Evaluating Egg Folate Deposition Throughout the Production Cycle of Laying Hens

A Thesis

Presented to

The Faculty of Graduate Studies

of

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by

Tyra Meaghan Dickson

In partial fulfillment of requirements

for the degree of

Master of Science

Department of Animal Science

University of Manitoba

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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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

MASTER OF SCIENCE

Abstract

Two trials were designed to determine the optimal level of folic acid supplementation within laying hen diets to maximize egg folate content over time, evaluate production parameters and hen health as affected by dietary folic acid supplementation level, and explore the relationship between egg folate deposition and intestinal absorption.

In Experiment 1, Hy-Line CV20 laying hens (n=416/diet) received a barley-based ration, containing 0, 2, or 4 mg of crystalline folic acid/kg diet for eleven 28-day periods to determine the optimal level of folic acid supplementation to maximize egg folate content and consistency over the production cycle of the hen. Egg folate content significantly ($P<0.05$) increased (1.42, 2.93, and 3.40 μg/g yolk) as the dietary folic acid supplementation level increased (0, 2, and 4 mg/kg diet) respectively. Hens consuming the 4 mg folic acid/kg diet deposited yolk folate at a higher amount than those hens consuming 0 or 2 mg folic acid/kg diet.

In Experiment 2, Hy-Line CV20 laying hens (n=12/diet) received a rye (40% of grain)/wheat-based ration, containing 0, 0.5, 1, 2, or 4 mg folic acid/kg diet with and without enzymes (β-glucanase and xylanase) for six 7-day periods to determine the effect of dietary enzymes, and a viscous ration on the *in vivo* viscosity of laying hen jejunal digesta, and egg folate content. Egg folate content significantly ($P<0.05$) increased (1.3, 1.7, 2.1, 2.5, and 2.6 μg/g yolk) as the dietary folic acid supplementation level increased (0, 0.5, 1, 2, and 4 mg/kg diet) respectively. The addition of enzymes significantly ($P<0.05$) decreased the *in vivo* viscosity of laying hen jejunal digesta, and did not affect egg folate content or other production parameters.

These studies suggest that 4 mg folic acid/kg diet is required to optimize egg folate deposition and consistency (maintained at a level significantly higher than other dietary folic acid supplementation levels) of deposition throughout the production cycle of the laying hen. Further mechanistic research is required to confirm the control points of egg folate saturation.

Acknowledgements

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Foreward

This thesis is written in manuscript style. The two manuscripts will be submitted to the Poultry Science Journal. The authors of these manuscripts are T. Dickson, J.D. House, and W. Guenter, Department of Animal Science, University of Manitoba, Winnipeg, Manitoba, Canada, R3T 2N2.

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Abbreviations

5-MTHF, (CH_3THF)	5-methyl-tetrahydrofolate
CH_2THF	5,10 methylenetetrahydrofolate
10-formyl-THF	10-formyl-tetrahydrofolate
AD	Alzheimer's disease
BHMT	betaine homocysteine methyltransferase
CBS	cystathionine beta-synthase
COMA	committee on medical aspects of food and nutrition policy
CV	coefficient of variation
CVD	cardiovascular disease
DFE	dietary folate equivalents
DHFR	dihydrofolate reductase
DMG	dimethyl glycine
DNA	deoxyribonucleic acid
FDA	Food and Drug Administration
folyl conjugase	exocarboxypeptidase folyl γ-glutamyl hydrolase
GFT	glutamic acid formiminotransferase
HPLC	high pressure liquid chromatography
IEC-6	rat small intestine epithelial cells
MS	methionine synthase
MTHFR	methyltetrahydrofolate reductase
MTX	methyltrexate
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)

NO	nitric oxide
NSP	nonstarch polysaccharide
NTD	neural tube defect
NRC	National Research Council
PABA	para aminobenzoic acid
PRPP	(erythrocyte) phosphoribosylpyrophosphate
PteGlu	pteroylglutamate
RBP	riboflavin binding protein
RDA	recommended dietary allowance
RFC	reduced folate carrier
rpm	rotations per minute
SAH	S-adenosyl homocysteine
SAM	S-adenosyl-methionine
SEM	standard error of the mean
shRNA	short hairpin ribonucleic acid
THF	tetrahydrofolic acid
US	United States

1.0 Introduction

The increased recommended dietary intake (RDI) (Institute of Medicine, 1998) of folic acid has initiated the need for additional sources of folate for human consumption due to the fact that folic acid does not occur naturally in substantial amounts in foods. It is recommended that adult humans, in particular, women of childbearing age consume 400 μg of folic acid per day. There is documented evidence (Czeizel, and Dudas, 1992) that consumption of 400 μg of folic acid per day will reduce the occurrence of neural tube defects such as spina bifida and anaencephaly. Folic acid is a cofactor in the remethylation of homocysteine to form methionine (House, et al., 1999). It has also been found to lower plasma homocysteine levels, which have been associated with prevention of cardiovascular disease (Boushey, et al., 1995) and neuropsychological conditions such as Alzheimer's Disease (Seshadri, et al., 2002). Therefore adequate consumption of this vitamin is important to human health.

In its natural state, the quality of protein in eggs is considered to be sufficiently high enough to be used as the standard for measuring the quality of other food proteins. The egg contains all the nine essential amino acids, as well as all essential vitamins, except C, and minerals. Changing dietary habits initiated a drop in annual total egg consumption in Canada from 23 dozen per person in 1960 to 14.3 dozen in 1995 (Agriculture and Agri-food Canada, 2005). However, in the past few years egg consumption has increased and in 2002, it reached 15.4 dozen per person (Agriculture and Agri-Food Canada, 2005).

The supplementation of laying hen diets with folic acid leads to the production of folate-enriched eggs. However, the level of folate in eggs is directly related to plasma

folate concentrations, both of which appear to be governed by a saturable process. Investigation of factors regulating folate absorption and metabolism in the laying hen will provide further insight into the mechanisms of folate deposition in eggs.

Currently industry provides laying hens with the amount of folic acid requested by National Research Council (NRC, 1994), which is 0.25 mg/kg of feed. Previous research documents from trials that supplemented diets with 4 mg of crystalline folic acid/kg of diet, increased egg folate content three-fold that of a regular egg (House, et al., 2002). In terms of its nutritional value, one large egg collected from a folic acid-supplemented hen provided approximately 12.5% of the RDA for adult humans.

Research has shown that egg yolk containing folate is a more bioavailable source of folate than crystalline folic acid (House, et al., 2003). Thus, humans have an alternate means of increasing their folate intake in a nutritious way, consuming an egg, which is actually more effective than taking folic acid in the form of a pill.

The key to successful folic acid enrichment of eggs is to allow for maximization of egg folate content throughout the entire production cycle of the hen. Throughout the typical 11-month production cycle of the hen, changes occur within several production parameters including a decline in egg production and shell quality with time. Successful marketing of a folic acid-enriched egg, will require that individual eggs from a hen fed a folic acid supplemented diet will have the same folate content, irrespective of the time the egg was laid. Furthermore an adequate understanding of the egg saturation mechanism is crucial to allow for consistent egg folate deposition from a variety of feed ingredients. For example, it must be known that an egg from a hen fed a barley-based folic acid

supplemented diet contains the same amount of folate as an egg from a hen fed a corn-based folic acid supplemented diet.

The overall objective of this thesis is to investigate maximization of egg folate deposition. Two trials are designed to determine optimal level of folic acid supplementation within laying hen diets to maximize egg folate content over time, evaluate production parameters and hen health as affected by dietary folic acid supplementation level, and explore the relationship between egg folate deposition and intestinal absorption. Further details of specific objectives are presented in each of the two manuscripts that constitute this thesis.

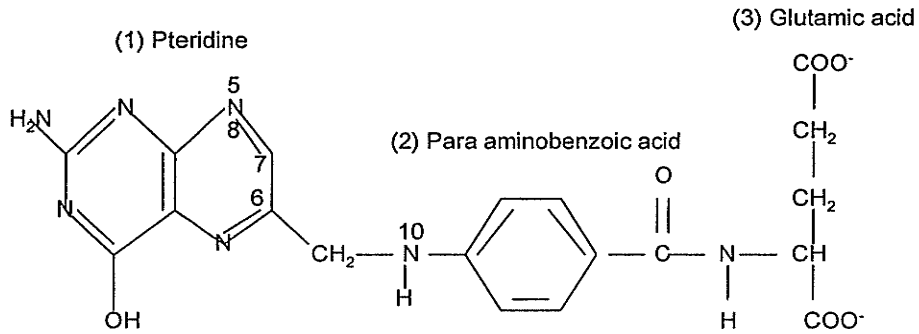
2.0 Literature Review

2.1 Folic Acid

2.1.1 Folic Acid Structure and Chemistry

Folate is the generic term for the water-soluble B-complex vitamin that includes various chemical structures with similar biological activity (Wagner, 1996). Folates are a family of compounds that have pteroylglutamate (PteGlu) as a common structure (Selhub and Rosenberg, 1996). Folic acid (**Figure 1**) consists of three subunits (1) pteridine linked to (2) para aminobenzoic acid (PABA) which forms pteroic acid, and (3) glutamic acid (Wagner, 1996). When one or more glutamic acid residues bond together, pteroylpolyglutamates are formed (Wagner, 1996). The mixture of folate derivatives differs by the oxidation state, one-carbon substitution of the pteridine ring and by the number of glutamate residues. The differences are associated with different physiochemical properties which together with food constituents could influence folate bioavailability. Most naturally occurring folates, are pteroylpolyglutamates, which contain one to six additional glutamate molecules joined in a peptide linkage to the γ-carboxyl of glutamate (Wagner, 1995).

Figure 1: Structure of folic acid



Source: Adapted from Selhub and Rosenberg, 1996

2.1.2 Folate Stability

Pteroylmonoglutamic acid is the most stable and oxidized form of folate, which rarely occurs in food, but is the form used in vitamin supplements and in fortified food products (Wagner, 1996). Folates (folates other than folic acid, pteroylglutamic acid, and 5-formyl-tetrahydrofolic acid) are sensitive to light, aerobic conditions, extremes of pH, and heat, especially boiling of foodstuffs, all of which result in losses of folate activity (Rosenberg and Godwin, 1971). Folate is heat labile; therefore a diet comprised exclusively of thoroughly cooked foods is likely to be low in folate (Herbert, 1987). Gujska and Majewska, (2005) found that in wheat and rye bread (without lactic acid) products there was a decrease in added folic acid from the flour to bread stage, of 12 % in wheat and 21 % in rye. Substantial losses in folic acid content can occur as a result of leaching and chemical degradation during cooking, especially in vegetables (Bailey, 1992). Total folate losses of 22% for asparagus and 84% for cauliflower have been observed (Combs, 1998).

2.1.3 Chemical Forms of Folate

Referring to **Figure 1**, naturally occurring folates are generally reduced to tetrahydrofolate (THF) with hydrogen at the 5,6,7, and 8 positions or to dihydrofolate with hydrogen at the 7 and 8 positions, and they have a one-carbon unit (methyl, methylene, methenyl, formyl, or formimino) at the N-5 or N-10 position, or both (Wagner, 1995). The chemical stability of each form is shown in **Table 1**.

Table 1: Chemical stability of various folate forms

Folate form	Chemical Stability
5-formyl tetrahydrofolic acid	heat stable
10-formyltetrahydrofolic acid	heat stable
5-methyl tetrahydrofolic acid	heat stable
methylenetetrahydrofolic acid	destroyed by acid
methenyltetrahydrofolic acid	quite stable at low heat

Source: Herbert, 1987

2.1.4 Functions of Folate

Folate functions as a coenzyme in the transfer of single carbon atoms in the metabolism of nucleic acid and amino acids (Wagner, 1995). Deoxyribonucleic acid (DNA) synthesis depends on a folate coenzyme for pyrimidine nucleotide biosynthesis involving the methylation of deoxyuridylic acid to thymidylic acid (Combs, 1998). Purine synthesis utilizes folate during the formation of glycinamide ribonucleotide and 5-amino-4-imidazole carboxamide ribonucleotide. Folate is also used for the generation of formate into the formate pool, including the utilization of formate. Folate coenzymes are involved in amino acid interconversions, which include the catabolism of histidine to glutamic acid, interconversion of serine and glycine, and the conversion of homocysteine to methionine (Wagner, 1996). The conversion of homocysteine to methionine serves as a major source of methionine for the synthesis of S-adenosyl-methionine, (SAM) which is an important *in vivo*-methylating agent (Wagner, 1996).

2.2 Folate Metabolism

2.2.1 Absorption, Transport, and Storage

Folates from food (polyglutamate derivatives) are hydrolyzed by an enzyme folyl conjugase (folyl gamaglutamylhydrolase) to monoglutamate forms in the gut (Wagner, 1984), before absorption across the intestinal mucosa has occurred. Conjugase activity occurs both intracellularly and in association with the brush border membrane in the mucosa of the proximal small intestine (Henderson, 1990). The monoglutamate form of folate is actively transported across the proximal small intestine by a saturable, pH-dependent process (Said, 2004). The optimal pH for folate transportation is 5.0-6.0, and folate absorption decreases with an increase in pH (Selhub and Rosenberg, 1996). The transport of folate across the human intestinal brush-border (including laboratory animals: rat and rabbit) membrane is accomplished by a specific carrier mediated process that is pH, but not Na^+ dependent, and electroneutral in nature (Said and Redha, 1987; Said et al., 2000). Folate uptake in intestinal epithelial cells is very low at a pH of 7.4, but increases approximately 8 fold at a pH of 5.5. Uptake at a pH of 5.5 is predominantly carrier-mediated, while uptake at pH 7.4 is non-mediated (Balamurugan and Said, 2006).

When vitamin supplements are consumed, it is the monoglutamate form of folate that is absorbed by a nonsaturable mechanism involving passive diffusion (Wagner, 1996). Monoglutamates, primarily 5-methyl-tetrahydrofolate, are present in the portal circulation. Prior to being stored in tissue, or used as a coenzyme, folate monoglutamates are converted to the polyglutamate form by the enzyme folylpolyglutamate synthetase. Most of the folate can be taken up by the liver, where it is metabolized to polyglutamate residues, and retained or released into the blood or bile.

The human liver is believed to contain 50 percent of the body stores of folate (Hoppner and Lampi, 1980). Since the estimated total body folate store is 12 to 28 mg, it

is subsequently estimated that the liver folate content would be approximately 6 to 14 mg. Sherwood et al., (1993) found that folate is more concentrated in the liver than other organs including the kidney or heart.

When released from tissues into circulation, folate polyglutamates are reconverted to monoglutamate forms by γ-glutamylhydrolase. Folate must be reduced enzymatically and resynthesized to the polyglutamate form to function in single-carbon transfer reactions (Wagner, 1996).

About two-thirds of the folate in the plasma is bound to protein. About half of the bound folate is bound to low-affinity protein binders, primarily albumin, and half is bound to high-affinity protein binders (Wagner, 1996).

2.2.2. Cellular Folate Transport

Cellular transport of folate involves two main systems; one is mediated by a binding protein occurring as different isoforms of the folate receptor. This carrier-mediated process transport system requires energy (glucose) and Na^+ for cellular folate uptake to take place (Combs, 1998). The second system involves a reduced folate carrier anion-exchange system (Combs, 1998) or a folate-binding protein-mediated system. This system is not saturated by folate under physiological conditions (Henderson, 1990); consequently folate influx into the tissues would be expected following any elevation in plasma folate after supplementation. Within cells, THF is methylated to yield 5-methyl-FH_4, which is bound to intracellular macromolecules (Burns and Jackson, 1982). Folate is held in cells by conversion to folyl polyglutamates; polyglutamation traps folates inside cells at concentrations greater than those of extracellular fluids (Anderson et al., 1992).

2.2.3 Subcellular Compartmentation of Folate Metabolism

The role of the folate coenzymes is to carry one-carbon units within the cytosol of a cell. The folate coenzymes play a role in 3 major biosynthetic pathways (Wagner, 1996). The pathways are as follows:

1) Pathway of purine biosynthesis in which folate provides a one-carbon unit at two different steps.
2) The pathway of thymidylate biosynthesis in which a one-carbon unit is transferred to deoxyuridylic acid from 5, 10-methylene tetrahydrofolate and simultaneously reduced to form the methyl group of thymidylic acid.
3) The formation of methionine from homocysteine and 5-methyltetrahydrofolate in a vitamin-B_{12}-dependent reaction. Usually it has been assumed that the concentration of the reduced folates used as substrates for these reactions are greater than the concentration of the enzymes which carry out the catalytic function; and that all enzymes that use the same folate-one-carbon derivative compete for a common pool.

2.2.4 Reduced Folate Carrier (RFC) System

The reduced folate carrier (RFC) is a major folate transporter in human and murine cells (Wang et al., 2005). It is a bidirectional transporter with the properties of an anion exchanger that generates transmembrane gradients by coupling folate transport mediated by RFC to the outward flow of organic phosphates that are present at high concentrations within the cell (Wang et al., 2005). Intestinal folate transport has been well characterized therefore, rat small intestinal epithelial (IEC-6) cells have been used as a model system for the study of this process at the cellular level (Wang et al., 2005). It is currently unclear whether or not the RFC is the only system responsible for folate transport into intestinal epithelial cells. Thus far, there have been no definitive studies

demonstrating the contribution of the RFC system toward folate uptake in intestinal epithelial cells (Balamurugan, and Said, 2006).

A short hairpin ribonucleic acid (shRNA) was used to selectively silence a rat RFC gene (via degradation of its mRNA). Silencing the RFC gene allowed the extent of folate uptake to be measured when the RFC transport system was not functioning. The findings clearly showed that the RFC system is the primary if not the only folate uptake mechanism operating in normal intestinal epithelial cells (Balamurugan and Said, 2006). Results also revealed that the highest distribution of complementary mRNA to the human small intestinal h1FC-1cDNA (intestinal folate carrier 1) is located in the upper half of the villi compared with those at the lower half of the villi and of the crypt. The finding furthermore suggests the importance of the folate carrier system in folate transport because epithelial cells of the upper half of the villi are known to be responsible for most of the nutrient absorption (Nguyen et al., 1997). Additionally, folate transport is higher across the brush-border membrane of the jejunum than the ileum (Said and Redha, 1987).

However, a study by Wang et al., (2005) revealed findings in contrast to Balamurugan and Said (2006). The authors used chemically mutagenized IEC-6 cells to deactivate the RFC system in the cells to test contribution of the RFC system to folate transport (Wang et al., 2005, Balamurugan and Said, 2006). Wang et al., (2005) suggests that folate transport via intestinal epithelial cells does not depend on the RFC system since there was no decline in folate influx when the chemical, methotrexate (MTX) (RFC inhibitant) was removed from the media the cells were consuming. Supposedly, removal of the MTX did not initiate a decline in folate influx due to the fact that the methyltrexate was not taken up by the RFC, thus intestinal epithelial cells were not disabled with

respect to folate uptake. Wang et al., (2005) did not suggest any molecular identity of the RFC system. This contrasting finding could have resulted from the fact that the methyltrexate did not fully inactivate the RFC system, due to incomplete mutagenesis, so folate was able to enter the intestinal epithelial cells at the regular rate.

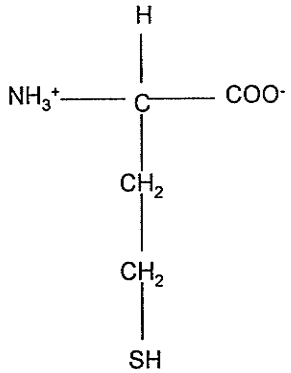
2.2.5 Excretion

The total urinary excretion of folates and metabolites is small (e.g., <1% of the total body stores per day). Folate conservation is effected by the reabsorption of 5-methyl-THF by the renal proximal tubule. Fecal concentrations of folate are usually higher due to the fact that most fecal folate originates from intestinal microflora (Combs, 1998).

2.2.6 Homocysteine Metabolism

Homocysteine (**Figure 2**) is an intermediate sulfur-containing amino acid that is produced from methionine during protein metabolism.

Figure 2. Homocysteine structure



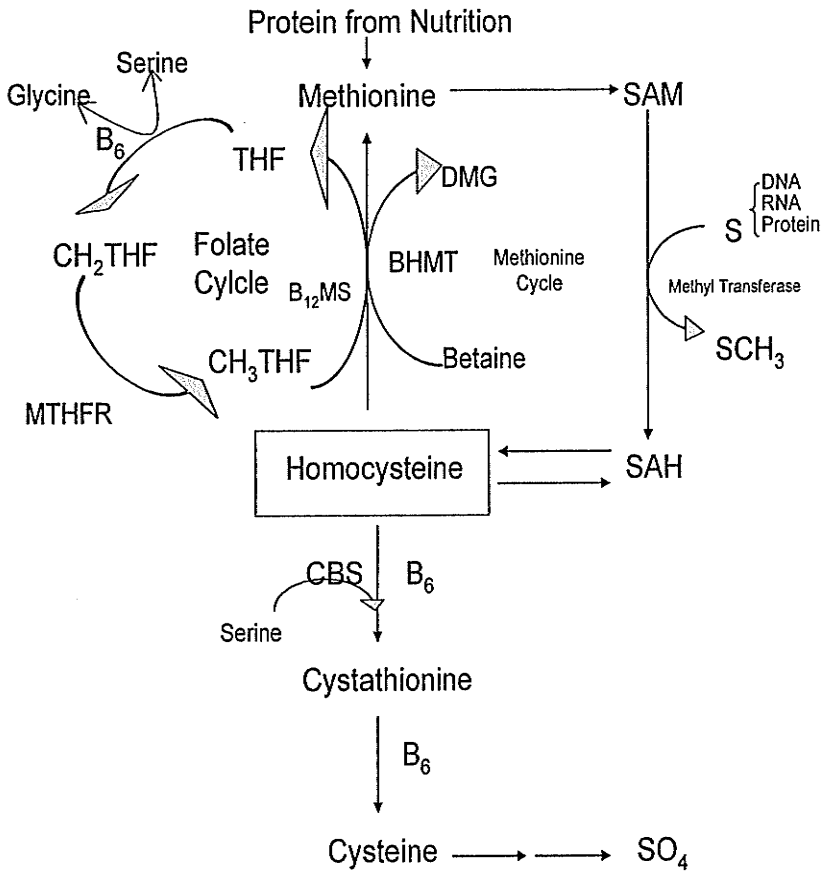
Source: Björklund and Gordon, 2006

There is no specific genetic code for homocysteine, and it is not present in naturally occurring proteins. All the homocysteine found in organisms is formed during

the metabolism of the essential amino acid methionine, in the methylation cycle (**Figure 3**) (Cook and Hess, 2005; Jacobsen, 1998).

