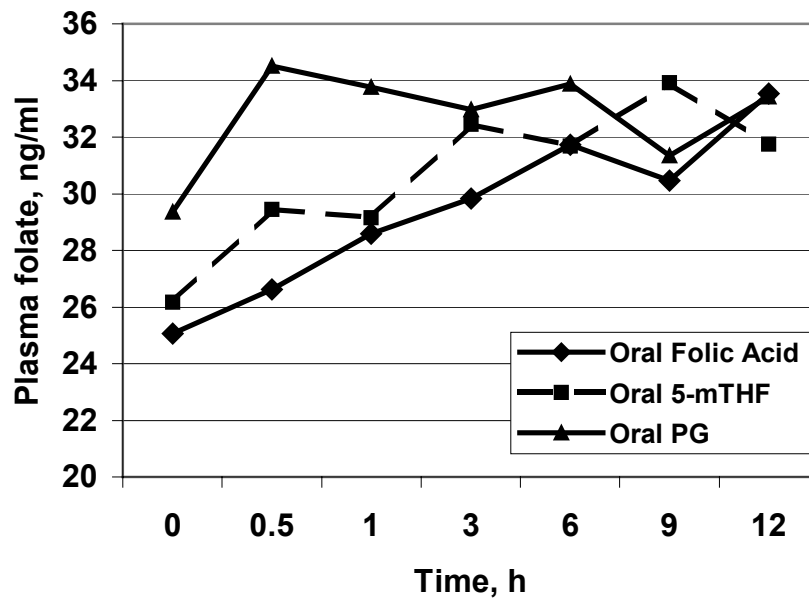
**Table 3.** Exponentials equations ( $y = a \cdot e^{-kt}$ ) for the plasma folate/time curves

Item	FA	5-mTHF	5-fTHF
Intercept	881.8	23.97	77.02
Exponent	-0.296	-0.0756	-0.513
F-statistic	541	28.5	21.6
P-value	0.0000	0.0059	0.0097
AUC, $\text{h} \cdot \text{ng} \cdot \text{ml}^{-1}$	2,979	31.71	150
Dose, mg	27.8	11.1	28.1
[AUC/Dose], $\times 10^{-6}$	107.2	2.86	5.34
Plasma clearance, l/h	9.3	350 <sup>a</sup>	187 <sup>a</sup>

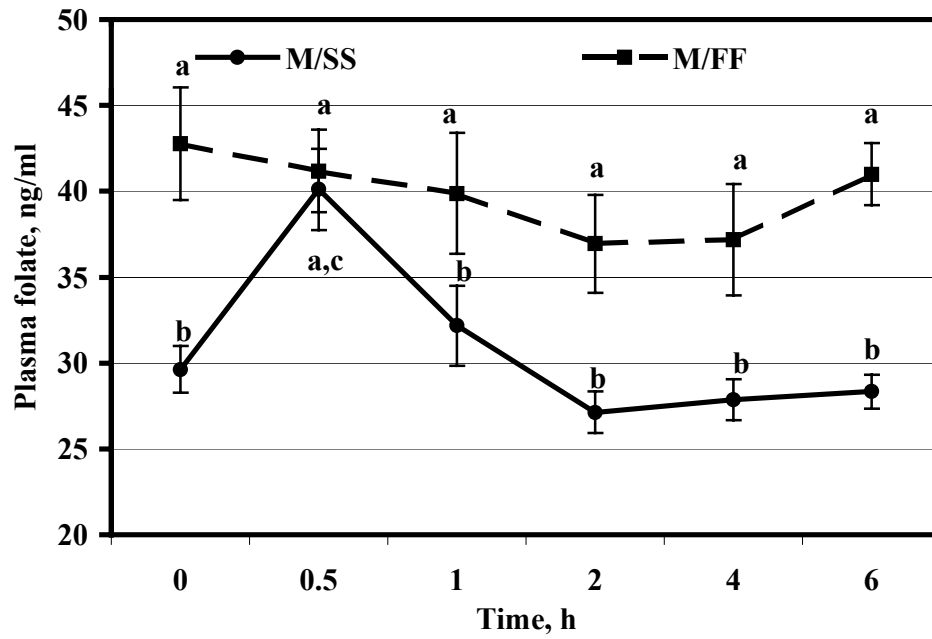
<sup>a</sup> Probably erroneous as explained in the text