Production of homocysteine from methionine, an essential amino acid is the primary purpose of homocysteine metabolism. Homocysteine is remethylated to methionine, which is catalyzed by methionine synthase (MS), which uses B_{12} as a cofactor and methyltetrahydrofolate as a substrate in the presence of 5-methyltetrahydrofolate (CH_3THF). 5-methyletrahydrofolate is the product of 5,10-methylenetetrahydrofolate (CH_2THF) reduction by methyltetrahydrofolate reductase (MTHFR). 5,10 MTHF serves as a substrate for the reversible synthesis of serine from glycine (Selhub and Rosenberg, 1996). This reaction is important because in the opposite direction a new carbon unit from serine is acquired by folate coenzymes for use in a number of syntheses. Under methionine deprivation, homocysteine is disposed via two methionine conserving pathways (Selhub and Rosenberg, 1996). Homocysteine can also be remethylated from betaine to DMG (dimethyl glycine) by betaine: homocysteine methyltransferase (BHMT) in the liver and kidney. In the methionine cycle, dietary methionine is converted to S-adenosylmethionine (SAM), which serves as a methyl group donor substrate for methyltransferases. The other product of this reaction is S-adenosyl homocysteine (SAH), which is hydrolyzed by SAH hydrolase to homocysteine and adenosine. The methionine and folate cycle enzymes are widely distributed. When methionine is in excess, homocysteine is directed to the trans-sulfuration pathway that irreversibly converts homocysteine to cysteine. The first enzyme in this pathway is B_6-dependent cystathionine beta-synthase (CBS).

Figure 3: Homocysteine metabolism



Source: Modified from Cook and Hess (2005), Jacobsen (1998), and Selhub and Rosenberg (1996)

THF – tetrahydrofolic acid

BHMT – betaine homocysteine methyltransferase

CH_2THF - 5,10 methylenetetrahydrofolate

SAM – S-adenosyl-methionine

CH_3THF - 5-methyl-tetrahydrofolate

SAH – S-adenosyl-homocysteine

MTHFR – methyltetrahydrofolate reductase

CBS – cystathionine beta-synthase

$B_{12}MS$ – B12 dependent methionine synthase

DMG – dimethyl glycine

Cystathionine is converted to cysteine by B_6-dependent cystathionase. Cysteine is further catabolized to inorganic sulfate, which is excreted in the urine. The transsulfuration pathway has somewhat limited tissue distribution (liver, kidney, pancreas, and brain).

2.3 Human Nutrition

2.3.1 Recommended Dietary Allowance

The minimum daily folate requirement is defined by Herbert (1977) as the "requirement to sustain a state of well-being, without medication, in which there is no biochemical malfunction induced by an inadequate dietary supply of the vitamin and correctable by increasing the supply". The primary indicator used to estimate the Recommended Dietary Allowance (RDA) for folate is erythrocyte folate in conjunction with plasma homocysteine and folate concentrations (Institute of Medicine, 2000). Folic acid does not occur naturally in substantial amounts in most foods, but due to its stability and commercial availability, it is the form that is used in vitamin supplements, fortified foods, and vitamin premixes.

The concept of dietary folate equivalents (DFEs;1 DFE = 1 μg food folate or 0.6 μg folic acid) for folate was introduced in 2000 (Institute of Medicine, 2000). Folic acid added to or ingested with food is estimated to be approximately 85% available, whereas natural food folate is only 50% available (Pfeiffer, et al., 1997). The folate dietary recommended intakes (DRIs) for adults, pregnant, and lactating mothers are 400 (240 μg folic acid), 600, (360 μg folic acid) and 500 μg (300 μg folic acid) respectively (Said and Mohammed, 2006).

Eggs naturally contain approximately 22 μg folate per large egg (USDA, 2005), which is equivalent to 5.5% (22/400 μg) of the most recently established adult daily requirements for folate (Institute of Medicine, 1998).

2.3.2 History of Folate and Pregnancy

Researchers have studied the treatment of pregnancy-related folate deficiency and megaloblastic anemia (anemia characterized by many large immature and dysfunctional red blood cells, megaloblasts, in the bone marrow; associated with pernicious anemia) since megaloblastic anemia was successfully treated in pregnancy with a yeast extract (Wills, 1931). Studies conducted in the 1950s and 1960s led to the recognition that consuming supplemental folic acid reduced the prevalence of folate deficiency in pregnancy and prenatal folic acid supplementation in the second and third trimesters became a public health practice (Tamura and Picciano, 2006).

In 1970, the US Food and Nutrition Board recommended folic acid supplementation (200-400μg/day) for pregnant women, which became a common practice in developed countries and substantially reduced pregnancy-induced folate deficiency, which can lead to megaloblastic anemia (Food and Nutrition Board, 1970). The level of recommended folic acid supplementation was reduced to 270 μg/day in 1989 primarily because of data showing that this amount was typically ingested by healthy folate-replete adults (Food and Nutrition Board, 1989). The third National Health and Nutrition Examination Survey dietary data (1989-1991) indicated that the mean folate intake of US women of childbearing age was approximately 230 μg/day, (Alaimo, et al., 1994) which is well below the recommended level.

In the 1990s the relationship between maternal folate status and fetal malformations, particularly neural tube defects (NTDs) was confirmed when research proved that periconceptional folic acid supplementation was found to reduce both the recurrence (MRC Vitamin Study Research Group, 1991) and the occurrence (Czeizel and Dudas, 1992) of NTDs. These discoveries led to the mandated folic acid supplementation, periconceptional folic acid supplementation, and folic acid fortification of staple foods, such as flour in the United States and Canada (Tamura and Picciano, 2006). The recommended folic acid intake was subsequently increased to 600 DFEs/day in 1999, after the bioavailability of food folate and folic acid was considered.

In the United Kingdom, the Committee on Medical Aspects of Food and Nutrition Policy (COMA) has recently recommended that all flour be fortified with folic acid at 240 μg/100 g (Institute of Medicine, 2000). In Costa Rica and Chile, the levels of fortification are 40-180 and 220 μg folate/100 g product respectively (Tamura and Picciano, 2006). Neural tube defect reductions after fortification were reported to be 35% and 40% in Costa Rica and Chile respectively (Chen and Rivera, 2004; and Hertrampf and Cortés, 2004). Food folate fortification recently began in Brazil in the summer of 2004 (Tamura and Picciano, 2006). Data from a number of studies (Willoughby and Jewell, 1966; Hansen and Rybo, 1967; and Chanarin, et al., 1968) suggest that 200-300 μg folic acid/day is needed in addition to dietary folate to maintain normal folate status and to prevent folate deficiency in pregnancy. In the past few years in countries with folic acid food fortification, it has become feasible for pregnant women to achieve this level of intake (Tamura and Picciano, 2006).

Aside from the basic functions of folate with respect to cellular metabolism, folate plays a role in maintaining homocysteine at a level low enough to lower incidence of neural tube defects in human babies, and cardiovascular disease, and inflammatory diseases in adults.

2.3.3 Neural Tube Defects

Published data has shown that periconceptional folate supplementation in females that become pregnant will prevent the occurrence of neural-tube defects. Neural tube defects are most commonly thought to occur due to failure of the sides of the neural tube to rise, or a failure of the neural tube margins to seal, or some combination of the two (Björklund and Gordon, 2006). Neural tube defects are the most common major congenital malformation of the central nervous system. They develop as a result of a disturbance of the embryonic process of neurulation, and are midline defects that affect neural tissues, their coverings anywhere along the neuraxis or both. Neurulation is the first organogenetic process, which begins at approximately 21 days post-fertilization, and is complete by 28 days. Therefore, neurulation is ongoing before a female may first recognize her pregnancy (Wagner, 1996).

Published data indicating that folic acid can help avert NTD's when given at high dose levels of 4.0 mg per day has led to folic acid fortification of grain products (140 μg folic acid per 100 g flour) which was mandated by the Food and Drug Administration in the US in 1996, and was fully in place by January 1, 1998, resulting in population wide increases in serum folate concentrations (Centres for Disease Control and Prevention, 2002). This implemented regulation produced a 20% decrease in rate of NTDs (Honein,

et al., 2001; Williams et al., 2005). Health Canada also implemented mandatory folic acid fortification (150 μg folic acid/100 g of flour) in 1998 (Health Canada, 1998).

It is hypothesized that the mechanism explaining the formation of NTDs involves metabolism of folic acid for DNA synthesis. Folic acid provides a critical methyl group to create a high energy methylating intermediate, SAM. Folic acid provides a methyl (CH_3) group for homocysteine, which is then converted to methionine. The enzyme S-adenosylmethionine synthase (also known as methionine adenosyltransferase) joins a methionine to an adenosine triphosphate by linking the sulfur of the methionine to the 5' carbon of the ribose unit, which forms SAM. SAM is the most important methylating reagent in the cell, required in enormous quantities for proper construction and functioning of DNA, proteins, and lipids. As developing embryos are rapidly creating new cells, they are especially sensitive to reduced levels of folates. Ectoderm and early neural epithelial tissue may be the most sensitive of all embryonic tissues. The neural plate epithelium expresses extremely high levels of messenger RNA for folate receptors when compared to other embryonic tissues such as the developing heart (Rosenquist, et al., 1996; Rosenquist and Finnell, 2001; Björklund and Gordon, 2006).

SAM is converted to S-adenosyl homocysteine (SAH) when the methylation reaction occurs. SAH is a feedback inhibitor of all the enzymes that create SAM, and can therefore be viewed as a potentially toxic waste product (Björklund and Gordon, 2006). The ratio of SAM to SAH is tightly regulated in each cell type. Following the methylation reaction, the adenosyl portion of SAH is detached, leaving homocysteine. Homocysteine must be quickly condensed to cysteine via the transsulfuration pathway, or recycled back to methionine (remethylation), or exported from the cell, to avoid causing

elevated SAH levels (Björklund and Gordon, 2006). A build up of homocysteine in the cells results in the reversal of the hydrolase reaction that changes SAH to homocysteine, causing an increase of SAH (Björklund and Gordon, 2006). This increase of SAH will inhibit the production of SAM, reducing methylation reactions throughout the cell and impeding normal cellular functions, such as cell differentiation, leading to neural tube defects.

2.3.4 Homocysteine and Cardiovascular Disease

Higher than normal plasma homocysteine levels are associated with an increased risk of cardiovascular events. Homocysteine accumulation (called hyperhomocysteinaemia, >10 μmol of homocysteine/L) depends on potential blocks in the metabolizing pathways (Cook and Hess, 2005). Folate (vitamin B_9), pyridoxine (vitamin B_6), and cobalamine (vitamin B_{12}) function as cofactors or substrates for methionine- homocysteine metabolism. Homocysteine is widely accepted as a major independent risk factor for cardiovascular, cerebrovascular, and peripheral vascular disease (Jacobsen, 1998). Folic acid is the most important dietary determinant of homocysteine (HOPE investigators, 2006). Daily supplementation with 0.5 to 5.0 mg typically lowers plasma homocysteine levels by about 25 percent (HOPE investigators, 2006). In plasma normally only about 1% of homocysteine exists in the free reduced form. About 70% of the plasma homocysteine is bound to albumin. The remaining 30% of plasma homocysteine forms disulphides, predominantly with cysteine or as the homocysteine dimer homocystine. Relatively little is known about the formation of different homocysteine species *in vivo* (Bolander-Gouaille, 2002). The sum of all forms is termed total homocysteine.

The most widely accepted mechanism of action to explain atherogenesis is as follows: At low plasma concentrations homocysteine is rapidly scavenged by nitric oxide (NO), (which is produced by endothelial NO synthase) resulting in S-nitroso homocysteine. The nitroso-compound has nitric oxide functionality including potent vasodilatation, regulation of glucose metabolism and oxidative modification of low-density lipoproteins, inhibition of proliferation of vascular smooth muscle cells, platelet aggregation and leukocyte adherence (Cooke and Scherrer, 2002; Stamler et al., 1992). At higher plasma concentrations, hyperhomocysteinemia is responsible for endothelial dysfunction, platelet activation, and thrombus formation in vivo (Celermajer et al., 1993; Harker et al., 1974; Van den Berg et al., 1995; Welch et al., 1998). When added to plasma, homocysteine is rapidly auto-oxidized and forms homocysteine-thiolactone, which is homocysteine mixed disulfides and directly or indirectly reactive oxygen species (Alvarez-Maqueda et al., 2004). The latter have been involved in a number of atherothrombogenic effects such as vasoconstriction, oxidation of low-density lipoproteins, lipid peroxidation, proliferation of vascular smooth muscle cells and prothrombotic states. Homocysteine appears to also alter mitochondrial function and to cause mitochondrial-mediated cellular apoptosis (Austin et al., 1998; Cook and Scherrer 2002; Mercie et al., 2000; and Olszewski and McCully, 1993).

A substantial amount of epidemiological evidence suggests an association between cardiovascular risk and moderately increased plasma homocysteine levels (Eikelboom et al., 1999). The epidemiologic analysis presented by Yang et al., 2006 does not establish causality, but the trends observed were consistent with the hypothesis that folic acid fortification is contributing to a reduction in stroke deaths. The benefits of

folic acid fortification appeared to benefit all members of the population regardless of ethnic, social, and economic barriers that have prevented the benefits of many advances in prevention and treatment for women, ethnic minorities, the poor, and the uninsured (Yang et al., 2006).

The homocysteine/CVD (cardiovascular disease) association seems to be strong, dose related, independent of other risk factors and biologically plausible, however not all study results are consistent, and it remains to be proven that reducing elevated plasma homocysteine levels via folic acid supplementation will in turn reduce the risk of CVD.

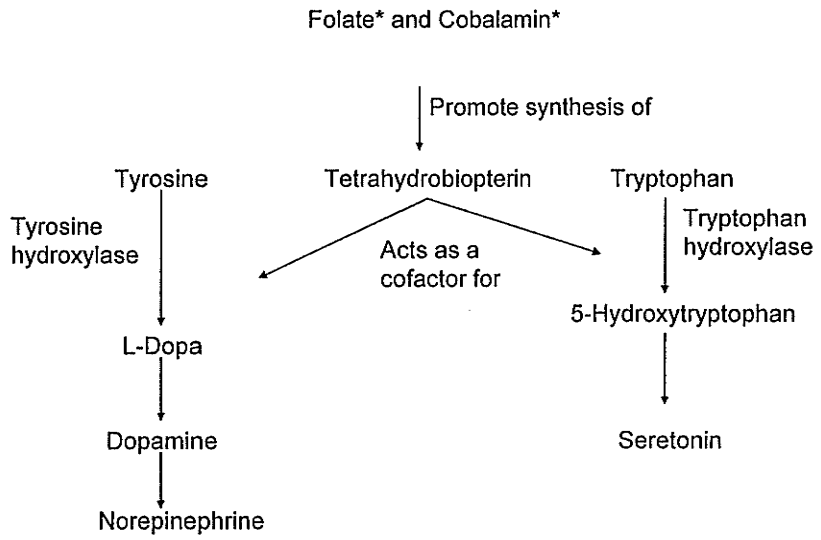
2.3.5 Inflammatory Conditions

Low serum folate levels have been related to Alzheimer's disease, and all types of dementia in addition to vascular diseases (Wang et al., 2001). Alzheimer's disease (AD) accounts for more than 70 percent of all cases of dementia (Seshadri, et al., 2002; Brookmeyer et al., 1998). Following a decade of research evaluating the relationship between vascular disease and Alzheimer's, it is now recognized that subjects with cardiovascular risk factors have an increased risk of both vascular dementia and Alzheimer's disease (Seshadri et al., 2002; Hofman, et al., 1997; Breteler, 2000; Snowdon, et al., 1997).

Vascular dementia is a term that is currently used to define any type of dementia which results from cerebral blood vessel disease (Micieli, 2006); for example people that suffer from cognitive dysfunction and behavioural changes following a stroke. Low folate levels have been found to be associated with specific areas of cognitive functioning, such as episodic recall and recognition (Wang et al., 2001, Hassing et al., 1999; Wahlin et al., 1996). Some studies have also shown that folate treatment has a

positive effect on memory deficits (Wang et al., 2001; Fioravanti, et al., 2002; La Rue, et al., 1997). Various mechanisms of action have been proposed to explain folate's relation to psychiatric conditions. In the brain, folate participates in the synthesis of monoamine neurotransmitters. Folate may influence the rate of synthesis of dopamine, norepinephrine, and serotonin by promoting the synthesis of tetrahydrobiopterin (BH_4), a cofactor in the hydroxylation of phenylalanine and tryptophan (Hutto, 1997) (**Figure 4**).

Figure 4: Synthesis of Norepinephrine and Seretonin Involving Folate and Cobalamin



*Cobalamin and folate indirectly promote the synthesis of monoamine neurotransmitters.

Source: Hutto, 1997

The interaction of folate with S-adenosylmethionine (SAM) is related to psychiatric conditions. Folate facilitates the production of SAM by promoting the conversion of homocysteine to methionine. SAM accepts or donates one-carbon units, like folate does, and it independently influences the rate of serotonin and possibly norepinephrine and dopamine synthesis (Hutto, 1997). Clinical trials have shown that SAM has demonstrated antidepressant activity (Hutto, 1997; Kagan et al., 1990).

Both folate and vitamin B_{12} deficiencies may result in similar neurologic and psychiatric disturbances, possibly indicating their involvement in the same biochemical pathway (Bottiglieri, 1996). Folate deficiency may affect the central monoamine neurotransmitter metabolism and lead to an increase in depressive disorders. There is also sufficient evidence that impaired methylation has some etiological significance in depression, dementia, and some demyelinating disorders (Bottiglieri, 1996).

2.4 Sources of Folate

Folates are most commonly found in animal liver, mushrooms, green leafy vegetables such as spinach and collard greens (Combs, 1998). With respect to feedstuffs for animals, oilseed meals including soybean meal and animal by-products are important sources of folate (Combs, 1998). Please refer to **Table 2** for a more extensive list of selected foods and their folate contents.

Food analyses have shown the predominant forms of folate found in food are 5-methyltetrahydrofolate (5-MTHF) and 10-formyltetrahydrofolate (10-FTHF) (Combs, 1998).

Folate is an essential micronutrient for humans and other mammals, therefore it cannot be synthesized *in vivo*, and must be obtained from exogenous sources via intestinal absorption. The intestine is exposed to two major sources of folate: a dietary source where the vitamin is absorbed in the small intestine, and a large intestine bacterial source, where folate is synthesized by the normal microflora and absorbed by the large intestine. The intestine plays a crucial role in controlling and regulating body folate homeostasis (Said et al., 2000). Interference with intestinal absorption could lead to a vitamin deficiency. Interferences could occur in a variety of conditions including

congenital defects in the digestive/absorptive systems, intestinal resection and diseases, drug interactions, and chronic use of alcohol (Said and Mohammed, 2006).

Table 2. Food sources of folate

Description	Weight (g)	Common Measure	Content per Measure (µg)
Cereals ready-to-eat, Whole Grain TOTAL	30	3/4 cup	807
Rice, white, long-grain, parboiled, enriched, dry	185	1 cup	797
Cornmeal, degermed, enriched, yellow	138	1 cup	501
Turkey, all classes, giblets, cooked, simmered, some giblet fat	145	1 cup	486
Wheat flour, white, bread, enriched	137	1 cup	395
Chicken, broilers or fryers, giblets, cooked, simmered	145	1 cup	373
Lentils, mature seeds, cooked, boiled without salt	198	1 cup	358
Cowpeas, common (blackeyes, crowder, southern), mature seeds, cooked, boiled, without salt	172	1 cup	358
Orange juice, frozen concentrate, unsweetened, undiluted	213	6-fl-oz can	330
Bread, Indian, fry, made with lard (Navajo)	160	10-1/2 inch bread	314
Beans, pinto, mature seeds, cooked, boiled, without salt	171	1 cup	294
Chickpeas(garbanzo beans, bengal gram), mature seeds, cooked, boiled without salt	164	1 cup	282
Fast foods, french toast sticks	141	5 sticks	275
Okra, frozen, cooked, boiled, drained, without salt	184	1 cup	269
Spinach, cooked, boiled, drained, without salt	180	1 cup	263
Beans, black, mature seeds, cooked, boiled, without Salt	172	1 cup	256
Asparagus, frozen, cooked, boiled, drained, without salt	180	1 cup	243
Bread crumbs, dry, grated, seasoned	120	1 cup	226
Beef, variety meats and by-products, liver, cooked, pan-Fried	85	3 oz	221
Noodles, egg, cooked, enriched	160	1 cup	221
Spinach, canned, drained solids	214	1 cup	210
Spaghetti with meat sauce, frozen entrée	283	1 package	204
Bagels, plain, enriched, with calcium propionate (includes onion, poppy, sesame)	89	4 inch bagel	201
Soybeans, green, cooked, boiled, drained, without salt	180	1 cup	200
Collards, cooked, boiled, drained, without salt	190	1 cup	177
Snacks, pretzels, hard, plain, salted	60	10 pretzels	172

Table 2. Food sources of folate (continued from previous page)

Description	Weight (g)	Common Measure	Content per Measure (µg)
Turnip greens, cooked, boiled, drained, without salt	144	1 cup	170
Broccoli, cooked, boiled, drained, without salt	156	1 cup	168
Macaroni, cooked, enriched	140	1 cup	167
Spaghetti, cooked, enriched, without added salt	140	1 cup	167
Leavening agents, yeast, baker's, active dry	7	1 package	164
Beef Macaroni, frozen entrée	240	1 package	158
Brussels sprouts, frozen, cooked, boiled, drained, without salt	155	1 cup	157
Lettuce, iceberg (includes crisphead types), raw	539	1 head	156
Lima beans, large, mature seeds, cooked, boiled without salt	188	1 cup	156
Noodles, egg, spinach, cooked, enriched	160	1 cup	150
Fast foods, shrimp, breaded and fried	164	6-8 shrimp	136
Beets, cooked, boiled, drained	170	1 cup	136
Collards, frozen, chopped, cooked, boiled, drained, without salt	170	1 cup	129
Peas, split, mature seeds, cooked, boiled, drained, without salt	196	1 cup	127
Papayas, raw	304	1 papaya	116
Corn, sweet, yellow, canned, cream style, regular pack	256	1 cup	115
Chicken, liver, all classes, cooked, simmered	256	1 cup	113
Seeds, sunflower seed kernels, dry roasted, with salt Added	32	1/4 cup	76
Raspberries, frozen, red, sweetened	250	1 cup	65
Cauliflower, raw	100	1 cup	57
Potato, baked, flesh and skin, without salt	202	1 potato	57
Bread, rye	32	1 slice	48
Muffins, corn, dry mix, prepared	50	1 muffin	45
Wild rice, cooked	164	1 cup	43
Plantains, cooked	154	1 cup	40
Mushrooms, shiitake, cooked, without salt	145	1 cup	30
Bananas, raw	150	1 cup	30
Cheese, cottage, creamed, large, or small curd	210	1 cup	25
Egg, yolk, raw, fresh	16.6	1 large	24

Source: Adapted from USDA, 2005

2.5 Animal Nutrition

2.5.1 Folate Intake

The nutritional status of animals with respect to adequate folate intake is assessed through an estimation of folate concentrations in serum or red blood cells (National Research Council, 1987). Additionally, animal growth rates, and maintenance of normal hematological responses have been used to measure folate adequacy (National Research Council, 1987). Research trials have determined that folate requirements range from 0.25 to 1.0 mg/kg of diet for chickens and 1 to 6 mg/kg of diet for rats and guinea pigs. Sulfa drugs, which are often added to commercial chick diets, increase the folate requirement (National Research Council, 1987). There are currently no folate requirements determined for ruminant animals and horses (National Research Council, 1987). The swine folate requirement is less than 1 mg/kg of diet.

The current estimated requirement for folic acid for laying hens, based on experiments conducted in the 1950s using productivity as the response criteria is between 0.21 to 0.31 mg/kg (NRC, 1994). This folic acid requirement level is well below the estimated level of folate in an unsupplemented layer diet, which is approximately 1.0 mg/kg of diet (Hebert, 2005) depending on the type of grain used in the diet.

Folate supplementation may provide health benefits for laying hens consuming the diets. Research performed by Hebert (Thesis, 2005) showed that one of two strains of laying hens (Hy-Line W98) benefited from the increased folate supplementation by decreasing their plasma homocysteine content. Mortality throughout the eleven month trial was significantly lower (p-value = 0.0013) in the 4 mg/kg folic acid supplemented diet (2.24%) than the 0 mg/kg folic acid supplemented diet (2.56%). The number of

mortalities in the trial was perhaps too low to make conclusions regarding the relationship between folic acid supplementation and mortality. In light of homocysteine being an independent risk factor for cardiovascular disease in humans (Jacques, et al., 1999) it is possible that homocysteine may also be related to mortality in laying hens. To address this issue post mortems should be conducted on mortalities to determine cause of death throughout another long term folic acid/laying hen trial.

2.5.2 History of Folate Research in Poultry

Folic acid, the term which was originally used by Mitchell et al. in 1941 to refer to a growth factor in spinach leaves, was isolated in 1943 by a team of scientists, followed by the identification and synthesis of pteroylglutamic acid in 1945 (Stokstad, 1992). Much of the initial work with folate was done to determine the amount required in poultry diets to ensure healthy birds. Scientific work progressed to more than ensuring adequate poultry health, but rather striving to optimize bird health in addition to healthy chick development thus promoting life long bird performance. The 1990s introduced a new wave of folate studies, with the objective of using folate in hen diets as a means of production optimization, and responding to consumer demand with respect to developing a functional food.

2.5.3 Hatchability and Chick/Poult Development

Schweigert et al., (1948) found young turkey poults require a dietary source of folic acid or else deficiency results, which is associated with retarded growth, moderate anemia, cervical paralysis (Kratzer et al., 1956), and high mortality. Deficiency of folic acid in the hen's diet results in a high incidence of embryonic deaths on the 20th day of development (Sunde et al., 1950a). Embryos appeared to be normal, but died soon after

pipping the air cell. Some embryos exhibited bending of tibiotarsus; other less frequently observed symptoms were syndactyly (a union of two or more toes), deformed mandibles and parrot beak (Sunde et al., 1950a).

Tolomelli et al., (1981) concluded from his research that total folate activity of the whole egg did not change significantly during incubation and at zero time, total folate activity was represented only by monoglutamate forms. The embryo is dependent on folic acid present in the egg for its metabolic needs, and only the monoglutamate forms can easily pass through the membranes. Following absorption, the embryo converts the vitamin from monoglutamate to the polyglutamate form, the form that is more easily retained by the tissues (Tolomelli et al., 1981). It was also found that more folic acid was deposited in eggs of hens that had been supplemented with folic acid. In general, higher mortality and slower growth rate occurred when young poults and hens were fed diets low in folic acid than when they were fed diets supplemented with folic acid. Jukes et al., (1947) found that the inclusion of 0.8 mg of folic acid/kg of diet appeared to be adequate to maintain healthy turkeys and chickens.

Sunde et al., (1950b) found that about 0.25 mg of folic acid/kg of diet was required for maintenance of body weight of the laying hen. Zero point two five mg/kg may be slightly suboptimal, due to the fact that there was a decline in egg production and weight within the middle of the experiment (Sunde et al., 1950b). There was a rapid decline in hatchability of eggs from hens receiving 0.25 mg of folic acid/kg of diet; primarily due to the fact the chicks did not have the strength to break the egg shell during pipping (Sunde et al., 1950b). Data showed 1.0 mg of folic acid/kg diet was required in the maternal diet to assure survival of chicks for a 3-week period, when fed a ration low

in folic acid (Sunde et al., 1950b). About 0.5 mg/kg of folic acid was required for good hatchability (Sunde et al., 1950b). An injection of 5 μg or more of folic acid into the egg prior to incubation resulted in an increase in hatchability (Sunde et al., 1950a). Lee et al., (1965) concluded that folacin requirements of the turkey breeder hen were approximately 1.23 mg/kg of diet for optimum hatchability and progeny performance.

Robel (1993) stated that based on his data in addition to previous data (Schweigert, et al., 1948; Ferguson et al., 1961; and Sirbu et al., 1981) hatchability and poult weight are related to folic acid in the egg. Due to the role of folic acid in cellular development, higher supplemental folic acid levels may be required for normal embryonic development. The supplemental folic acid treatment (5.51 mg folic acid/kg of diet) resulted in the deposition of 144 μg folic acid/82 g of egg, in contrast to the 104 μg folic acid/egg by hens fed 2.64 mg folic acid/kg of diet. It is possible that greater folic acid transfer to the egg advanced nutrient utilization by extending folic acid functions in intermediary amino acid metabolism and biosynthesis of nucleotides for greater embryonic cellular development (Robel, 1993). Regardless of the stage of egg production, there were no significant differences ($P>0.05$) in hatchability of fertile eggs injected with folic acid as compared with eggs injected with the vitamin carrier solution and control (uninjected) eggs (Robel, 1993). No statistically significant differences in average hatchability of turkey eggs from hens fed the practical corn-soybean meal based diet with or without supplemental folic acid were observed (Robel, 1993).

2.5.4 Factors Affecting Folic Acid Requirements for Poultry

When an antibiotic, sulfasuxidine (succinylsulfathiazole) was included in the basal diet, the chicks receiving no supplemental folic acid developed a more severe

deficiency, and under these conditions, 25μg of synthetic folic acid per 100g of diet was not sufficient to overcome the folate deficiency in the chicks. A comparison of the activity of synthetic folic acid with and without the addition of 1 per cent sulfasuxidine in the diet indicated that folic acid was about 3 times as effective without the antibiotic (Luckey et al., 1946). In 1947, Taylor determined that 25 μg/100 g of diet was the folate requirement for optimum chick survival to 6 weeks and 35 μg/100 g for hemoglobin formation at 6 weeks of age. Forty-five micrograms of folic acid/100g of diet was the optimum level to obtain growth and hemoglobin formation at 4 weeks of age. The requirement was only 12 μg/100 g for normal rates of egg production for yearling hens.

Folic acid requirement of chicks was found to be increased when chicks were fed high protein and high fat diets (March and Biely, 1956). Practical diets containing soybean meal are thought to contain adequate folic acid (Saxena et al., 1954, Ryu, et al., 1995). Folic acid supplementation increased chick performance only when supplements of methionine and choline were not present. Labile methyl group requirements, to be met by methionine, or choline or both, should be determined only when adequate folic acid is present in the diets (Pesti et al., 1991). Dietary supplemental folic acid makes more methyl groups available to remethylate homocysteine to form methionine *in vivo*.

Adding folic acid to the diets of broiler chicks, for example, has been shown to cause large increases in growth (Ryu et al., 1995). The broiler chickens in the experiment by Ryu et al., (1995) required 1.45 mg/kg total folic acid in their diet for maximum performance (with no supplemental methionine or choline), approximately 3 times the NRC (1984) estimated requirement (0.55 mg/kg), but similar to levels suggested by Whitehead and Rennie, (1989). Whitehead and Rennie (1989) determined that 1.5 – 2.0

mg folate/kg was needed to maximize the activity of the histidine oxidation metabolic pathway in broilers.

Due to the changes in environment and genetics of modern strains of rapidly-growing broilers, vitamin requirements of broiler birds have changed. Wong et al., (1977) estimated the requirement of broilers to be between 0.27 and 0.44 mg/kg, depending on the source of the protein in the feed. NRC (1984) requirements for folate in broiler diets was set at 0.55 mg/kg, and has not since been altered (NRC 1994). Rennie et al., (1993) suggested that the measurement of erythrocyte phosphoribosylpyrophosphate (PRPP) and dihydrofolate reductase (DHFR) activity may be a useful criterion of folate status in broilers. PRPP is the required substrate for those reactions in cells in which adenine, hypoxanthine, and guanine are directly converted to ribonucleotides. It is also a required substrate in the ten-step biosynthetic pathway for the *de novo* synthesis of inosine monophosphate which is used to synthesize purines. Folate is required at two stages in this pathway in the form of 10-formyltetrahydrofolate (Rennie, et al., 1993). DHFR is a key enzyme in folate metabolism, catalyzing the reduction of dihydrofolic acid to tetrahydrofolic acid (THF) with NADPH (nicotinamide adenine dinucleotide phosphate) providing reducing power (Rennie, et al., 1993). The results of these trials suggested that the functions of all folate-dependent pathways tested were normalized by dietary folate supplements of 1 mg/kg.

Whitehead et al., (1997) also examined the metabolism of folate in chicks. The folate-dependent step in oxidation of histidine is catalyzed by GFT (glutamic acid formiminotransferase). There was a significant decrease in histidine oxidation for the

basal diet containing no added folate. Additionally, the oxidation was less with the diets containing 1.0 and 1.5 mg folate/kg of diet than with the diet containing 4 mg.

Benefits of the addition of folic acid to broiler diets extend beyond the increased biochemical pathway utilization. For broilers fed folic acid supplemented wheat and maize-based diets bodyweight and feed conversion efficiency were improved, with optimal responses occurring at supplemental levels of 1.5 mg folic acid/kg of diet (Whitehead et al., 1997). However, it was recommended to use 2.5-3.0 mg/kg diet since diets are often pelleted and processed to decrease incidence of Salmonella, which may decrease available folic acid (Whitehead et al., 1997).

There is adequate evidence that the NRC (1984, 1994) folic acid recommendation for broiler chicks of 0.55 mg/kg is reasonable for slow-growing chicks fed purified or semi-purified diets (March and Biely, 1956; Creek and Vasaitis, 1963; Wong et al., 1977) and thus represents the true minimum requirement. However, the data presented by Ryu et al., (1995) with faster growing broilers fed practical ingredient-based diets shows that the requirement may be higher under specific circumstances, particularly when low levels of choline are fed.

Low levels of an important nutrient such as choline in the diet can create a sudden demand for methyl groups to support biosynthesis (Rees et al., 2006). Choline and its metabolite, betaine, are involved in one-carbon (methyl donors) metabolism for methylation of homocysteine to methionine and are utilized for the synthesis of cell membrane phospholipids (Shaw et al., 2004). Choline is a precursor for the formation of phosphatidylcholine (lecithin, membrane constituent), betaine (a methyl donor), and acetylcholine (a neurotransmitter) (Shronts, 1997). Methionine, methyl-folate, and

vitamin B_{12} are so closely intertwined with choline in methyl-group metabolism that a disturbance in one affects the others (Shronts, 1997). Choline is in great demand as a methyl donor, and when folate is not available for methyl transfer reactions, other methyl transfer reactions such as the production of choline via SAM are hampered (Shronts, 1997). Methylation of DNA can be influenced by dietary contributions of methyl donors such as choline (Shaw et al., 2004), which is important in rapidly growing animals including broilers.

2.5.5 Preventing Folic Acid Deficiency in Poultry

In 1946, Luckey determined that the addition of 25 μg of synthetic folic acid/100g (0.25 mg/kg of diet) to the basal ration consistently prevents reduced growth, poor feathering condition, and low hemoglobin and hematocrit values which occurred in chicks fed the basal ration. In 1977, Wong et al., concluded that 0.36 mg total folacin per kg of diet was adequate for normal growth and normal hematocrit values, as well as for the prevention of leg weakness and cervical paralysis in growing chicks. NRC (1971) recommended 0.9 mg folic acid/kg of diet to meet the nutritional requirement of poults.

Maxwell et al., (1988) noted that anemia was one indicator of folic acid deficiency in chicks. Anemia was characterized by decreases in hemoglobin concentrations and numbers of erythrocytes, leucocytes and thrombocytes, together with increases in erythrocyte size and proportion of heterophils relative to lymphocytes. Macrocytic anaemia is caused by defective DNA synthesis and inadequacies in pathways involving cobalamin or folate. He concluded that the abnormalities responded to increasing levels of dietary folic acid, but a supplemental level between 2 and 4 mg/kg of diet was needed for all the responses to reach a plateau. This information was contrasting

to the chick growth requirement estimated by the (US) National Research Council (1984) to be 0.55 mg/kg.

2.6 Folate Deposition

2.6.1 Yolk Biosynthesis and Deposition

The rate of development of yolk cells in the hen from the primordial germ cells to the completed ovum occurs in 3 phases: 1) Before the bird reaches maturity 2) After a rest period 3) Final deposition of the yolk occurs 7-11 days before ovulation (Burley and Vadehra, 1989)

In sexually mature laying hens, when vitellogenesis occurs, the follicle enlarges due to massive cytoplasmic deposition of nutrient reserves for use by the prospective embryo (Wallace and Ho, 1972). During this phase under estrogenic influence, the maternal liver responds with *de novo* synthesis and secretion of several yolk-specific proteins which are transported through the circulation to the developing ovarian follicles to be selectively chosen (sequestered by the oocyte surface by micropinocytosis) (Wallace and Jared, 1976, and Gruber et al., 1976). Micropinocytosis is the incorporation of macromolecules or other chemical substances into cells by membrane invagination and the pinching off of relatively minute vesicles.

2.6.2 Folate Deposition in the Egg Yolk

The ovary is the site of assembly of the primordial germ cells in the embryo (Burley and Vadehra, 1989; Mine and Kovacs-Nolan, 2004), which are later transformed into oocytes. Each oocyte becomes a follicle after being covered with the granulose and theca cell layers. Yolk constituents are synthesized in the liver and are transported to the follicular walls via the blood. The follicle undergoes a rapid development during which

most of the yolk is deposited prior to ovulation. When sufficient yolk has accumulated, (12-14 days) the ovulated ovum enters the oviduct (Burley and Vadehra, 1989; Okubo et al., 1997). Yolk riboflavin binding protein (RBP) is synthesized in the liver in response to estrogen secreted into the bloodstream where it picks up riboflavin and transports riboflavin to the developing follicle where it is deposited in the yolk as a protein-vitamin complex (White and Merrill, 1988). Due to the similarities between RBP and folate binding protein (FBP) that have been noted, it is well justified to believe that folate is transported into the yolk via a similar method (Henderson, 1990; White and Merrill, 1988). The amino acid sequence of RBP is homologous with bovine milk folate-binding protein (Zheng et al., 1988). A search of the protein sequence data bank revealed a significant similarity of RBP to a fragment of bovine milk folate-binding protein (Svendsen, et al., 1979,). The recognition of homology between RBP and FBP establishes another family of high affinity, soluble, transport proteins (White and Merrill, 1988). The structures of both RBP and FBP contain phosphoryl groups, which are necessary for the transport of serum RBP into the follicle (Miller et al., 1982a). This discovery of the vitamin-binding protein is one of a number of nutrient-transport proteins now known for the chicken egg (White, 1987). Folates and flavins are functionally distinct in their catalytic function in cells, however, they are structurally similar in that they both have a pterin ring system. Therefore, it is possible that an ancestral protein had the capacity to bind riboflavin and folate, or that a few amino acid replacements could interconvert the binding specificities (White and Merrill, 1988).

2.6.3 Nutrient Transport System

The hen has the ability to utilize the nutrient transport system in partitioning nutrients to fulfill maternal and embryonic requirements. Therefore, there must be a biological capacity of each transport system. The mechanism by which the flavins are bound to the riboflavin binding proteins has been physically and chemically evaluated. Nowak and Langerman (1982) studied the thermodynamics of the binding of flavin mononucleotide and flavin adeninedinucleotide by the binding proteins of the hen's egg yolk and albumen. The amount of binding protein limits the amount of vitamin deposited in the egg (Sherwood et al., 1993). Therefore in a folate-deficient situation there would be fewer folate binding proteins and less folate deposited in the egg yolk (Sherwood et al., 1993). Data presented by White et al., (1986) demonstrated that the amount of riboflavin in an egg is limited by the amount of riboflavin-binding protein, and that even at a high riboflavin intake, little if any unbound riboflavin is found in the egg. The deposition of riboflavin in yolk involves the direct transfer of the vitamin-protein complex from the plasma (White et al., 1986). Results from Miller et al., (1982b) also suggested that apo- (riboflavin-binding protein) is deposited in the yolk. Considering the similarities of riboflavin-binding proteins, and folate-binding proteins, it can be speculated that egg folate deposition is dependent on the amount of folate-binding proteins present.

The folate content of egg yolk saturates with increasing dietary folate. This saturation is not due to limits on the system that transfers folate from plasma across the oocyte vitelline membrane to yolk because yolk folate concentrations are proportional to plasma folate concentrations over the entire dietary range that was studied (Sherwood, et al., 1993). It was observed by House et al., (2002) that egg folate concentrations

increased by 2 to 4 fold when as much as 32 mg of crystalline folic acid/kg of diet was fed to laying hens. Egg folate levels reached a plateau between 2 and 4 mg crystalline folic acid/kg of diet, above which no further significant increases in egg folate content occurred until folate dietary concentration levels reached 32 mg/kg of diet.

House et al., (2002) speculated that there was a biphasic response to egg folate deposition due to the fact that there was a significantly higher egg folate concentration present with 32 mg of dietary folic acid/ kg of diet. Therefore, it could be speculated that above a critical inclusion level for dietary folate, folate accumulation in the egg yolk may surpass the saturable processes via another mechanism. However, it was recently determined that a biphasic response does not exist for egg folate content (Hebert et al., 2005); as increasing dietary folate levels up to 128 mg of folic acid/kg diet did not lead to consistent increases in egg folate content over and above those levels achieved at plateau.

2.6.4 Intestinal Absorption of Viscous Feedstuffs

When blood plasma folate in the hen is maximized, so is the egg folate deposition, therefore other critical points exist within the process of folate metabolism that must be considered to elucidate the mechanism responsible for maximal folate deposition. Perhaps the critical control point is before the plasma folate step, when folate is absorbed in the intestine.