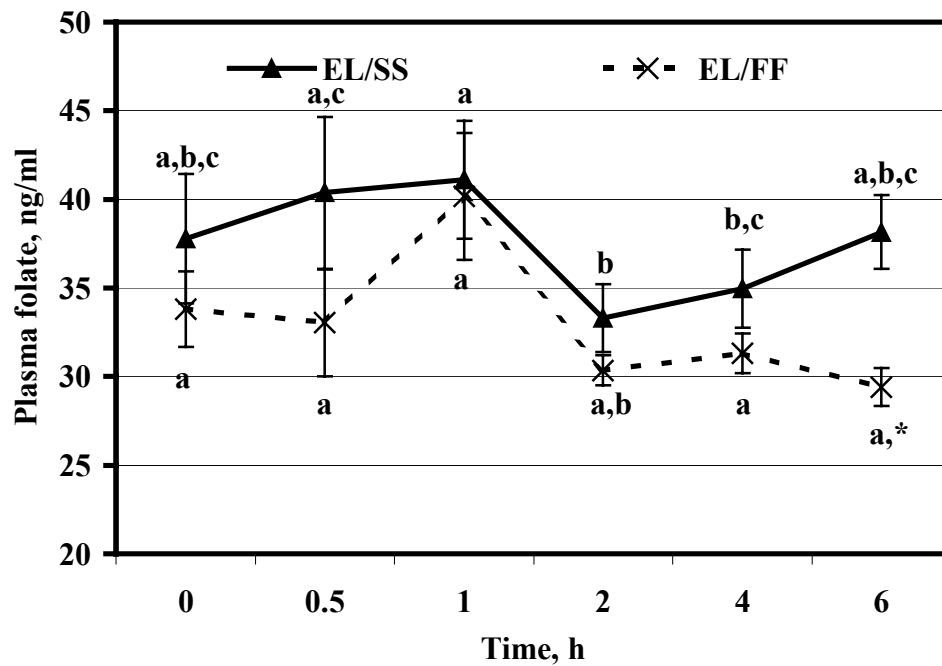
**Figure 1.** Response of plasma folate to i.v. administration of synthetic folic acid (FA, 0.05 mg/kg BW), 5-formyltetrahydrofolate (5-fTHF, 0.05 mg/kg BW) and 5-methyltetrahydrofolate (5-mTHF, 0.02mg/kg). <sup>a,b,c,d,e</sup> Means with unlike superscripts differ ( $P < 0.05$ ).



**Figure 2.** Response of plasma folate to i.v. administration of synthetic folic acid (FA, 0.05 mg/kg BW), 5-formyltetrahydrofolate (5-fTHF, 0.05 mg/kg BW) and 5-methyltetrahydrofolate (5-mTHF, 0.02mg/kg). Plasma folate was similar at all time periods between and within treatment.



**Figure 3.** Response of plasma folate in mares at maintenance after consumption of 1.82 kg of starch/sugar (M/SS) or fiber/fat (M/FF) supplement. <sup>a,b,c</sup> Means within and between groups with unlike superscripts differ ( $P < 0.05$ ).



**Figure 4.** Response of plasma folate in mares in early lactation after consumption of 1.82 kg of starch/sugar (EL/SS) or fiber/fat (EL/FF) supplement. <sup>a,b,c</sup> Means within and between groups with unlike superscripts differ ( $P < 0.05$ ). \* EL/SS higher than EL/FF at 6 h ( $P < 0.05$ ).

## CHAPTER VII

### General Summary

The main objectives of the present studies were to assess the effects of lactation, growth, moderate exercise, and anti-folate drug administration on folate status, in addition to evaluating the bioavailability of natural and synthetic forms of folate in the horse. The present studies evaluated plasma and RBC folate, and plasma homocysteine in horses in various physiological states and references ranges including the mean and a 95 % confidence interval for each variable are presented in Table 1.

Folate status was only moderately lowered after 12 wk of moderate exercise conditioning and during late lactation compared to horses administered anti-folate drugs. Administration of PYR and SDZ after 9 wk impaired folate status sufficiently to cause a decline in plasma folate and a subsequent increase in plasma homocysteine, which was not observed to occur during the other studies. Attempts at increasing folate status with oral folic acid supplementation during moderate exercise period and with FA or PG during anti-folate drug administration were unsuccessful, which can be partially explained by a low efficiency of absorption of 11 % for FA.

Initial attempts to use a combination of indicators to assess folate status including plasma folate, RBC folate, milk folate (when applicable), and plasma homocysteine were unsuccessful based on the fact that no correlation between RBC folate and the other indicators was observed. Therefore, assessment of folate status was based primarily on concentrations of RBC folate. Since plasma homocysteine was not negatively correlated to RBC folate as observed in humans, the use of plasma homocysteine as a functional indicator of folate status in the horse is not recommended.