Nonstarch polysaccharides (NSPs) must be considered when feeding poultry because NSPs are poorly utilized, and some soluble NSPs have been shown to have anti-nutritive properties in poultry diets (Fengler and Marquardt, 1988; Bedford and Classen, 1992; Choct and Annison, 1992). It has been demonstrated (Burnett, 1966) that the highly viscous water-soluble β-glucans in barley and oats are the primary growth-

depressing factors limiting the bioavailable energy of these cereal grains. Barley β-glucans increase digesta viscosity and consequently decrease absorption of nitrogen and carbohydrate (Burnett, 1966; Hesselman and Aman, 1986). It is well established that NSPs impair nutrient digestion in poultry (Choct and Annison, 1992). For example, the high amounts of soluble arabinoxylans in rye and β-glucans in barley are responsible for the cereals' poor nutritive values for poultry (Antoniou et al., 1981; Campbell, et al., 1989). It is believed that the viscous nature of the NSPs is the primary cause for their anti-nutritive effect in poultry. The anti-nutritive effect is caused by the fact that the increased quantity and viscosity of the intestinal contents causes a decrease in the rate of diffusion of substrates and digestive enzymes. Due to the increase in viscous intestinal volume, the insufficient amount of substrates and digestive enzymes cannot bind to the entire mucosal surface of the small intestine. As a result, viscous materials are not properly hydrolyzed, which leads to malabsorption (Edwards et al., 1988; Ikegami et al., 1990). The gel-like environment caused by increased viscosity, reduces mixing of dietary components with pancreatic and other intestinal secretions, which is required for proper digestion (Ward, 1996). Additionally, the movement of sugars, amino acids, and other nutrients to the mucosal sites for digestion is impeded, which results in depressed digestion and absorption of fats, proteins, and carbohydrates (Fengler et al., 1988). Viscous polysaccharides, such as soluble pentosans and β-glucans, might also directly bind with digestive enzymes and reduce their activity (Ikeda and Kusano, 1983). Arabinoxylans are the major NSP in wheat, and they are known to give rise to highly viscous conditions in the small intestine of wheat-fed chickens (Antoniou and Marquardt,

1982; White et al., 1983; Classen and Bedford, 1991; Salih et al., 1991, Teitge et al., 1991) in addition to reducing nutrient digestion in the small intestine.

The adverse effects of soluble NSPs on growth decrease when these polymers are partially hydrolyzed by using enzymes known as glycanases (Hesselman and Aman, 1986; Classen et al., 1988; Bedford and Classen, 1992). These enzymes cause a partial depolymerisation of the NSPs which reduces the viscosity of the gut contents, resulting in increases in nutrient absorption (Bedford et al., 1991; Choct and Annison, 1992). For example, enzyme supplementation (xylanase) to rye diets reduces viscosity of intestinal content, and accelerates the rate of passage of digesta through the gastrointestinal tracts of birds (Dänicke et al., 1997; Lázaro et al., 2003). Enzymes improve poultry performance by increasing feed intake and nutrient digestibility (Dänicke et al., 1997; Lázaro et al., 2003).

2.7 A New Era of Folic Acid Use

2.7.1 Fortification of the Egg

A large proportion of the significant studies quantifying nutrient requirements for hatchability were conducted prior to 1950. Since that time there have been marked changes in dietary ingredients, dietary specifications, bird type, management, and incubation techniques to meet the needs of a more sophisticated industry. Leeson et al., (1979) pioneered the idea of different requirements for different strains of birds when he determined that Rhode Island Reds need greater quantities of vitamins for optimum hatchability. However, no significant ($P>0.05$) differences were observed for egg production in Rhode Island Reds compared to the Single Comb White Leghorns (Leeson, et al., 1979).

In 1992 and 1993, Naber and Squires evaluated vitamin transfer of riboflavin, vitamin A, and B_{12} to eggs. Their results along with previous research indicated that the riboflavin and vitamin A dietary status of laying hens is not a problem in commercial flocks. However, some of the flocks sampled demonstrated marginal vitamin B_{12} status to support hatchability and maximum egg size.

Sherwood et al., (1993) studied folate metabolism and deposition in eggs by laying hens. The folate contents of egg yolk and blood plasma from the hens were estimated with an isotope-dilution radioligand-binding assay and it was found that the folate content of the egg yolk saturates with increasing dietary folate.

More recently Leeson and Caston (2003) enriched eggs with folic acid (10 μg/egg; 2.5% of DRI) by feeding hens folic acid supplemented diets including 3 mg of folic acid/kg of diet. They also attempted to enrich eggs with other vitamins, including vitamin A, vitamin D_3, vitamin E, vitamin K, thiamin, riboflavin, pyridoxine, biotin, niacin, pantothenic acid, and vitamin B_{12}. Dietary concentrations of vitamins were chosen with the expectation of producing an egg with sufficient vitamins to achieve 50% of daily recommended intake (DRI). Vitamin B_{12} showed the best response, increasing from 36 to over 100% DRI, 50% of DRI was recorded for vitamin K and biotin, and vitamins D_3 and E were enriched to 11 and 25% of DRI respectively.

House et al., (2002) looked at the folate enrichment of table eggs by determining the potential for folate-fortification of eggs from hens receiving barley-based diets. The studies provided strong evidence of the sensitivity of egg folate concentrations to dietary folate levels. By adding crystalline folic acid to cereal based laying hen diets, it was possible to increase the folate content of eggs by two-to four-fold. The data from this

study was in agreement with the work of Sherwood et al., (1993), with respect to the relationship between dietary folate and plasma, and egg yolk folate concentrations. The two authors have stated that saturation of egg folate content is not due to limitations in transport processes from plasma to the egg yolk, as plasma and yolk folates are proportionate across the range of dietary folic acid levels investigated (0, 1, 2, 4, 8, 16, and 32mg/kg of diet) (House et al., 2002). They concluded that the regulatory processes controlling plasma folate levels might be at the point of metabolic control. Through the use of brush-border membrane vesicles derived from rat jejunum, folate transport has been shown to be a saturable process (Said et al., 2000). Hebert et al., (2005) designed a study to determine the optimal dietary folic acid level required for maximal egg folate deposition and to determine the potential for differences in how two different strains responded. Results from the short term trial found that increasing folate levels up to 128 mg of folic acid/kg did not lead to consistent increases in egg folate content over and above those achieved at the folate plateau, (between 2 and 4 mg/kg of diet).

2.7.2 Development of the Folate Enriched Egg

In 1996, the US Food and Drug Administration (FDA) issued a regulation to be effective by January, 1998, requiring that all flour, rice, pasta, cornmeal, and other grain products be enriched with 140 μg of folic acid per 100 g, in addition to the thiamine, riboflavin, niacin, and iron, already present in these grains (Fed. Regist., 1996). The goal of this folic acid fortification was to increase the intake of folate by women of childbearing age in response to the recommendation of the Public Health Service that "all women of childbearing age in the United States who are capable of becoming pregnant should consume 0.4 mg of folic acid per day for the purpose of reducing their risk of

having a child affected by spina bifida or another neural tube defect (NTD)." Folic acid fortification may have a beneficial effect on cardiovascular disease because of the relation between inadequate folate intake and higher circulating homocysteine concentrations (Tucker et al., 1996 and Boushey et al., 1995). Folate is also a cofactor in the remethylation of homocysteine to form methionine (House et al., 1999). Therefore, the adequate consumption of folate is very important.

The entire issue of folic acid supplementation arose from the fact that folate deficiency is the most frequent vitamin deficiency in developed countries: 5-25% of women present megaloblastic anemia or megaloblastic bone marrow (Zittoun and Cooper, 1989). Women of childbearing age were unable to take in adequate folate to meet recommended dietary allowances by consuming a normal well-balanced diet. The need for folic acid supplements or folic acid-supplemented food thus presented itself. Grain products were chosen for enrichment as a method of folate transfer because it was thought they were the most widely consumed food.

Aside from folate-enriched grain products, vegetables including okra, spinach, asparagus, and orange juice are foods that are naturally high in folate (270-240 μg of folate/serving). However, these vegetables are not as readily available year round as other foods may be. Alternatively, folic acid supplement pills may be taken by people. The development of the folate-enriched egg has offered an alternative vehicle for folate delivery (House et al., 2002; Sherwood et al., 1993). A folate-enriched egg could supply people with 12.5% of their recommended dietary allowance of folate intake (45-50 μg of folate/large egg) (House et al., 2002). Other advantages of using the egg as a method of folate delivery include that the folate level is not as high as the level in folate

supplements, which are known to mask macrocytic anemia of B_{12} deficiency, which will lead to neuropathy if not corrected (Rothenberg, 1999). It has been found that egg yolk folate is equally or more bioavailable than crystalline folic acid (House et al., 2003). Globally, over the past 20 years, the consumption of grain products has been gradually declining, and it is expected this trend will continue (United Nations, 2002). In western countries, the decline in carbohydrate consumption is attributed to lifestyle changes that reflect reduced physical activity at work and during leisure time; and in developing countries, people continue to face food shortages and nutrient inadequacies (Ferro-Luzzi and Martino, 1996).

The fact that House et al., (2002) found that the egg folate level saturated at a level above 2 mg/kg diet led Hebert to perform a long-term (11-month) trial comparing 2 strains of laying hens in addition to 2 possible folic acid supplementation levels (0 and 4 mg) to determine if folate could be deposited in the egg throughout an entire production cycle.

The level of crystalline folic acid supplementation is crucial for the successful production of folate-enriched eggs. Using the lowest possible supplementation level that will consistently deposit the maximum amount of folate in the egg will ensure successful, least cost egg production with optimal human nutritional benefits. Therefore the level of dietary folic acid supplementation must be finalized, and the mechanism responsible for egg folate maximization must be elucidated. In a short term trial Hebert et al., (2005) found that there was no significant difference in egg folate content with respect to levels of crystalline folic acid supplementation between 2 and 128 mg/kg of diet. Perhaps it is possible to maximize egg folate content and achieve consistent folate deposition over a

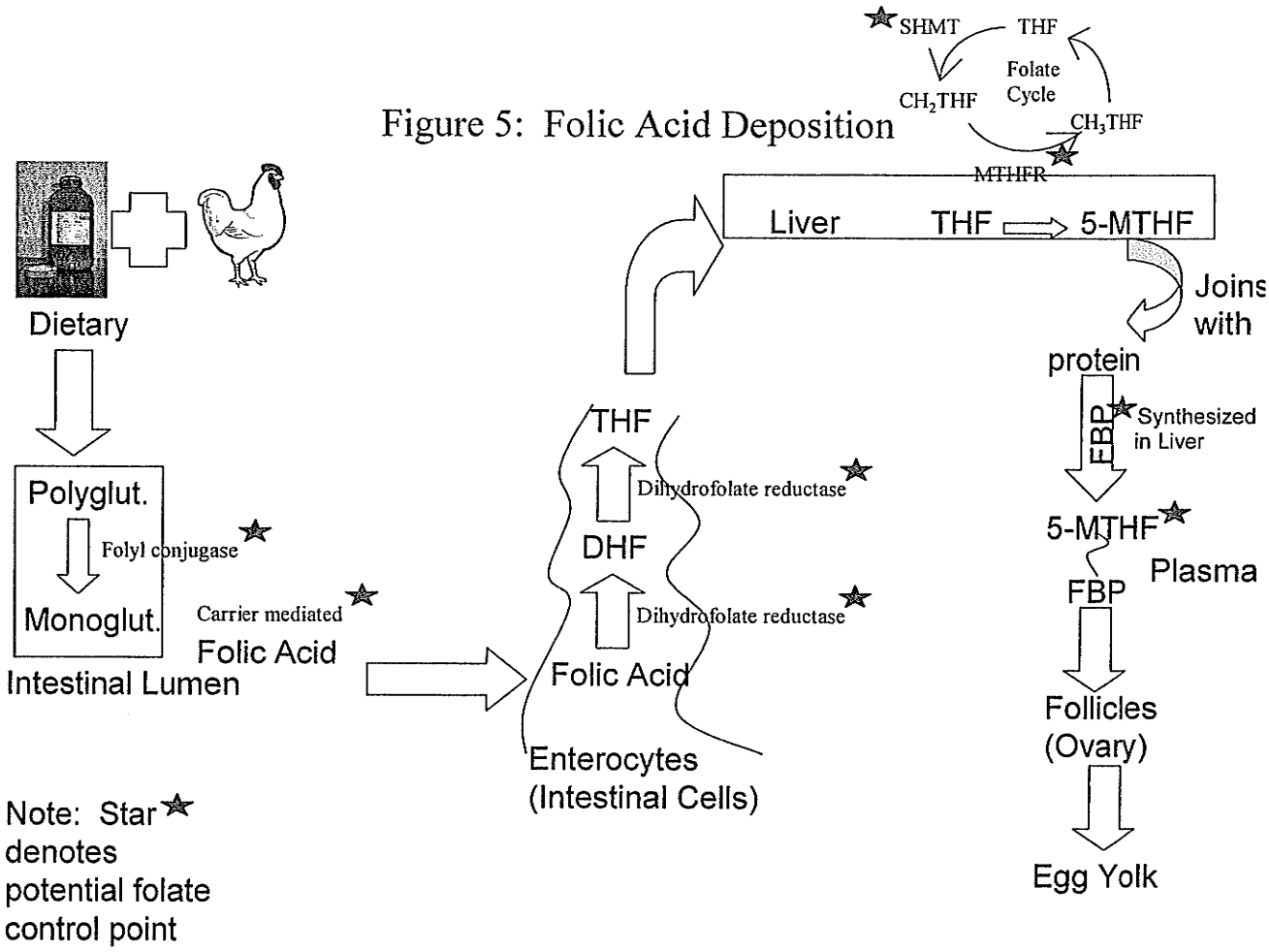
production cycle with only 2 mg/kg rather than 4 mg of supplemental crystalline folic acid/kg of diet.

2.7.3 Mechanism Responsible for Maximizing Egg Folate Content

Dietary folate is absorbed from the gut via a membrane-bound folate transport system (Said et al., 2000), metabolized in the liver cells to 5-MTHF and then is released into plasma (Henderson, 1990). There is little available information on the metabolism and deposition of folate in chickens. There are potential folate control points that exist throughout the metabolic pathway from folic acid (fed to the hen in a crystalline form) to egg folate deposition. These control points are denoted by a star throughout the hen metabolic pathway (**Figure 5**).

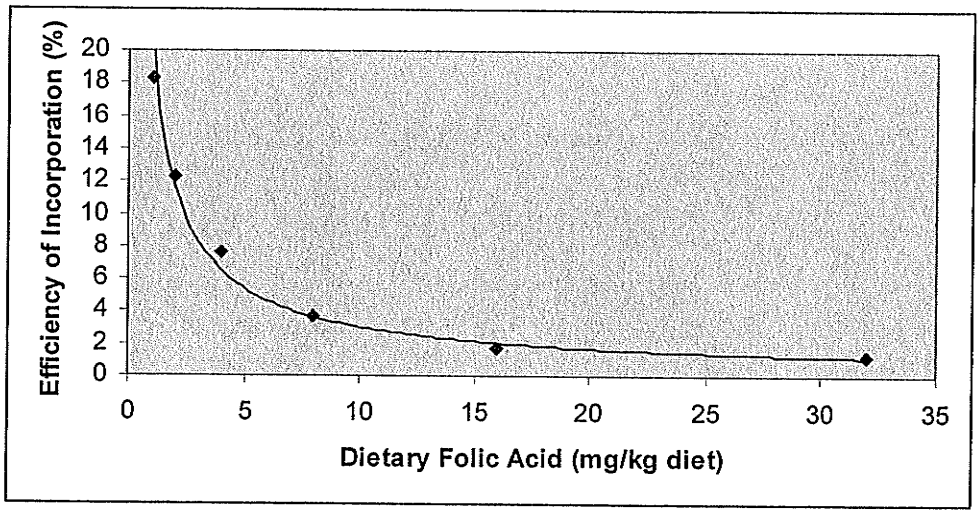
With respect to egg folate saturation, it has been proven that with increasing dietary folic acid supplementation plasma folate concentrations and egg yolk folate content are increased. Due to the fact that both the plasma and yolk folate saturate at the same dietary folic acid level, the system that transfers folate from plasma across the oocyte vitelline membrane to the yolk does not appear to be contributing to the saturation. Perhaps there is a limit of folate that the plasma can sustain due to the fact that the developing embryo would not require anymore folate. It is possible that metabolic systems prior to folate being released to the plasma are responsible for limiting supply of folate to plasma. Absorption of folate by the intestine (conversion of food folate from polyglutamate to monoglutamate form), or metabolic conversion of monoglutamate folate to reduced methyl folate (5-MTHF) within intestinal cells prior to release of folate into portal circulation then deposition in the egg yolk could be possible areas of folate control.

Figure 5: Folic Acid Deposition



The efficiency of dietary folic acid incorporation into eggs of laying hens can be illustrated by **Figure 6.**

Figure 6. Efficiency of incorporation into eggs of supplemental folic acid contained in barley-based laying hen rations.



Source: (House, 2001)

*Efficiency was calculated as the difference in the average folate content between the eggs from hens fed folic acid supplemented diets and the baseline folate content of the basal diet eggs, divided by the average folic acid intake per treatment (intake = crystalline folic acid content per diet), expressed as a percentage.

After 4 mg dietary folic acid/kg diet there is a dramatic drop in the efficiency of folate incorporation into the egg. This drop in efficiency of folate incorporation into the egg implies that egg folate is a saturable process. Hebert et al., (2005) did not find a dramatic rise in plasma folate concentration when dietary folic acid was supplemented at 0, 2, 4, 8, 16, 32, 64, and 128 mg/kg. This finding implies that folate uptake at the gut is mediated by a membrane-bound folate transport system (Anderson et al., 1992; Henderson, 1990) rather than a plasma folate-binding transport protein. Said et al., (2000) referred to a carrier-mediated folate uptake system across the brush-border

membrane, derived from rat jejunum, which was shown to be saturable. The study showed that in folate deficient rats, the activity of the enzyme folate hydrolase, which hydrolyzes polyglutamyl forms of folate to monoglutamates prior to intestinal absorption, was up regulated. Additionally, the hydrolysis of polyglutamates to monoglutamates and the transport of the resulting monoglutamates are up regulated in folate deficient rats (Said et al., 2000). Folic acid uptake by jejunal brush-border membrane vesicles of folate-deficient rats was significantly ($P<0.01$) higher than the folic acid uptake of pair-fed control rats, to ensure maximum absorption of dietary folates by the animal under folate-deficient conditions (Said et al., 2000). Therefore, intestinal uptake could be responsible for regulating folate which is eventually deposited in the egg. To test this theory, a model must be created so that egg-folate deposition can be measured when intestinal uptake of folic acid is impaired.

Nonstarch polysaccharides in certain cereals, most notably β-glucans in barley and arabinoxylans in wheat and rye, which are associated with the endosperm cell wall of the grain, cannot be hydrolysed by enzymes produced by the laying hen (Rotter et al., 1990). NSPs increase the viscosity of the gut, which reduces digesta passage time, and impairs diffusion of endogenous digestive enzymes to the substrates and mixing of the gut digesta (Antoniou et al., 1981; Antoniou and Marquardt, 1982). The viscous conditions of the intestine may negatively impact the amount of folate available for uptake by intestinal cells.

Laying hens may be fed a wheat or rye-based diet, to create such conditions, and enzyme may also be added to some diets. The egg folate content may be measured to determine whether or not exogenous enzymes will increase intestinal folate uptake, by

increasing digestibility of the diet, thus making folate more available to the intestinal cells.

3.0

MANUSCRIPT 1

Effect of Dietary Folic Acid Supplementation on Egg Folate Content Throughout the Production Cycle of Laying Hens.

3.1 Abstract

Previous research has shown that supplementing laying hen diets with 4 mg folic acid/kg diet leads to folate-enriched eggs (3 fold increase) over a short term feeding period. As consistent egg folate deposition is the key to successful marketing, the current study was designed to address the following objective: Could a lower level of folic acid supplementation (2 versus 4 mg/kg diet) maximize egg folate content over the full production cycle? To address this objective, 1248 Hy-Line CV20 laying hens (n = 416 per diet), in a completely randomized design, received a barley-based ration containing 0, 2, or 4 mg/kg of crystalline folic acid for eleven 28-day periods.

Response criteria included production parameters and measurement of egg folate content. Data was analyzed as a repeated measures experiment using the mixed procedure. The analysis revealed a significant ($P<0.0001$) main effect of folate supplementation on egg folate content. Significant egg folate content differences ($P<0.0001$) were evident between each of the three rations. As the level of supplemental crystalline folic acid increased in the diets, the egg folate content significantly increased. Average egg folate levels over 11 periods were 1.4, 2.9, and 3.4 micrograms/gram of yolk for respective rations containing 0, 2, and 4 mg of crystalline folic acid/kg of diet. Production parameters including egg production, egg size, feed consumption, feed efficiency, and egg shell quality were not significantly ($P>0.05$) affected by dietary folic acid supplementation. This study confirms that egg folate content and consistency are maximized throughout the production cycle when crystalline folic acid is supplemented to the hen's diet at 4.0 mg/kg diet.

3.2 Introduction

The development of the folate enriched egg (House et al., 2002; Sherwood et al., 1993) is an effective method of delivering a highly bioavailable folate source to the human population. Aside from fulfilling basic nutritional needs, folate plays a role in maintaining homocysteine at a level low enough (non-hyperhomocysteinemic) to lower incidence of neural tube defects in human babies (Czeizel and Dudas, 1992), cardiovascular disease (Eikelboom et al., 1999) and inflammatory diseases in adults (Wang et al., 2001).

Based on findings by Hebert (2005) and House et al., (2002), supplementing laying hen diets with 4 mg crystalline folic acid/kg diet can increase egg folate up to three-fold that of an unsupplemented hen egg. This level of supplementation exceeds the National Research Council (1994) requirements of 0.25 mg folic acid/kg feed for laying hens by sixteen fold.

House et al., (2002) determined that additions of folic acid above 2 mg/kg diet yielded no further significant increases in egg folate content until dietary concentrations reached 32 mg/kg. Hebert et al., (2005) followed up this possibility of a biphasic reaction with a short term trial during which laying hens were fed diets supplemented with levels of folic acid up to 128 mg/kg diet. Hebert (2005) did not find evidence of the biphasic response, as increasing folic acid levels up to 128 mg/kg diet did not lead to consistent increases in egg folate content over and above those achieved at plateau, (2-4 mg/kg diet).

Hebert's (2005) short term trial prompted a long term production trial involving supplementation of laying hen diets with 0 and 4 mg folic acid/kg diet, to evaluate the effects of age and rate of production on folate deposition of two different strains of laying

hen. Naber and Squires (1993) had noted a decrease in riboflavin and vitamin A deposited in the eggs of laying hens over 42 weeks of production, and Robel (1983) noted a decrease in the efficiency of pyridoxine, vitamin B_{12}, calcium, and folic acid transfer to turkey eggs over a 70-week trial. The production trial performed by Hebert (2005) showed consistent egg folate deposition, after period one (1 period = 28 days) of the trial, until period nine. Hebert (2005) found that plasma homocysteine concentrations tended to decrease with increasing dietary folate supplementation in the short term trial. The Hy-Line W98 strain of laying hens exhibited a decrease in plasma homocysteine levels when supplemented with 2 mg folic acid/kg diet. Due to the facts that: plasma homocysteine concentrations had decreased with increasing dietary folate supplementation, the Hy-Line W98 strain of laying hens exhibited a decrease in plasma homocysteine, and homocysteine is an independent risk factor for cardiovascular disease (Jacobsen, 1998), it was speculated that reduction in hen plasma homocysteine due to folate supplementation could result in improved hen livability. However, causes of mortalities were not closely documented in the trial.

Based on the above findings, a long term production trial was performed, to determine if a lower level of folic acid supplementation (2 mg/kg diet versus 4 mg/kg diet) is sufficient to maximize egg folate content and consistency throughout the production cycle. Additionally, post mortems were performed, and careful documentation of mortalities were recorded throughout the production cycle, to determine if cardiovascular abnormalities are attributed to any of the hen mortalities.

3.3 Hypotheses and Objectives

3.3.1 Hypotheses

Alternative Hypothesis:

1) Egg folate level will be affected by dietary folate treatment, laying hen age, and rate of production.
2) Production performance of Hy-Line CV20 laying hens will be affected by dietary folic acid level.
3) Dietary folic acid level will affect laying hen mortality.

Null Hypothesis:

1) Egg folate level will not be affected by dietary folate treatment, laying hen age, and rate of production.
2) Production performance of Hy-Line CV20 laying hens will not be affected by dietary folic acid level.
3) Dietary folic acid level will not affect laying hen mortality.

3.3.2 Objectives

The purpose of this research was to determine the optimal level of folic acid supplementation within laying hen diets to maximize egg folate content over time. This was accomplished through the following objectives:

i) To determine if a lower level (<4 mg/kg) of folic acid supplementation is adequate to maximize egg folate content over the full production cycle.
ii) To determine consistency of egg folate deposition at various periods throughout the laying hen production cycle.

iii) To determine the age and rate of production effects of laying hens on egg folate content.

iv) To determine the production performance of Hy-Line CV20 laying hens fed three different levels of folic acid.

v) To determine the effect of three levels of supplemental folic acid on laying hen mortality.

3.4 Materials and Methods

3.4.1 General

Single-Comb White Leghorn Hy-Line Commercial Variety (CV20) laying hens (Manitoba Perfect Pullets, Rosenort, Manitoba, Canada), the newer version of the W36 strain were used in this experiment. The CV20 strain is a higher producing, lower feed consuming strain of birds. Laying hens were approximately 21weeks old at the start of the trial. Hens were kept in confinement housing under semi-controlled environmental conditions and exposed to 16-hour photoperiod, with 8 hours of darkness. Birds were housed four per cage, the cage dimensions were 40.64 cm by 40.64 cm, providing 413 cm^2 (64 in^2) per bird. Feed and water were available *ad libitum* for all hens. Animal care approval was received from the University of Manitoba's Animal Care Protocol Review Committee, and the birds were managed in accordance with recommendations established by the Canadian Council on Animal Care (1984).

3.4.2 Diet and Experimental Protocol

The basal diet was a barley-based ration (**Table 3**).

Table 3. Composition of basal barley-based laying hen ration.

Ingredient	%
Barley (11.9% CP)	56.75
Soybean Meal (45.0 % CP)	13.40
Canola Meal (37.58% CP)	10.00
Fishmeal (60% CP)	2.00
Vegetable Oil	6.05
Limestone	9.47
Biophos (monocalcium phosphate)	0.83
Vitamin premix[1]	1.00
Mineral premix[2]	0.50
Calculated nutrient Composition	
Crude Protein	17.90
Metabolizable energy, kcal/kg	2700.00
Calcium	4.01
Phosphorus (total)	0.40
Folate[3] mg/kg	0.90

[1]Provided (per kg of diet): 8255 IU vitamin A; 3500 IU vitamin D_3; 30.0 IU vitamin E; 0.55 mg vitamin K_3 (menadione with Na-bisulfite); 0.011mg vitamin B_{12}; vitamin B_{58} complex (contains/kg: 4.50 mg riboflavin, 8.24 mg pantothenic acid, 12.55 mg niacin, and 164.80 mg choline); 0.100 mg biotin; 10 mg niacin; 2 mg thiamine (B_1) (mononitrate); 3 mg pyridoxine (B_6) (HCl); 1037 mg choline chloride (contains 520,800 mg choline/kg); 900 mg dl-methionine; 125 mg ethoxyquin (anti-oxidant).
[2]Provided (per kg of diet): 70 mg MnO (manganese oxide); 66 mg ZnO (zinc oxide); 50 mg $FeSO_4$ (ferrous sulfate); 8.8 mg $CuSO_4$ (copper sulfate); 0.3 mg $NaSeO_3$ (sodium selenite); 0.33 mg $Ca(IO_3)_2$ premix (calcium iodate premix contains: 2.0 g calcium iodate and 43 g iodized salt); 0.67 mg iodized salt.
[3]Measured by Covance Laboratories Inc., Madison, WI via Official Methods of Analysis of AOAC International (2000)17th ed., AOAC International, Gaithersburg, MD, USA, Official Methods **960.46, 992.05**.

The diet was formulated to meet the requirements of laying hens consuming approximately 100 g of feed/day (NRC, 1994). In accordance with industry standards (BASF, 2000), the basal diet included no crystalline folic acid. Folate content in the basal diet was 0.9075 mg/kg diet, therefore actual dietary folate concentrations in the 2 and 4 mg/kg diet supplementation levels were approximately 2.9075 (2.0 +0.9075) and 4.9075μg/g yolk (4.0 +0.9075) respectively.

For two weeks prior to the commencement of the study, 1248 healthy hens in production received a standard barley-soybean meal based ration with no additional folic acid supplementation.

In order to study the parameters of the production cycle of hens laying fortified eggs, the 1248 hens were grouped into 4 birds/cage, and 26 cage units/treatment. Each replicate was randomly assigned to receive one of three dietary folic acid levels (26 replicates/treatment:416 hens): the basal diet (no added folic acid), the basal diet plus 2 mg crystalline folic acid/kg of diet (Sigma Chemical Co., Oakville, ON, Canada), or the basal diet plus 4 mg crystalline folic acid/kg diet. Following a 2-week adaptation period to the treatments, the long term (eleven 28-day periods) trial commenced.

Information on mortality, body weight, feed consumption, egg production, egg weight, and egg shell quality (specific gravity) was recorded. Any mortalities that occurred throughout the duration of the trial were immediately recorded, birds were weighed, and post mortems were performed to determine the cause of death. For laying hens that died of urolithiasis, cause of death was verified by post mortem analysis by a veterinary pathologist (for further details on post mortems and urolithiasis diagnosis, please see appendix). Individual body weight of all hens was taken on the initial and

final day of the trial to determine average body weight gain. Residual feed was weighed back at the end of each period to determine feed consumption for each replicate. Average daily feed intake as well as feed efficiency was then calculated. Egg production was recorded daily for each replicate and an average egg production rate (hen-day percent) was calculated for each production period.

Thirty eggs (1 egg/replication; 10 replications/ treatment) were randomly collected per treatment per day during two consecutive days on day 17 and 18 of each period. The eggs were weighed to give an average egg weight for the period and processed for egg folate determination.

Measurements of egg quality, including egg weight, yolk weight, and specific gravities of individual eggs laid by the hens were determined following the sixth period and in period ten during the trial. Specific gravity was performed halfway through the trial (sixth period) to test for egg shell quality, and in period ten because as the hens near the end of their production cycle their shell quality begins to deteriorate. For three consecutive days all the eggs from each replicate were weighed and specific gravity was determined (Holder and Bradford, 1979). No further egg quality measurements such as albumen height (Haugh Unit measurement) were necessary, because previous research has shown that folic acid does not affect interior egg quality (Hebert, 2005).

3.4.3 Extraction of Egg Yolk Folate

All chemicals used in the extraction and analysis of folate were purchased from Sigma Chemical Co. (Oakville, ON, Canada). It has been previously shown that: 1) 99.0% of folate is found in the yolk fraction of the egg (Sherwood et al., 1993) 2) Folate is bound to the yolk as the 5-MTHF form (Seyoum and Selhub, 1998) 3) Folate is present

as the monoglutamate form, which is readily absorbed (Seyoum and Selhub, 1998). Further preparation of samples is not required to deconjugate glutamate residues or to quantify other forms of folate.

On sample days, the eggs were collected and labeled with cage and ration numbers. The eggs were then weighed and placed in a large metal pot. Cold water was added to cover the eggs with one inch of water. The pot was covered with a lid, and the element was set at high until, the water began to boil. Once the water was boiling the element temperature was reduced by half to ensure a gentle boil for 10 minutes. The water was then drained from the pot and the eggs were submersed in ice water for 5 minutes. The yolks were then separated from the albumen, and individually bagged; yolk was crushed, and stored at –20 °C until analyzed.

The egg yolk folate extraction procedure is described by House et al., 2002. In brief, approximately 0.5 g of egg yolk was weighed into glass tubes with lids. Ten milliliters of ascorbate buffer (pH 7.8) (see appendix for preparation) was added to each tube. The tubes were capped with nitrogen gas, caps were secured, vortexed and placed in a boiling water bath for 60 minutes. The tubes were then centrifuged at 4,000 x g for 30 minutes (Beckman). The supernatant from each tube was removed and placed in a corresponding 25 ml volumetric flask. Another 10ml of ascorbate buffer was added to each tube. The tubes were vortexed and centrifuged as before. The supernatants were then pooled, and brought to a final volume of 25 ml with ascorbate buffer. The extracts were then mixed thoroughly. A sample from each flask was placed into a microcentrifuge tube and stored at –80°C until analyzed.

3.4.4 Analysis of Egg Yolk Folate Content

As previously described by Vahteristo et al., (1997), the concentration of 5-MTHF in the egg yolk extracts was determined by reverse-phase high pressure liquid chromatography (HPLC) with fluorescence detection (Shimadzu, Mantech, Guelph, ON, Canada). An external standard curve with purified 5-MTHF was used to quantify egg folate concentrations. The inter- and intra-assay coefficient of variance for determinations was <2%, and recovery of 5-MTHF added to egg yolk was 98.9%. The content of egg folate was expressed as micrograms of folic acid per egg.

3.4.5 Measurements of Egg Quality

Specific gravity was determined by the saline flotation method as described by Holder and Bradford (1979). A series of four solutions were prepared, with saline concentrations including 1.070, 1.075, 1.080, and 1.085 specific gravities. The density of each solution was confirmed with a hydrometer prior to use and specific gravity was determined by sequentially placing eggs in solutions of increasing salinity. Both the eggs and solutions were at 15°C. Eggs that did not float in the 1.085 specific gravity solutions were assigned a specific gravity of 1.090.

3.4.6 Calculations

Hen-Days = Cumulative Number of days each hen was present during the period.

$$\textit{Hen-Day Production (\%)} = \frac{\text{(Total Number of Eggs Laid in the Period)}}{\text{(Number of Hen-Days)}} \times 100$$

$$\textit{Feed Consumption (grams/hen/day)} = \frac{\text{Total Amount of Feed Added-Feed Weigh Back During the Period (g)} \quad \text{at the End of the Period (g)}}{\text{Number of Hen-days in the Period}}$$

Feed Efficiency (g feed/ g egg) = Feed Consumption (g/hen/day)
Egg mass (g/hen/day)

Egg Mass = Average Egg Weight (g) for the Period x Hen-day Production (%)

Percent Yolk of Egg Weight = Average Yolk Weight (g) x 100
Average Egg Weight (g)

Cumulative Mortality (% of total hens housed) =

Number of Hens Dead Since Beginning of the Trial x 100
Number of Hens Housed at the Beginning of the Trial

Total Uncollectable Eggs (% of total eggs laid) =

Total Number of Uncollectable Eggs for the Period x 100
Total Number of Eggs Laid During the Period

Efficiency of Folate Deposition in Egg (%) =

Hen-Day Production (%) x Mean Egg Folate (µg/egg)

Mean Feed Consumption (g/hen/day) x Folate in Ration (mg/kg) (Endogenous + Supplemental)

3.4.7 Statistical Analysis

Statistical analysis was performed with the Statistical Analysis System (SAS Institute Inc., 1998) using the mixed procedure. A completely randomized, repeated measures design was used to analyze the data for the six experimental periods (period 1, period 3, period 5, period 7, period 9, and period 11) throughout the eleven experimental periods of the production trial. The type of mixed procedure used was compound

symmetry with heterogeneous variance across periods, allowing unequal variance in all periods, which proved to be the most accurate method available for the data. The fit statistics computed by SAS indicated that compound symmetry with heterogeneous variance across periods was the method that best fit analysis of entered data. Folate data was analyzed as a repeated measures design. Performance data collected were subjected to type three tests of fixed effects followed by a least square difference test using the pdmix 800 program, which determined differences between means. To enable the SAS program to recognize the hen-day production values that were >1, an arc sin transformation needed to be performed. Differences with an alpha level of $P<0.05$ were considered to be statistically significant. Mortality data was analyzed using a Chi Square Test.

3.5 Results

The main effects of ration, period, and their interactions for different performance traits are shown in **Table 4**.

Significant ($P<0.05$) ration differences existed for feed efficiency, percent yolk of egg weight, yolk folate content, and egg folate content. Significant ($P<0.05$) period differences were found for all measured performance traits. Significant ration by period interactions ($P<0.05$) were observed for hen-day production, feed consumption, yolk folate content, egg folate content, and body weight.

The cumulative mortality of hens consuming three levels of folate enriched diets is shown in **Table 5**. Cumulative mortality (% of total hen housed) was analyzed using a chi square test. The chi square test showed that there was a significant difference in

Table 4. P-values of main effects and interactions of ration*period on different performance traits.

Parameters	Ration	Period	Ration*Period
Hen-day Production (%)	0.2475	<0.0001	0.0127
Feed Consumption (g/h/d)	0.1628	<0.0001	0.0027
Feed Efficiency (g feed/g egg)	0.0362	<0.0001	0.2096
Egg Weight (g)	0.0518	<0.0001	0.8253
Yolk Weight (g)	0.8754	<0.0001	0.8267
% Yolk of Egg Weight	0.0265	<0.0001	0.8110
Yolk Folate Content (µg/g yolk)	<0.0001	<0.0001	0.0039
Egg Folate Content (µg/egg)	<0.0001	<0.0001	0.0057
Total Uncollectable Eggs (% of total eggs laid)[1]	0.6108	<0.0001	0.8624
Specific Gravity[2]	0.6373	<0.0001	0.8440
Body Weight[3]	0.3333	<0.0001	0.0212
Cumulative Mortalities (% of total hen-housed)		0.0453	

[1]Includes cracked and shell-less eggs.
[2]Compares eggshell quality measurements at period 6 and period 10 of production.
[3]Birds weighed at day 1 and final day (after 11 periods) of trial.
*Note: Shading signifies significance (P<0.05)

Table 5. Cumulative mortality of laying hens receiving diets with or without crystalline folic acid supplementation – Main effects of the ration[1].

	Parameters
Dietary Folic Acid Supplementation (mg/kg diet)	Cumulative Mortality[2] (% of total hen housed)
0	1
2	2.1
4	1.1
p-value	0.0453

[1]Data are presented as least squares means

[2]Analyzed using chi square test

P<0.05 denotes significance

mortality among the three levels of dietary folic acid supplementation. There were significantly more mortalities from hens consuming the 2 mg folic acid/kg diet. Due to the fact that the number of laying hen mortalities was relatively low (n=53 mortalities), Tukey's test could not be used to compare mortalities among the three dietary treatment levels. Therefore a chi square test was used to determine if there was a significant difference in hen mortality among the three treatment levels, consequently, it cannot be stated whether there were significantly more mortalities from the 2 mg folic acid/kg diet compared to the 0 mg folic acid/kg diet level or the 4 mg folic acid/kg diet level.

The cumulative mortalities for 0, 2, and 4 mg of folic acid/kg diet were 1.0, 2.1, and 1.1 percent respectively. The breakdown of causes of mortality for each ration throughout the trial is shown in **Table 6.** Approximately 50% of the mortalities in each ration can be attributed to urolithiasis (please see **Figure 10**).

The performance data of laying hens, including the main effects of the ration: feed efficiency and percent yolk of egg weight is shown in **Table 7.**

The feed efficiency of the birds on the 4 mg folic acid/kg diet was significantly ($P = 0.0362$) better, than the feed efficiency of the birds on the 2 mg folic acid/kg diet, (1.76 versus 1.82 and 1.84 respectively). Compared to the 0 and 4 mg folic acid/kg diet, the 2 mg folic acid/kg diet had significantly ($P = 0.0265$) higher percent yolk of egg weight (~ 26% versus ~25%).

The performance data of hens throughout the production cycle –as main effects of period is shown in **Table 8.**

Table 6. Causes of mortality for each ration throughout 11-period trial.

Dietary Folic Acid Supplementation (mg/kg diet)	% of Ration Mortalities	Cause of Mortality
0	53.80	urolithiasis
	46.20	other[1]
2	46.20	urolithiasis
	53.80	other
4	42.90	urolithiasis
	57.10	other

[1]Other refers to other causes of mortality in laying hens excluding urolithiasis. For example sick birds, calcium shock, etc.

Table 7. Performance summary of Hy-Line CV20 laying hens-Main effects of ration[1].

	Supplementation Level (mg/kg diet)				
Parameters	0	2	4	SEM	P-values
Feed Efficiency (g feed/g egg)	1.82[a,b]	1.84[a]	1.76[b]	0.0203	0.0362
% Yolk of Egg Weight	25.34[a,b]	25.68[a]	25.17[b]	0.1300	0.0265

[1]Data are presented as least squares means

a,b,c means within each parameter with differing letters are significantly different ($P<0.05$)

Table 8. Performance summary and egg quality measurements of laying hens throughout the production cycle-Main effects of period[1].

	Parameters						
Period	Feed Efficiency (g feed/g egg)	Egg Weight (g)	Yolk Weight (g)	% Yolk of Egg Weight	Cumulative Mortality (% of total hen housed)	Total Uncollectable Eggs[2](%)	Specific Gravity[3]
1	1.93^{a}	52.3^{f}	11.4^{g}	21.8^{f}	0.32	0.37^{b,c,d}	
2	1.82^{b,c}	53.7^{f}	12.6^{f}	23.5^{e}	0.72	0.31^{b,c,d}	
3	1.71^{d,e}	58.3^{e}	14.2^{e}	24.3^{d}	1.36	0.28^{c,d}	
4	1.70^{d,e}	59.4^{d,e}	14.4^{e}	24.2^{d,f}	2.16	0.37^{b,c,d}	
5	1.69^{e}	62.4^{b,c}	15.9^{c,d}	25.5^{c}	2.4	0.21^{d}	
6	1.75^{c,d,e}	61.6^{b,c,d}	15.8^{c,d}	25.7^{c}	2.72	0.28^{c,d}	1.0810^{a}
7	1.86^{a,b}	58.8^{e}	15.5^{d}	26.3^{b,c}	3.13	0.54^{a,b}	
8	1.92^{a}	60.2^{c,d,e}	16.2^{c,d}	26.9^{a,b}	3.37	0.60^{a}	
9	1.77^{a,b,c,d,e}	61.8^{b,c}	16.5^{b,c}	26.8^{a,b}	3.53	0.46^{a,b,c}	
10	1.87^{a,b}	62.6^{b}	17.2^{a,b}	27.4^{a}	3.77	0.48^{a,b}	1.0789^{b}
11	1.83^{a,b,c,d}	64.7^{a}	17.4^{a}	26.9^{a,b}	4.25	0.67^{a}	
p-value	<0.0001	<0.0001	<0.0001	<0.0001	0.2125	<0.0001	<0.0001

[1]Data presented as least squares means
[2]Includes cracked and shell-less eggs
[3]Compares shell quality measurements at period 6 and period 10 of production
a,b,c means within each parameter with differing letters are significantly different (P<0.05)

There was a significant ($P<0.0001$) period effect for feed efficiency. Feed efficiency significantly improved in period three, remained relatively constant throughout periods four to six, declined in periods seven and eight, and remained fairly constant through period eleven.