In conclusion, horses administered PYR and SDZ are at a higher risk of incurring an impaired folate status compared to pregnant mares and foals maintained on pasture and moderately exercised horses fed hay and concentrate. Future studies investigating the administration of PYR and SDZ to horses should focus on the potential negative effects of increased plasma homocysteine and alternative folate sources that would maintain folate status. In addition, the

common practice of supplementing horses with oral FA in vitamin supplements appears to be of little benefit to horses engaged in routine submaximal exercise or administered PYR and SDZ. The low bioavailability of FA indicates a need for further research on the potential benefits of alternative sources of folate, including 5-fTHF and 5-mTHF to maintain or increase folate status in the horse.

**Table 1.** Reference ranges for plasma folate, RBC folate, milk folate, and plasma homocysteine assessed in present studies

Item	95 % Confidence		Item	95 % Confidence	
	Mean	Interval		Mean	Interval
TB Foals			Horses in moderate exercise regimen		
Plasma folate (ng/ml)			Plasma folate (ng/ml)		
Foaling	13.5	1.8 - 28.2	Baseline	21.6	12.7 - 30.5
1 to 3 mo	11.2	1.7 - 19.6	2 to 6 wk	17.6	10.1 - 25.2
4 to 6 mo	22.2	11.9 - 32.4	8 to 12 wk	16.7	7.1 - 26.2
RBC folate (ng/ml)			RBC folate (ng/ml)		
Foaling	753.6	579.8 - 927.4	Baseline	587.18	353.1 - 821.3
1 to 3 mo	761.1	602.6 - 919.6	4 wk	569.36	364.1 - 774.6
4 to 6 mo	720.9	536.8 - 905.0	8 wk	586.22	419.2 - 753.2
Plasma homocysteine (umol/L)			Plasma homocysteine (umol/L)		
Foaling	12.98	1.0 - 24.9	10 wk	485.33	361.9 - 608.8
1 to 3 mo	4.94	2.8 - 7.1	Geldings administered PYR and SDZ		
4 to 6 mo	4.38	3.0 - 5.7	Plasma folate (ng/ml)		
TB Mares			Baseline		
Plasma folate (ng/ml)			0 to 5 wk		
Foaling	20.6	15.8 - 25.4	6 to 10 wk		
1 to 3 mo	18.3	11.5 - 25.2	11 to 15 wk		
4 to 6 mo	17.2	11.5 - 23.0	Plasma homocysteine (umol/L)		
RBC folate (ng/ml)			Baseline		
Foaling	693.2	548.0 - 838.5	4 to 9 wk		
1 to 3 mo	752.6	607.0 - 898.2	12 to 15 wk		
4 to 6 mo	656.8	501.4 - 812.3	Plasma homocysteine (umol/L)		
Milk folate (ng/ml)			Baseline		
Foaling	217.7	155.8 - 279.7	4 to 9 wk		
1 to 3 mo	173.1	123.2 - 223.0	12 to 15 wk		
4 to 6 mo	181.6	141.8 - 221.4	Plasma homocysteine (umol/L)		
Plasma homocysteine (umol/L)			Baseline		
Foaling	5.2	1.9 - 8.6	4 to 9 wk		
1 to 3 mo	5.3	1.6 - 9.0	12 to 15 wk		
4 to 6 mo	6.3	2.5 - 10.1	Plasma homocysteine (umol/L)		

## CHAPTER VIII

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## VITA

Amy Lynn Ordakowski, daughter of Mrs. Carol Slicher and Mr. Paul Ordakowski, was born on March 6, 1973 in Baltimore, Maryland. She graduated from Chesapeake High School in Pasadena, Maryland in 1991. She received her Bachelor of Science degree in Biology from James Madison University in May, 1995. Shortly thereafter, she was employed as a research technician in the Division of Behavioral Biology of the Department of Psychiatry at The Johns Hopkins School of Medicine in Baltimore, MD.

In August of 1996, she pursued graduate studies in equine nutrition in the Department Animal and Poultry Sciences at Virginia Polytechnic Institute and State University where she was a member of Phi Sigma National Biological Honor Society. She received her Master of Science degree in Animal and Poultry Sciences with emphasis on equine nutrition in August of 1998. Her Master's Thesis was titled: *Alkanes as Internal Markers to Estimate Digestibility in Horses*.

In August of 1998, she was awarded a John Lee Pratt Fellowship to pursue a doctoral degree in Equine Nutrition from the Department of Animal and Poultry Sciences Department of Virginia Polytechnic Institute and State University under the supervision of Dr. David S. Kronfeld. She is an active member of the American Society of Animal Science, the Equine Nutrition and Physiology Society, and Sigma Xi. She was recently appointed to the Lecturer in Equine Science/Equine Extension Specialist position in the Department of Animal and Avian Sciences at the University of Maryland.

Amy L. Ordakowski