A significant ($P<0.0001$) increase in egg weight throughout the production cycle was observed. Egg weight rapidly increased from periods one to three (52.3, 53.7, and 58.3 g respectively). There was a further increase in egg weight in period five (62.4 g), a significant decrease (58.8 g) in period seven, followed by a steady increase in egg weight until period eleven (60.2 g, 61.8 g, 62.6 g, and 64.7 g). Yolk weight changes ($P<0.0001$) generally followed the same pattern as egg weight increasing from 11.4 g to a maximum of 17.4 g in period 11. The percent yolk of egg weight significantly ($P<0.0001$) increased each period one to three (21.8, 23.5, and 24.3%; respectively). There was a further significant increase in the percent yolk of egg weight in period five. As expected egg and yolk weight increased with maturity of the hen. In period six and seven, the percent yolk of egg weight remained constant (25.7 and 26.3% respectively). Throughout the production cycle, the percent yolk of egg weight increased to a maximum of ~ 27% where it plateaued.

Cumulative mortality (53 birds) significantly increased from period one (0.32%) to period two (0.72%), with another significant increase from period three (1.36 %) to four (2.16 %). Cumulative mortality steadily increased in each period to a total of 4.25 % for the 11 periods (please see appendix for additional information on detailed causes of hen mortalities).

Egg quality was significantly affected throughout the production cycle. Total uncollectable eggs were significantly ($P<0.0001$) higher in the second half of the production cycle, periods seven to eleven, than the first half of the production cycle, periods one to six. The percent of uncollectable eggs was the lowest in periods three, five, and six.

Specific gravity significantly ($P<0.0001$) decreased from 1.0810 in the middle (period six) of the production cycle to 1.0789 at the end (period ten) of the production cycle.

Ration by period interactions were significant ($P<0.05$) for egg production (**Figure 7**), feed consumption (**Figure 7**), yolk folate content (**Figure 8**), egg folate content (**Figure 8**), and hen body weight (**Figure 9).**

Significant ration by period interactions for hen-day production and feed consumption can be addressed by noting that throughout the production cycle egg production increased to a peak then decreased (**Figure 7**). A significant ($P<0.05$) increase in egg production was observed between periods one and two. Egg production remained over 91% for periods two, three, four, and five, decreasing to 89.44% in period six, and gradually declining over period seven, eight, nine, ten, to 78.85% in period eleven. There were significant differences ($P<0.05$) in hen-day egg production (**Figure 7**) among 0, 2, and 4 mg folic acid/kg of diet in periods three, four, and eleven. In period three, hens on the 2 mg/kg folic acid level had significantly lower egg production (94.33 %) than hens on the 0 and 4 mg/kg folic acid level (95.85 and 95.79 % respectively). In period four, hens on the 2 mg/kg folic acid level had significantly lower egg production (91.81 %) than the hens on the 4 mg/kg folic acid level (93.27 %). In period eleven, the

hen-day production of hens consuming the 4 mg folic acid/kg diet (80.3) was significantly higher than the hen-day production of hens consuming the 0 mg folic acid/kd diet (77.5). Hens consuming the 4 mg/kg folic acid level produced more eggs overall than hens consuming 2 and 0 mg/kg folic acid levels (111 407 compared to 109 032 and 111 282 eggs respectively). This resulted in 267.5, 262.1, and 267.8 overall eggs per hen housed over a 308-day period for hens consuming 0, 2, and 4 mg folic acid/kg diet, respectively (calculations based on the average number of eggs laid by hens in each of the 3 supplementation levels of dietary folic acid; calculations not shown). From period five until period eleven inclusive, hens consuming 4 mg folic acid/kg feed produced more eggs each period than hens consuming the 0 and 2 mg folic acid/kg diet (data based on average number of eggs laid by hens in each of the 3 supplementation levels in each period; calculations not shown). Therefore, hens consuming the 4 mg folic acid/kg feed had improved laying persistency compared to the hens consuming the 0 and 2 mg folic acid/kg feed.

There was a significant ($P<0.05$) general increase and decline in feed consumption throughout the production cycle (**Figure 7**). Feed consumption (FC) increased significantly from period one (87.34 g/h/d) through period three (95.02 g/h/d) and variable feed consumption increased to 97.2 g/h/d by period 5 with a general decline to 93.7 g/h/d in period 11.

There were significant differences ($P<0.05$) in feed consumption (**Figure 7**) among 0, 2, and 4 mg folic acid/kg of diet in periods three, four, five, and eight. In period three, hens fed 4 mg folic acid/kg diet consumed significantly more feed than hens fed 0 or 2 mg folic acid/kg diet (95.9 g versus 94.6 or 94.5 g respectively). In period

four, hens fed the 0 mg folic acid/kg diet consumed significantly less feed than hens fed the 2 or 4 mg/kg diets (93.1 g versus 94.4 or 94.2 g respectively). In period five, hens fed 4 mg folic acid/kg diet consumed significantly more feed than hens fed 0 mg/kg diet (98.0 g versus 96.3 g respectively). In period eight, hens fed 4 mg folic acid/kg diet consumed significantly more feed than those fed 0 or 2 mg folic acid/kg diet (97.3 g versus 95.7 or 95.3 g respectively).

The yolk folate content was similar in all periods (2.45-2.58 μg/g) except period three which was significantly higher **(Figure 8)**. Yolk folate increased in period three from 2.58 to 2.95 μg/g of yolk. There were significant differences ($P<0.0001$) in yolk folate content (**Figure 8**) between each of the supplementation levels: 0, 2, and 4 mg folic acid/kg of diet in periods one, five, seven, and eleven. In periods three and nine there was no significant difference in yolk folate content between hens fed 2 and 4 mg folic acid/kg diet. In period three, yolk folate content for hens fed 2 and 4 mg folic acid/kg diet was 3.54 and 3.67 μg/g yolk respectively versus 1.64 μg/g yolk for hens fed the basal diet. In period nine, yolk folate content for hens fed 2 and 4 mg folic acid/kg diet was 2.96 and 3.15 μg/g yolk respectively versus 1.32 μg/g yolk for hens fed the basal diet. Yolk folate content significantly (<0.05) increased as the supplementation level of folic acid increased from 0 to 2 to 4 mg folic acid/kg of diet (1.42, 2.93, and 3.40 μg/g yolk respectively). Yolk folate content of eggs from the hens consuming the 4 mg folic acid/kg diet remained 3.40 ± 0.46μg folic acid/g yolk; CV~13.50% throughout the entire production trial.

Figure 7. Significant ration*period interaction for hen-day production and feed consumption of Hy-Line CV20 laying hens over 11 periods.

	Folic Acid Supplementation (mg/kg diet)			
Period	0	2	4	SEM
1	$88.1^{k,l}$	$86.6^{l,m}$	$86.0^{l,m}$	0.0074
2	96.2^{a}	$94.7^{a,b}$	95.9^{a}	0.0054
3	95.9^{a}	$94.3^{b,c}$	95.8^{a}	0.0047
4	$93.0^{c,d,f}$	$91.8^{f,g,h}$	$93.3^{b,c,d,e}$	0.0052
5	$92.1^{e,g,h}$	$91.3^{g,i}$	$92.5^{d,e,f,g}$	0.0054
6	$89.1^{j,k}$	$89.3^{j,k}$	$90.0^{i,j}$	0.0054
7	$86.6^{l,m}$	$86.2^{l,m}$	$87.9^{k,l}$	0.0074
8	$83.2^{n,o,q,r}$	$83.5^{n,o,p}$	$84.9^{m,n}$	0.0083
9	$81.6^{p,s}$	$81.4^{q,r,s}$	$82.8^{o,p,q}$	0.0075
10	$79.4^{t,u}$	$81.0^{q,r,s}$	$81.1^{r,s,t}$	0.0072
11	77.5^{v}	$78.8^{u,v}$	$80.3^{s,t,u}$	0.0079

1	87.6^{s}	86.6^{s}	$87.8^{q,s}$	0.5659
2	$93.6^{k,m,n,o,p,r}$	92.5^{o}	$93.4^{m,n,o,r}$	0.3970
3	$94.6^{e,f,g,h,i,j,k,l,m,n,o}$	$94.5^{f,i,j,k,l,m,n,p}$	$95.9^{b,c,d,e,f,g,i,j,k,l}$	0.4678
4	$93.1^{n,o}$	$94.4^{i,j,k,l,m,n,p}$	$94.2^{g,h,i,j,k,l,m,n,o,p}$	0.4131
5	$96.3^{a,b,c,d,e,f,g,h,i}$	$97.2^{a,b,d}$	98^{a}	0.4386
6	$96.5^{a,b,c,d,e,f,g,h,i}$	$96.6^{a,b,c,d,e,g,h}$	$96.7^{a,b,c,d,e,f}$	0.4572
7	$94.6^{b,c,d,e,f,g,h,i,j,k,l,m,n,o}$	$96.1^{a,b,c,d,e,f,g,h,i,j,k,m}$	$96.4^{a,b,c,d,e,f,g,i,j,k,l}$	0.6370
8	$95.7^{a,b,c,d,e,f,g,h,i,j,l}$	$95.3^{c,e,f,g,h,i,j,k,l,m,n}$	$97.3^{a,b,c}$	0.4384
9	$92.3^{n,o}$	91.6^{o}	$93.3^{h,m,n,o,p}$	0.7148
10	$93.6^{j,k,l,m,n,o,p}$	$93.1^{l,n,o,p,r}$	$94.6^{d,e,f,g,h,i,j,k,l,m,n,o}$	0.5890
11	$94.4^{a,b,c,d,e,f,g,h,i,j,k,l,m,n,o,q}$	$92.2^{a,b,c,d,e,f,g,h,i,j,k,l,m,n,o,q,s}$	$94.6^{a,b,c,d,e,f,g,h,i,,j,k,l,m,n,o}$	1.6907

Data reported as least squares means +/- standard error

Ration*period effect on hen-day production $P<0.0127$

Ration*period effect on feed consumption $P<0.0027$

a,b,c means within each parameter with differing letters are significantly different ($P<0.05$)

All means are composed 13 replicates

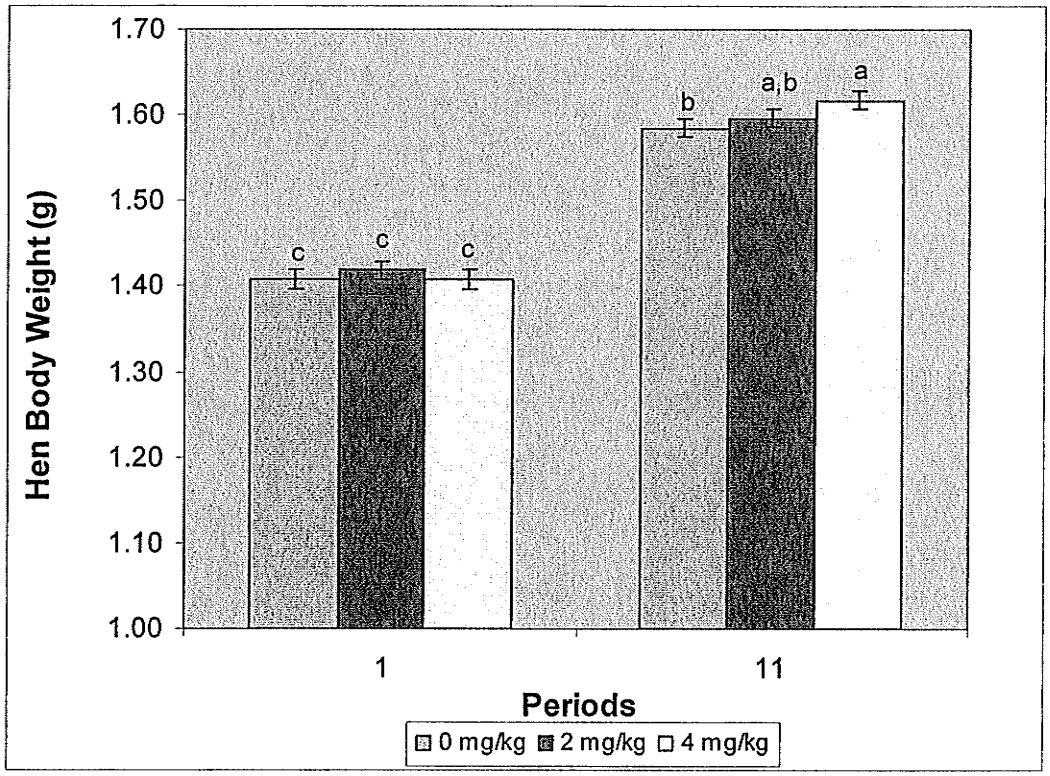
Figure 8. Significant ration*period interaction for yolk and egg folate content of Hy-Line CV20 laying hens.

	Folic Acid Supplementation (mg/kg diet)			
Period	0	2	4	SEM
1	$1.34^{h,i}$	2.85^{f}	$3.54^{a,b,c}$	0.0977
3	1.64^{g}	$3.54^{a,b}$	3.67^{a}	0.0846
5	$1.35^{g,h,i}$	2.69^{f}	$3.36^{a,b,c,d,e}$	0.1771
7	$1.58^{g,h}$	2.86^{f}	$3.27^{c,d}$	0.1013
9	$1.32^{h,i}$	$2.96^{e,f}$	$3.15^{d,e}$	0.1094
11	1.26^{i}	2.71^{f}	$3.38^{b,c,d}$	0.1031

1	14.99^{h}	$33.02^{e,f}$	40.5^{d}	1.3794
3	23.75^{g}	$50.33^{a,b,c}$	$53.88^{a,b}$	1.1878
5	$21.27^{g,h}$	$42.46^{c,d,e}$	$52.39^{a,b,c}$	2.6438
7	$25.32^{f,g}$	$43.97^{c,d}$	$52.64^{a,b,c}$	1.7466
9	22.35^{g}	$48.33^{a,b,c}$	$50.68^{a,b,c}$	1.6647
11	$22.27^{g,h}$	$46.45^{b,c,d}$	57.91^{a}	2.1733

Data reported as least squares means +/- standard error
Ration*period effect on yolk folate content $P<0.0039$
Ration*period effect on egg folate content $P<0.0057$
a,b,c means within each parameter with differing letters are significantly different ($P<0.05$)
All means are composed of 13 replicates

Figure 9. Significant ration*period interaction for body weight of Hy-Line CV20 laying hens over 11 periods.



Data reported as least squares means +/- standard error
Ration effect P=0.3333
a,b, means within period with differing letters are significantly different (P<0.05)
All means are composed of 416 replicates

As expected, the egg folate content (**Figure 8**) followed the same pattern as did the yolk folate concentration. Egg folate content significantly (<0.05) increased as the supplementation level of folic acid increased from 0 to 2 to 4 mg folic acid/kg of diet (21.66, 44.09, and 51.33 μg/egg respectively). Yolk folate content is a more accurate way of measuring folate in the egg due to the fact that it measures folate on a μg/g yolk basis rather than a μg/egg basis. Folate measured on a μg/egg basis will vary with the size of the egg, which is not an accurate representation of the yolk folate content. For example if one egg was larger than another egg, it would contain more folate, due to the fact that the larger egg has more yolk in it. This would give the false impression that the larger egg contained more folate than the smaller egg. Per gram of yolk, the smaller egg could contain the same amount of folate as the the larger egg, however since it is smaller, with less yolk, the smaller egg would appear to contain less folate when compared to the larger egg.

Body weight of hens significantly ($P<0.05$) increased from 1.41 kg at the beginning (period one) of the production cycle to 1.60 kg at the end (period eleven) of the production cycle (**Figure 9**). There were significant differences ($P<0.05$) in hen body weight (**Figure 9**) between the hens consuming the 0 and 4 mg folic acid/kg of diet in period eleven. Hens fed the 4 mg folic acid/kg diet weighed significantly more than hens fed the basal diet (1.62 versus 1.59 kg respectively).

It is noteworthy that the type of statistical program used to analyze the data was compound symmetry which allowed heterogeneous variance across periods, or unequal variance across periods. Therefore, the standard error of the mean was different for each

period. The program allowed for data points to be analyzed more accurately as they were, without the data points requiring transformation prior to analysis.

3.6 Discussion

The focus of the 11-period production trial was to build on egg folate-fortification work done by Sherwood et al. (1993), House et al. (2002), and more recently Hebert (2005) and determine the effect of three levels of folic acid supplementation on consistency of egg folate deposition as well as the effect of folic acid supplementation on egg production and bird health. Additionally, it was to be determined if a lower level of folic acid supplementation (2 versus 4 mg/kg diet) would be adequate to maximize egg folate content over the hen's full production cycle.

3.6.1 The Effect of Dietary Folate and Hen Age on Consistency of Egg Folate Content

As expected, hen age (period) had a significant effect on each performance parameter. For example, egg production increased to a peak, then declined; egg size and yolk size gradually increased over the hen's production cycle; and shell quality declined as the hens aged. This is due to physiological changes that naturally occur in a hen, or the egg throughout the production cycle (Ledur et al., 2002; Fletcher et al., 1983).

In period three, during peak hen-day production, the yolk folate content of eggs from hens consuming the 4 mg folic acid/kg diet also peaked, then significantly decreased (compared to the eggs from hens consuming the 2 mg folic acid/kg diet), and remained relatively constant for the rest of the production cycle (data from figure 6). When hens were supplemented with 2 and 4 mg folic acid/kg diet, yolk folate content peaked in period three, during peak hen-day production for both folic acid

supplementation levels. For the eggs from the 2 mg folic acid/kg diet (data from figure 6), following the peak in yolk folate content, the yolk folate content significantly decreased, and remained relatively constant for the remaining periods. For the eggs from the hens consuming the 4 mg folic acid/kg diet (data from figure 7), following the peak in yolk folate content, the yolk folate content decreased slightly, and remained constant for the remaining periods. Yolk folate content of eggs from hens consuming the 4 mg folic acid/kg diet remained at 3.40 $\pm$ 0.46 µg folic acid/g yolk; CV~13.50% (calculations not shown) throughout the entire production trial. The variability of egg folate concentration was greater for the 0 and 2 mg folic acid supplementation levels. Yolk folate content of eggs from hens consuming the 0 and 2 mg folic acid/kg diet was 1.42 $\pm$ 0.22 µg folic acid/g yolk; CV~15.87%; and 2.93 $\pm$ 0.49 µg folic acid/g yolk; CV~16.94% respectively. The simultaneous yolk folate content and hen-day production peaks may be explained by the fact that during this time period (period three) the hens are also the most efficient, therefore, the vitamin transfer is the greatest. Hebert's (2005) yolk folate data from an 11-period production trial (egg folate values only up to period nine), also showed that yolk folate content peaked with hen-day production, then declined slightly and remained constant for the remaining periods.

Contrary to the more recent findings in chickens, data from a 23-week trial performed by Robel (1983) suggested that vitamin and mineral levels deposited in the egg are related to the aging process of the hen. Yolk folate content of turkey eggs, from turkeys fed folic acid-supplemented diets decreased over time. In hens supplemented with riboflavin for 42 weeks, egg riboflavin concentrations decreased with the age of the hens (Naber and Squires, 1993). The reason for the phenomenon was not stated (Naber

and Squires, 1993), and it is possible that there is a species difference with respect to egg folate levels in chickens compared to turkeys. Perhaps older birds may have a decreased ability to absorb vitamins, or may have a lower efficiency of folate (vitamin) transfer to the ovaries and magnum via folate-binding proteins. Riboflavin studies have shown that riboflavin transfer across membranes (White et al., 1986) or the hormonal induction of liver riboflavin-binding protein synthesis decreases with age.

3.6.2 The Effect of Dietary Folate and Hen Age on Production Performance

Feed efficiency and percent yolk of egg weight were parameters significantly affected by diet. Feed efficiency was significantly better in the hens consuming the 4 mg folic acid/kg diet than the 0 and 2 mg folic acid/kg diets (see figure 7), possibly due to the fact that the overall mean egg production of hens consuming 4 mg folic acid/kg diet was higher than the mean egg production of hens consuming 0 and 2 mg folic acid/kg diet. Generally, feed efficiency is expected to improve with the maturity of the hen, since the hen is eating only for maintenance and production, and not growth (Renden, 1987). It is possible that less folate is required by laying hens as they mature, due to the same reason that feed efficiency is expected to improve, as hens age, they require less folate to synthesize tissue for growth. As hens age they are eating more for maintenance and production rather than growth and tissue (bone, muscle, and fat) synthesis.

Percent yolk of egg weight was significantly higher in the eggs of hens consuming 2 mg folic acid/kg diet possibly due to the fact that the overall mean egg weight for this treatment was lower than egg weight from hens consuming 0 or 4 mg folic acid/kg diet. The lower egg weight of hens consuming 2 mg folic acid/kg diet, and relatively similar yolk weight would result in a greater percent yolk of egg weight.

Significant ration by period effects were evident for hen-day production, feed consumption, yolk folate content, egg folate content, and body weight. There was no difference in feed consumption of hens consuming 4 mg folic acid/kg diet compared to hens consuming 0 or 2 mg folic acid/kg diet. Perhaps there was no significant difference between yolk folate content in eggs from hens consuming 2 and 4 mg folic acid/kg diet in period three because of the increased efficiency of vitamin deposition by the hen during peak egg production. Therefore, more folate may have been deposited during period three in the egg yolk of the hens consuming 2 mg folic acid/kg diet, compared to yolk folate deposition from 2 mg folic acid/kg diet in other periods. Body weight of hens consuming the 4 mg folic acid/kg diet was greater possibly due to the fact that the hens consuming that ration also consumed more feed.

Specific gravity, a measure of egg shell quality, was not significantly affected by dietary folic acid. In an experiment in which folic acid and vitamin B_{12} was removed from the diet of hens during the latter stages of the egg production cycle, there were no adverse effects on performance, except that egg weight was significantly reduced, and because of reduced egg weight, egg shell quality (specific gravity) was significantly increased (Keshavarz, 2003).

Egg weight increases with the age of hens (Ledur et al., 2002; Fletcher et al., 1983), and the proportion of the yolk is less in small eggs than in larger ones. Egg size increases proportionally with yolk size, and the solids content of eggs from older hens, which lay larger eggs, may be significantly different than those from younger birds (Rossi and Pompei, 1995). In an experiment with twenty flocks (various strains) Rose et al., (1966) found significant differences in the percentage of solids in the egg throughout

the hen's production cycle. Differences in the percentage of solids were attributed to differences in the length of time the hen had been laying, egg size, and genetics of the hen (Rose et al., 1966).

In general, the percentage of total uncollectable eggs was higher at the beginning, and end of the 11-period production cycle. This could be attributed to the increased incidence of shell-less eggs at the beginning of the production cycle when the hen is younger, and the laying system is still becoming synchronized, and the large egg size at the end of the production cycle. As a result egg quality decreased with age of the birds. Ledur et al., (2002) also noted similar findings. Reduced egg shell quality associated with aging is due to the fact that as hens age, they lay larger eggs. As hens age, absolute daily retention of calcium (Keshavarz and Nakajima, 1993) and shell weight remain constant (Roland et al., 1975). The same amount of calcium must be distributed over a larger surface area, resulting in a thinner, poorer quality egg shell. Specific gravity describes shell strength and relates to the egg's resistance to breakage (Grunder et al., 1989). Since specific gravity decreased in latter production, this would contribute to the increased number of total uncollectable eggs, and increased number of cracked eggs at the end of the production cycle.

3.6.3 The Effect of Dietary Folate on Efficiency of Egg Folate Deposition The folate level of the basal barley-based diet was 0.9075 mg folate/kg of the barley diet (measured by Covance Laboratories Inc., Madison, WI). Thus it is evident that even without dietary supplementation laying hens receive 3.63 times (0.9075/0.25 mg/kg diet) the NRC recommended folate requirement. Considering the folate content in the basal diet (0.9075 mg/kg diet) the actual dietary folate concentrations in the 2 and 4 mg/kg diet

supplementation levels were approximately 2.9075 (2.0 +0.9075) and 4.9075μg/g yolk (4.0 +0.9075) respectively.

Efficiency of folate deposition decreased with increasing folic acid supplementation. For example when no dietary folic acid was supplemented, 1.42 μg/g yolk was deposited in egg yolk, however when 2 mg/kg crystalline folic acid was supplemented, 2.94 μg/g yolk was deposited in the egg yolk, which is a 207% increase in egg folate content. In comparision, when 4 mg/kg crystalline folic acid was supplemented in the diet, 3.40 μg folate/g yolk was deposited in the egg there was a 116% (3.40/2.94) increase in egg folate content. Therefore after doubling the crystalline folic acid supplementation rate, the percent of increased folate deposition was halved. The efficiencies of folate deposition for the 0, 2, and 4 mg/kg folic acid supplementation levels were 22.25, 14.11, and 9.72 % respectively.

Perhaps this decrease in folate efficiency of deposition is due to the fact that as the egg folate nears its saturation point, slightly higher than 2 mg/kg folic acid dietary supplementation (House et al., 2002), the mechanism responsible for egg folate saturation becomes less effective, resulting in a decreased efficiency of deposition. Any increase in dietary folic acid supplementation caused a drop in efficiency and content of egg folate deposition, supporting the suggestion (Sherwood et al., 1993) that there may be a limited number of folate binding proteins available for transporting folate into the egg. For example, perhaps protein transcription is decreased and less folate binding proteins are synthesized, thus folate cannot be mobilized and deposited in the egg as efficiently as when there are more folate binding proteins available.

3.6.4 The Effect of Dietary Folate on the Livability of Hens

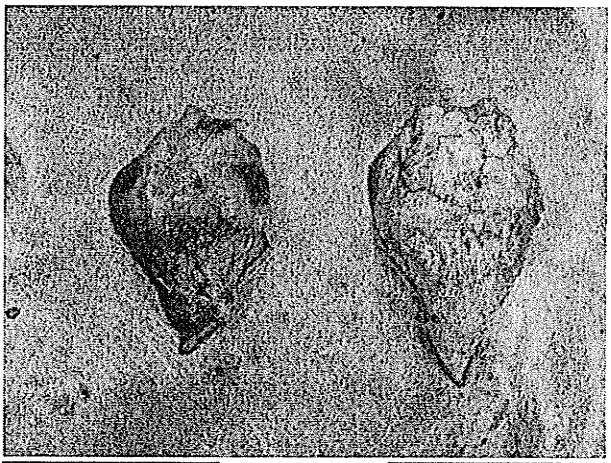
An eleven-month laying hen trial involving two strains of laying hens led Hebert (2005) to speculate whether or not folic acid could improve the overall livability of laying hens. Within the trial it was found that more hens died from the 0 mg folic acid/kg diet than from the 4 mg folic acid/kg diet. However, the low mortality of birds within the trial, and a Chi Square Test used to verify significance of the finding, proved that in fact there was no significant difference in mortality between diets.

Post mortems were performed on all mortalities during the current 11-period laying hen trial, to verify causes of death, and compare mortalities among all three levels of folic acid supplementation (0, 2, and 4 mg folic acid/kg diet). Hebert (2005) found that plasma homocysteine concentrations tended to decrease with increasing dietary folate supplementation. The Hy-Line W98 strain also exhibited a significant decrease in plasma homocysteine levels when supplemented with 2 mg folic acid/kg diet. Due to the fact that plasma homocysteine concentrations tended to decrease with increasing dietary folate supplementation, and the Hy-Line W98 strain exhibited a significant decrease in plasma homocysteine levels when supplemented with 2 mg folic acid/kg diet, it is speculated that a reduction in hen plasma homocysteine due to folate supplementation could result in improved hen livability. In the short term, hen-day production may not be affected by increased folate supplementation; however long term, it is possible that a folate deficiency could impact DNA synthesis and reduce responsiveness to an immune challenge (Steinberg, 1984).

Mortality throughout this trial was 4.5%. In a commercial operation mortality of 4-5% is common throughout the production cycle (Hy-Line Inc., 2004; 2003). A Chi Square test revealed a significant difference between mortality in birds consuming 2 mg

folic acid/kg diet and the other two diets. However, the mortalities were not caused by cardiovascular incidents in the hens. A closer evaluation of causes of death showed that ~50% of the mortalities within each ration could be attributed to urolithiasis. Urolithiasis is a condition in which kidney function has decreased to the point where uric acid (nitrogenous waste) accumulates in the blood and body fluids (Hy-Line, 2003). The laying hens that died of urolithiasis had white chalky deposits over the heart, liver, and abdominal cavity, and the cause of death was verified by a veterinary pathologist (**Figure 10**).

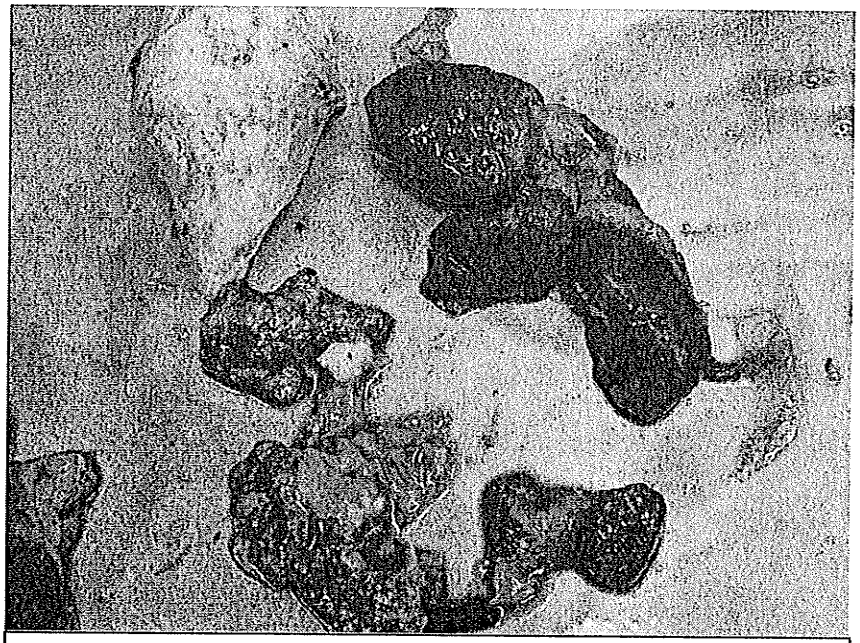
Figure 10. Pictures of urolithiasis in laying hens



Heart from hen without urolithiasis.

Heart from hen with urolithiasis. Note the chalky, white residue.

Figure 10. Pictures of urolithiasis in laying hens (continued from previous page)



Heart and other organs from laying hen that died due to urolithiasis. Note the chalky white residue, and chunky white pieces in the organs.

Aside from the previously stated trial by Hebert (2005), there has been no data collected with respect to evaluating homocysteine concentrations and cardiovascular incidents in laying hens. In a review of epidemiologic studies on the association between homocysteine level and risk for cardiovascular disease as well as the potential benefits of homocysteine-decreasing therapies, it was concluded that although simple, inexpensive, nontoxic therapy with folate and vitamins B_6 and B_{12} is highly effective at reducing plasma homocysteine levels in humans, it remains to be demonstrated that decreasing homocysteine levels reduces cardiovascular morbidity and mortality (Eikelboom et al., 1999). Furthermore, in observational studies whereby it was assessed whether folic acid,

B_6, and B_{12} supplementation reduced the risk of major cardiovascular events in patients with vascular disease, it was found that supplementation did not reduce the risk of major cardiovascular events in patients with vascular disease (HOPE-2 investigators, 2006). Even though folic acid supplementation may reduce plasma homocysteine concentrations in hens (Hebert, 2005), subsequent protection from cardiovascular incidents may not occur.

In summary, hens consuming the 4 mg folic acid/kg of diet deposited yolk folate at a higher rate throughout their production cycle than those hens consuming 0 and 2 mg folic acid/kg diet. The 4 mg folic acid/kg diet offered the additional benefit of improving egg production in period 11. Aside from hen-day production, other performance parameters of the laying hens were not affected by crystalline folic acid supplementation. Though there was a significant difference among mortalities between different rations, this was not attributed to dietary folic acid supplementation. Urolithiasis was the cause of the majority of laying hen deaths, and urolithiasis was most likely caused by including too much calcium in the pullet (immature laying hen) diets. This long term trial supports the use of 4 mg folic acid/kg diet as the optimal level of folic acid supplementation within laying hen diets to maximize quantity of egg folate deposition over time.

4.0

MANUSCRIPT 2

Effect of Folic Acid Supplemented Rye/Wheat Diets With and Without Enzyme on Folate Deposition in the Egg Yolk.

4.1 Abstract

Previous research has shown that egg folate deposition is a saturable process. One potential regulatory mechanism exists at the level of intestinal folate absorption. Feed constituents can influence nutrient absorption: diets high in non-starch polysaccharides (NSPs) are poorly digested, leading to increased intestinal viscosity and potentially reduced folate absorption. The objectives of the current trial were 1) to determine the effect of the addition of dietary enzymes to a rye/wheat-based diet on egg folate content, and 2) to determine the effect of rye/wheat-based rations supplemented with crystalline folic acid on the egg folate content. To address these objectives, 120 Hy-Line CV20 laying hens (n = 12 per diet) received a 40% (of grain) rye/wheat ration containing 0, 0.5, 1.0, 2.0, or 4 mg of crystalline folic acid/kg of feed with or without a β-glucanase and xylanase enzyme cocktail (2000 units/kg of diet) for six 7-day periods. Response criteria included measurement of *in vivo* viscosity and egg folate content. Data was analyzed as a completely randomized design, which revealed that the enzyme had a significant ($P<0.0001$) main effect on *in vivo* viscosity. Folate data was analyzed as a repeated measures design using the mixed procedure which showed that the addition of enzymes to the diet had no significant ($P>0.05$) effect on egg folate levels. Egg folate content significantly ($P<0.0001$) increased 2.3-fold from an unsupplemented diet without enzyme (1.2 μg of folate/g of yolk) to a diet supplemented with 4 mg folic acid/kg without enzyme (2.6 μg of folate/g of yolk). Average egg folate levels over the five periods were 1.3, 1.7, 2.1, 2.5, and 2.6 μg/g of yolk for respective rations containing 0, 0.5, 1.0, 2.0, and 4 mg of crystalline folic acid/kg of diet without enzyme. While folate

levels in the egg increased as the amount of supplemental dietary crystalline folic acid increased, the intestinal viscosity did not affect egg folate deposition.

4.2 Introduction

It has been previously shown (House et al., 2002; Hebert 2005) that a linear increase in egg folate levels was observed when crystalline folic acid was added from 0 to 2 mg/kg to a laying hen diet, after which egg folate levels appeared to reach a plateau. Manuscript One showed that 4 mg folic acid/kg diet is needed to maximize egg folate content and consistency throughout the production cycle of laying hens. The mechanism responsible for egg folate saturation has yet to be determined.

Sherwood et al., (1993), (using a radio-isotope dilution assay) determined that both yolk and plasma folate concentrations became saturated with increasing dietary folate. Data from House et al., (2002) and Sherwood et al., (1993) support the suggestion that saturation of egg folate content is not due to limitations in transport processes from plasma to the egg yolk. Both authors agree that the regulatory processes controlling plasma folate levels might be the point of metabolic control. In studies with rats and *in vitro* intestinal cell model systems, folic acid has been shown to be absorbed from the gut via a membrane-bound folate transport system (Said, 2004). This process has been shown to be saturable in a number of model systems (Said, 2004), and thus represents a potential control point for plasma folate concentrations.

To test this theory, a model must be created so that egg-folate deposition can be measured when intestinal uptake of folic acid is impaired. Nonstarch polysaccharides in certain cereals, most notably β-glucans in barley and oats, and pentosans (arabinoxylans) in wheat and rye, which are associated with the endosperm cell wall of the grain, cannot

be hydrolysed by enzymes produced by the laying hen (Rotter et al., 1990). Nonstarch polysaccarides increase the viscosity of the gut contents, which reduces digesta passage time, and impairs diffusion of endogenous digestive enzymes to the substrates and mixing of the gut digesta (Antoniou et al., 1981; Antoniou and Marquardt, 1982). The viscous conditions of the intestine may negatively impact the amount of folate available for uptake by intestinal cells.

In a short term trial, Hebert (2005) found that dietary ingredients appeared to affect total egg folate content. For example, a corn-based diet resulted in eggs with significantly higher total folate content than a wheat-based diet.

To explore the relationship between egg folate deposition and intestinal folate absorption, a short term trial involving rye/wheat-based, rations and enzyme was performed.

4.3 Hypotheses and Objectives

4.3.1 Hypotheses

Alternative Hypothesis:

1) Egg folate content will be affected by the addition of dietary enzymes to a rye/wheat-based diet.
2) Egg folate deposition will be affected by increased gut viscosity.
3) *In vivo* viscosity of laying hen jejunal digesta will be affected by addition of enzymes to rye/wheat-based rations.

Null Hypothesis:

1) Egg folate content will not be affected by the addition of dietary enzymes to a rye/wheat-based diet.
2) Egg folate deposition will not be affected by increased gut viscosity.
3) *In vivo* viscosity of laying hen jejunal digesta will not be affected by addition of enzymes to rye/wheat-based rations.

4.3.2 Objectives

The purpose of this research was to explore one potential regulatory mechanism of egg folate saturation; the relationship between egg folate deposition and intestinal folate absorption. This will be accomplished through the following objectives:

i) To determine the effect of the addition of dietary enzymes to a rye/wheat-based diet on egg folate content.

ii) To determine the effect of rye/wheat-based rations supplemented with five levels of crystalline folic acid on the egg folate content.

iii) To determine the effect of the addition of dietary enzymes on *in vivo* viscosity of laying hen jejunal digesta.

4.4 Materials and Methods

4.4.1 General

Hens were kept in confinement housing under semi-controlled environmental conditions and exposed to a 16-hour photoperiod, with 8 hours of darkness. One hundred twenty Single-Comb White Leghorn Hy-Line Commercial Variety (CV20) laying hens sourced from Manitoba Perfect Pullets, (Rosenort, Manitoba, Canada) were housed individually; the cage dimensions were 25.4 cm by 40.64 cm, providing 1032 cm^2 per bird. Laying hens were approximately 36 weeks old, and within the sixth month of production. Feed and water were available *ad libitum*. Animal care approval was received from the University of Manitoba's Animal Care Protocol Review Committee, and the birds were managed in accordance with recommendations established by the Canadian Council on Animal Care (1984).

4.4.2 Diet and Experimental Protocol

The basal diet was a rye (40% of grain) and wheat ration (**Table 10**). The diet was formulated to meet the requirements of laying hens consuming approximately 100 g of feed/day (NRC, 1994). In accordance with industry standards (BASF, 2000), the basal diet included no crystalline folic acid.

For two weeks prior to the commencement of the study, 150 healthy hens of the Hy-Line CV 20 strain each received a standard barley-soybean meal ration with no additional folic acid supplementation. These hens were monitored for egg production,

Table 9. Composition of basal rye/wheat based laying hen ration.

Ingredient	%
Wheat (13.8 % CP)	36.85
Rye (37.58% CP)	25.00
Soybean (45.8% CP)	20.10
Fishmeal (60% CP)	2.00
Vegetable Oil	3.05
Limestone	9.52
Biophos (monocalcium phosphate)	1.09
Vitamin premix[1]	1.00
Mineral premix[2]	0.50
Calculated nutrient Composition	
Crude Protein	18.00
Metabolizable energy, kcal/kg	2700
Calcium	4.01
Phosphorus (total)	0.40
Folate[3] mg/kg	0.91

[1]Provided (per kg of diet): 8255 IU vitamin A; 3500 IU vitamin D_3; 30.0 IU vitamin E; 0.55 mg vitamin K_3 (menadione with Na-bisulfite); 0.011mg vitamin B_{12}; vitamin B_{58} complex (contains/kg: 4.50 mg riboflavin, 8.24 mg pantothenic acid, 12.55 mg niacin, and 164.80 mg choline); 0.100 mg biotin; 10 mg niacin; 2 mg thiamine (B_1) (mononitrate); 3 mg pyridoxine (B_6) (HCl); 1037 mg choline chloride (contains 520,800 mg choline/kg); 900 mg dl-methionine; 125 mg ethoxyquin (anti-oxidant).

[2]Provided (per kg of diet): 70 mg MnO (manganese oxide); 66 mg ZnO (zinc oxide); 50 mg $FeSO_4$ (ferrous sulfate); 8.8 mg $CuSO_4$ (copper sulfate); 0.3 mg $NaSeO_3$ (sodium selenite); 0.33 mg $Ca(IO_3)_2$ premix (calcium iodate premix contains: 2.0 g calcium iodate and 43 g iodized salt); 0.67 mg iodized salt.

[3]Measured by Covance Laboratories Inc., Madison, WI via Official Methods of Analysis of AOAC International (2000) 17th ed., AOAC International, Gaithersburg, MD, USA, Official Methods **960.46, 992.05**.

and the 120 highest producing hens were selected for the experiment. Information on body weight, feed consumption, egg production, and egg weight was recorded.

Individual body weight of all hens was taken at the initial and final day of the experiment to determine if the diets had any effect on the body weight of the hens. Mortalities were recorded as they occurred. Residual feed was weighed back at the end of each period to determine feed consumption for each replicate and average daily feed intake so that feed efficiency could be calculated. Egg production was recorded daily for each replicate and an average egg production rate (hen-day percent) was calculated for each production period.

The selected hens were randomly assigned to one of ten diets: the basal 25% rye/wheat diet plus or minus enzyme containing 0, 0.5, 1.0, 2.0, or 4.0 mg of crystalline folic acid/kg of feed each (Sigma Chemical Co., Oakville, ON, Canada) (n = 12 hens per treatment). The folate level of the basal (not supplemented with folic acid) rye/wheat-based diet was 1.01 mg (measured by Covance Laboratories Inc., Madison, WI). Considering the folate content in the basal diet (1.01 mg/kg), the actual folate concentrations were 1.01, 1.51, 2.01, 3.01, and 5.01 mg/kg diet for the respective levels of crystalline folic acid supplementation: 0, 0.5, 1.0, 2.0, and 4.0 mg/kg.

The enzyme cocktail (Canadian Bio-Systems Inc, Calgary, AB, Canada) consisted of β-glucanase and xylanase as the main enzymes, with broad spectrum side activities. The enzyme premix (β-glucanase plus xylanase) was supplemented at a level of 0.5% of the diet yielding 2000 activity units/kg of feed. The birds were given a two-week adaptation period followed by six 7-day periods. Ninety eggs (1 egg/replication; 9 replications/treatment) were randomly collected per treatment per day for two days (180

eggs total). The eggs were weighed to give an average egg weight for the treatment period and processed for egg folate determination.

4.4.3 Extraction of Egg Yolk Folate

The extraction of the egg yolk folate content was performed as described previously in Section 3.4.3. In brief, eggs were weighed, placed in boiling water for 10 minutes, cooled, and the yolks separated, weighed, and retained for analysis by storing at -20°C. Egg folate in the form of 5-MTHF, was extracted into an ascorbate buffer (pH 7.8) using a series of buffer additions, centrifugation, and supernatant collection.

4.4.4 Analysis of Egg Yolk Folate Content

The analysis of the egg yolk folate content was performed as described previously in Section 3.4.4. In brief, the extracts were analyzed for 5-MTHF via reverse-phase HPLC with fluorescence detection (Shimadzu, Mantech, Guelph, Ontario, Canada), using the method of Vahteristo et al. (1997). An external, standard curve with purified 5-MTHF was used to quantify egg folate concentrations. The inter and intraassay CV for determinations was <2%, and recovery of 5-MTHF added to egg yolk was 98.9%. The content of egg folate was expressed as micrograms of folic acid per egg.

Following the six week trial, when the birds were 42 weeks old, the hens were euthanized by cervical dislocation to verify the viscosity of the jejunal contents. The small intestine was removed from the end of the duodenal loop to the ileo-ceco-colic junction, and the digesta was collected from the jejunum portion of the small intestine to measure digesta supernatant viscosity. Digesta samples from six hens consuming each of the ten diets (n=6/diet; 60 hens in total) were pooled and mixed together prior to spooning digesta into the microcentrifuge tubes.

4.4.5 *In Vivo* Viscosity Measurement

Fresh jejunal digesta was spooned out of the respective sample bag, into a 1.0 ml microcentrifuge tube, in duplicate. Digesta was then centrifuged for 5 minutes at 9000 rpms. Half a milliliter of supernatant was pipetted into a metal, circular, disk-shaped dish so the viscometer could measure the resistance of the supernatant (viscosity). Viscosity values were reported in centipoises.

Jejunum digesta supernatant viscosity was measured with a Brookfield DVII viscometer. The spindle was S40, temperature of the water bath was 40°C to simulate the internal bird temperature, the speed used was 30 rotations per minute, and 0.5 ml of supernatant was measured at a time in duplicate.

4.4.6 Calculations

The collected production data was then used to calculate hen-day production, egg production, feed consumption, feed efficiency, and percent yolk of egg weight, based on the equations explained in the previous section, (section 3.4.6).

4.4.7 Statistical Analysis

A completely randomized design, with a 5 x 2 (folate level x enzyme as main effects) factorial arrangement of treatments was used to analyze the data for the 3 experimental periods (period 1, period 3, and period 5) throughout the 6-period trial. Statistical analysis was performed with the Statistical Analysis System (SAS Institute Inc., 1998). The mixed procedure was used, with compound symmetry with heterogeneous variance across periods, or allowing unequal variance in all periods, which proved to be the most accurate method available for the data. Folate data was analyzed as a repeated measures design. Performance data collected were subjected to type 3 tests

of fixed effects followed by a least square difference test using the pdmix 800 program, which determined differences between means. To enable the SAS program to recognize the hen-day production values that were >1, an arc sin transformation needed to be performed. Differences with an alpha level of $P<0.05$ were considered to be statistically significant. *In vivo* viscosity measurements were analyzed using the mixed procedure with a completely randomized design, allowing for differing variances across treatments.

4.5 Results

The main effects of ration, enzyme, and period, and their interactions for different performance traits are shown in (**Table 10**).

Significant ($P<0.05$) ration differences existed for feed consumption (**Table 11**).

Significant enzyme ($P<0.0001$) differences are shown only for *in vivo* viscosity (**Figure 11**). The mean viscosity of jejunal digesta *in vivo* of laying hens that consumed rye/wheat-based diets without enzyme was 15.2 centipoise (SEM = 1.118) whereas the mean viscosity of jejunal digesta *in vivo* of laying hens that consumed rye/wheat-based diets with enzyme was significantly lower, at 6.9 centipoise (SEM = 0.335).

Significant ($P<0.0001$) period differences were found for hen-day production, feed consumption, feed efficiency, egg weight, yolk weight, and percent yolk of egg (**Table 12**). Throughout the six weeks, egg production started at 92.55 % in period 1, significantly decreased in periods two (82.33 %), and three (77.46 %), then recovered and remained constant at ~89 % for the remaining three periods.

Feed consumption significantly ($P<0.0001$) dropped from 97.52 g/h/d in the first period to 80.60 g/h/d in the second period, and rebounded to 92.24 g/h/d, where it stabilized for the remaining three periods.

In period one, feed efficiency was 1.85, then it significantly ($P<0.0001$) worsened to 2.09 in period three, and significantly improved to 1.77 in period four, remaining relatively constant for the remaining two periods. In the last two periods hens were the most feed efficient.

Egg weight remained relatively constant from period one (58.43 g) to period five (59.7 g) inclusive, and significantly ($P<0.0001$) increased to 60.4 g in period six. Yolk weight changes generally followed the same pattern as egg weight, remaining constant from period one (14.42 g) to period five (14.92 g) inclusive, and significantly ($P<0.05$) increasing to 15.13 g in period six. The percent yolk of egg weight remained relatively constant for the first four periods, then increased to approximately 25 % in period five, and remained constant into period six.

Ration by enzyme interactions ($P<0.05$), and period by enzyme interactions were not observed for any parameters. However, it must be noted that when there are significant 3-way interactions no conclusive statements can be made about the main effects causing the interactions.

The yolk folate content and egg folate content with significant period by folate by enzyme interactions is shown in **Table 13**. Regardless of enzyme, most levels of supplemental folic acid resulted in an increase in egg yolk folate from period one to period three. Another general trend noted in **Table 13** was that the addition of enzyme tended to cause an increase in egg folate deposition. For example, for the 1 mg/kg dietary folic acid supplementation level without enzymes, 32.44g of folate (period 5) was deposited, and when enzyme was added to the diet, 35.83g of folate was deposited in the egg.

Table 10. P-values of main effects and interactions of ration, enzyme, and period on different performance traits.

Parameters	Ration	Enzyme	Period	Ration*Enzyme	Ration*Period	Period *Enzyme	Period*Ration*Enzyme
Feed Consumption (g/h/d)	0.0096	0.8548	<0.0001	0.2633	0.0734	0.6845	0.6946
Egg Production (hen-day)	0.8251	0.5064	<0.0001	0.1346	0.1481	0.2529	0.4408
CRD *In Vivo* Viscosity (cp)	0.7477	<0.0001	NA[1]	0.1781	NA[1]	NA[1]	NA[1]
Egg Weight (g)	0.6186	0.4854	0.0004	0.2127	0.6231	0.9260	0.1534
Yolk Weight (g)	0.3538	0.8748	<0.0001	0.0608	0.5209	0.9289	0.1678
Feed Efficiency (g feed/g egg)	0.5867	0.9731	<0.0001	0.1871	0.1601	0.9354	0.2617
Yolk of Egg (g yolk/g egg)	0.2842	0.2497	<0.0001	0.6041	0.7212	0.9013	0.9769
Yolk Folate (µg/g yolk)	<0.0001	0.6921	<0.0001	0.1111	<0.0001	0.2505	0.0020
Egg Folate (ug/egg)	<0.0001	0.8456	<0.0001	0.0993	0.0007	0.2650	0.0005
Body Weight (kg)	0.6710	0.4833	0.8875	0.8875	0.5243	0.9944	0.9856

[1]Not applicable for specific parameter
Shading signifies significance ($P<0.05$)

Table 11. Feed Consumption of laying hens receiving diets at different crystalline folic acid supplementation – Main effects of ration[1].

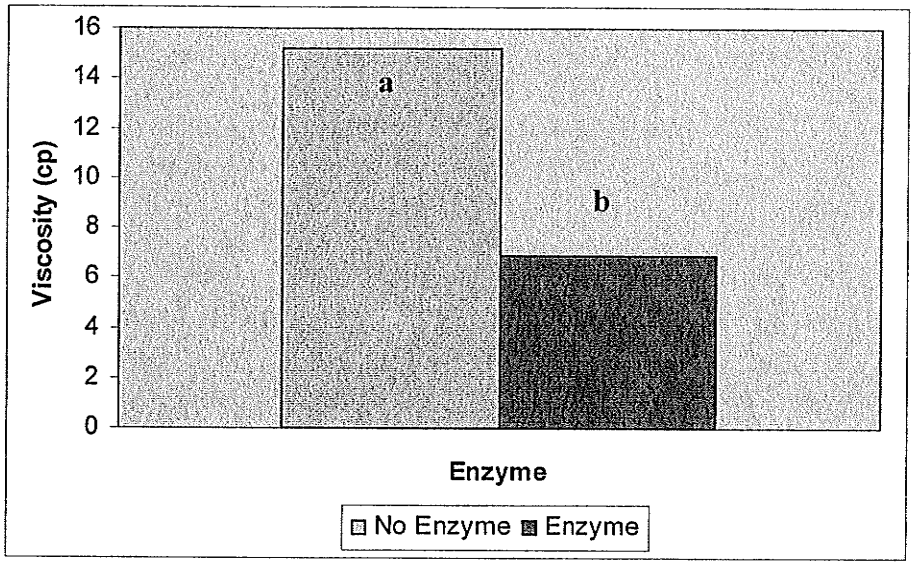
	Supplementation Level (mg/kg diet)						
Parameters	0	0.5	1	2	4	SEM	P-values
Feed[2] Consumption (g)	93.77[a]	93.10[a]	87.31[a]	87.56[a]	92.66[a]	1.67	0.0096

[1]Data are presented as least squares means

[2]F-test showed a significant difference between 1 and 2 mg folic acid/kg diet and 0, 0.5, and 4 mg folic acid/kg diet. To remain consistent with other data reported within the two manuscripts, data was reported using Tukey's Studentized Range Test which showed no significant difference among all 5 dietary folic acid supplementation levels with respect to the feed consumption parameter.

a, b, c, d means are compared within the row; means not followed by the same superscript are significantly different $P<0.05$

Figure 11. Significant enzyme effect on *in vivo* jejunal digesta viscosity of Hy-Line CV20 laying hens.



Data are reported as least squares means
The SEMs for without enzyme and with enzyme are 1.118 and 0.335 respectively
Enzyme effect $P<0.0001$
a,b,c means within enzyme parameter with differing letters are significantly different ($P<0.05$)
Both means are composed of 30 replicates (n=30 hens without enzyme in diet; n=30 hens with enzyme added to diet; 60 hens in total)

Table 12. Performance summary of Hy-Line CV20 laying hens throughout the 6-week trial-Main effects of period[1].

	Parameters											
Period	Hen-day Production (%)	SEM	Feed Consumption (g/hen/day)	SEM	Feed Efficiency (g feed/g egg)	SEM	Egg Weight (g)	SEM	Yolk Weight (g)	SEM	% Yolk of Egg Weight	SEM
1	92.55^{a}	0.0124	97.52^{a}	0.865	1.85^{b}	0.0386	58.43^{b}	0.489	14.42^{c,d}	0.1403	24.3^{c}	0.0016
2	82.33^{b}	0.0124	80.60^{c}	1.5565	1.74^{b,c}	0.0436	59.36^{a,b}	0.4415	14.57^{c,d}	0.1075	24.5^{c}	0.0015
3	77.46^{c}	0.0138	92.24^{b}	1.028	2.09^{a}	0.0598	58.71^{b}	0.7612	14.53^{c,d}	0.171	25.0^{a,b,c}	0.0041
4	88.35^{a}	0.0168	91.33^{b}	1.076	1.77^{b,c}	0.0411	58.77^{a,b}	0.8077	14.54^{c,d}	0.1792	24.6^{b,c}	0.0016
5	91.48^{a}	0.0149	90.86^{b}	1.05	1.64^{c}	0.0292	59.70^{a,b}	0.8052	14.92^{a,b,c}	0.2075	25.1^{a}	0.0017
6	89.75^{a}	0.0141	92.72^{b}	0.856	1.67^{c}	0.0226	60.40^{a}	0.6884	15.13^{a,b}	0.1936	25.1^{a,b}	0.002
p-value	<0.0001		<0.0001		<0.0001		<0.0001		<0.0001		<0.0001	

[1]Data are presented as least squares means

a,b,c means within each parameter with differing letters are significantly different (P<0.05)

Table 13. Yolk folate content (µg folate/g yolk) (a), and egg folate content (µg folate/egg) (b) of Hy-Line CV20 laying hens throughout periods 1, 3, and 5-Variable with significant (P<0.05) period*ration*enzyme[1] interaction.

	Dietary Folic Acid Supplementation (mg/kg)[2]										
(a)	0		0.5		1		2		4		
Period	-	+	-	+	-	+	-	+	-	+	SEM
1	$1.20^{n,o}$	1.08^{o}	$1.70^{h,l,j,k,m,n}$	$1.46^{j,k,n,o}$	$1.79^{g,j,k,m,n}$	$1.82^{f,l,j,k,m}$	$2.09^{d,e,f,g,h,i}$	$2.15^{c,d,e,f,g,h,i}$	$2.24^{c,d,e,f,g,h,i}$	2.97^{a}	0.11
3	$1.46^{i,j,k,m,n,o}$	$1.20^{j,k,l,n,o}$	$1.63^{e,f,g,h,l,j,k,n,o}$	$2.07^{c,d,e,f,g,h,l,j,k}$	$2.45^{a,b,c,d,e,f,h,i}$	$2.52^{a,b,c,d,e,g,h}$	$2.94^{a,b,c}$	$2.66^{a,b,c,d,e,f}$	$3.13^{a,b}$	3.25^{a}	0.19
5	$1.25^{k,n,o}$	1.00^{o}	$1.89^{e,f,g,h,l,j}$	$1.93^{e,f,g,h,l}$	$2.13^{c,d,e,f,g,h,i}$	$2.31^{b,c,d,e,g}$	$2.45^{a,b,c,d,e}$	$2.18^{c,d,e,f,g,h,i}$	$2.51^{a,b,c,d}$	$2.67^{a,b,c,d}$	0.11
(b)											
1	17.65^{m}	16.36^{m}	$23.94^{f,g,l,j,l,m}$	$21.03^{l,m,n}$	$25.19^{f,j,l,m}$	$25.61^{g,j,l,m}$	$29.67^{d,e,f,g,k,l}$	$28.69^{e,f,g,j,l}$	$31.13^{c,e,f,g,k}$	$41.25^{a,b}$	1.83
3	$22.18^{f,g,j,l,m}$	$18.49^{j,l,m}$	$24.09^{f,g,h,j,l,m}$	$31.61^{b,c,d,e,f,g,j}$	$35.79^{a,b,c,d,e,g,h,i}$	$36.16^{a,b,c,d,e,f}$	$44.03^{a,b}$	$35.93^{a,b,c,d,e,f,g}$	46.3^{a}	$44.62^{a,b}$	2.71
5	$19.28^{j,m,n}$	16.36^{m}	$27.91^{e,f,g,j,l}$	$29.74^{d,e,f,g,j,k}$	$32.44^{b,c,d,e,f,g}$	$35.83^{a,b,c,d,e,h}$	$37.06^{a,b,c,d,e}$	$30.82^{c,d,e,f,g,l}$	$39.59^{a,b,d}$	$40.52^{a,b,c}$	1.96

[1]Data are presented as least squares means

a,b,c means within each parameter with differing letters are significantly different (P<0.05)

[2]The -/+ designation denotes without and with (respectively) addition of enzyme to the ration

There was some variation, for example, for the 0 and 2 mg/kg supplementation levels, enzyme did not increase egg folate deposition. This variation in egg folate levels did not result in a significant difference between not adding enzyme to the diet, and the addition of enzyme. Enzyme only increased yolk folate content in period one, at the 4 mg/kg folic acid supplementation level. The fact that enzyme increased yolk folate content at the 4 mg/kg folic acid supplementation level, only in period one is important, because it is evident that the 4 mg/kg level can increase yolk folate content more quickly than any other level. Therefore, commercially it could be possible to feed the 4 mg/kg folic acid diet with enzyme to achieve maximum yolk folate levels. Without enzyme, the 4 mg/kg folic acid diet would need to be fed for five weeks to achieve the same yolk folate level that enzyme with the 4 mg/kg level achieved in one week. The 4 mg/kg folic acid supplementation level with enzyme resulted in significantly more egg folate than any other supplementation level only in period one.

Enzyme had a beneficial effect on various levels of dietary folic acid supplementation. For the 0.5 mg/kg level with enzyme, the yolk folate level significantly increased from period one to period five. For the 1 mg/kg level with enzyme, yolk folate significantly increased from period one to period three, and from period one to period five. In some of the levels of dietary folic acid supplementation, when yolk folate increased from one period to the next even when no enzyme was present. For example, for the 1 mg/kg level without enzyme, yolk folate significantly increased from period one to period three. For the 2 mg/kg level without enzyme, yolk folate content significantly increased from period one to period three, and for the 4 mg/kg level without enzyme,

yolk folate content significantly increased from period one to period three. Egg folate content followed the same patterns as did yolk folate content.

Egg folate content with significant period by ration by enzyme interaction, followed a pattern similar to the yolk folate content. The only difference was that in period one there was no significant increase in egg folate content from 0 mg/kg folic acid level with enzyme (16.36 μg/egg) to 0.5 mg/kg folic acid level without enzyme (23.94 μg/egg). The yolk folate content of Hy-Line CV20 laying hens throughout periods 1,3, and 5-Variable with significant ($P<0.05$) period by ration by enzyme interactions is graphed in **Figure 12** (please see appendix). At the 4 mg folic acid/kg diet level, enzyme had a consistent positive response to yolk folate content, which was significant in period one.

It is noteworthy that the type of statistical program used to analyze the data was compound symmetry, which allowed heterogeneous variance across the periods, or unequal variance across the periods. Therefore, the standard error of the mean was different for each period. The program allowed for data points to be analyzed more accurately as they were, without the data points requiring transformation prior to analysis.

4.6 Discussion

To determine the extent of small intestine participation in egg yolk folate regulation, a gut viscosity model was employed. The study is a follow-up of work done by Hebert (2005) on the affect of basal dietary ingredients on the folate-fortification of eggs. This short term study was conducted to explore one potential regulatory mechanism of egg folate saturation; the indirect relationship between intestinal folate absorption and egg folate deposition by measuring gut viscosity.

4.6.1 Effect of Folic Acid-Supplemented Rye/Wheat-Based Rations on Egg Folate Content

Feed consumption, was significantly affected by ration. The diets with the highest overall feed consumption, also had the heaviest eggs. It was expected that the yolk and egg folate content would be affected by ration, due to the fact that the more folic acid that was supplemented within the diets, the more folate was deposited in the egg yolks. Chickens do have taste buds; however, taste buds in addition to other organs of taste and smell are poorly developed, and have hardly any significance (Koch, 1973). It is therefore unlikely that chickens would prefer one ration over another, when the rations only differed by the level of folic acid supplementation. There has been no research done to test whether or not folic acid is an appetite stimulant.

Overall feed consumption, irrespective of diet significantly dropped ($P<0.05$) from 97.52 g/hen/day in period one to 80.60 g/hen/day in period two. Period two was during August 19-25th, the weather was especially warm (mean temperature = 27.4 °C) during this time period (Environment Canada, 2006), therefore the temperature in the barn was higher than normal. The warm barn conditions most likely caused the drop in feed consumption. As was reported by the National Academy of Sciences (1981) under commercial production systems, where temperature inside the barn may range from 20-37 °C, the feed intake of laying leghorns will decrease by 1.0-1.5 g per day per 1°C from 25 to 34 °C but by 4.2g per 1 °C from 32 to 36 °C (Davis et al., 1973) .

Egg production, feed consumption, and feed efficiency followed the same trend of inconsistent values for the first three periods of the trial followed by consistent values for the second half of the trial. The trend suggests that it took three weeks for the digestive

tracts of the hens to adapt to the viscous diet. The high temperatures during period two may also have contributed to inconsistent production parameter values. For example, since the mean outside temperature during period two was 27.4 °C (Environment Canada, 2006), the barn temperature was around 27 °C, and hens were consuming 3-4.5 grams/bird/day less than normal. Lower feed consumption would result in lower egg production, and poorer feed efficiency. Following period three, hens had increased their feed consumption, hen-day production increased, and feed efficiency had improved from period three. Period three production parameter values are poorer than period four because the hens were recovering from the effects of the hot weather. Following the period of hot weather, the hens increased their feed consumption, and consequently egg production and feed efficiency values also improved, contributing to the inconsistent values within the first half of the trial.

It is known that viscosity producing dietary cereals induce changes in the morphology of host intestinal tissues (Johnson and Gee, 1986), which results in enlargement of the small intestine, prolonged epithelial villi, and higher turnover of epithelial cells (Gee et al., 1996). These changes should also increase total bacterial colonization potential on host tissue surfaces and mucous layers due to a larger intestinal surface. All feed ingredients that are not digested by the animal are potential substrates for bacteria (Moir, 1991). Therefore, nondigestible NSPs such as in rye/wheat diets, inside the intestine of the laying hen, create an ideal opportunity for bacteria to grow. It is possible for diet to shift intestinal bacterial composition. For example in an experiment by Hübener et al., (2002), the wheat/rye diet shifted maximum bacterial growth so that NSP degrading bacteria that are normally found in the cecum increased in the ileum.

Xylanase supplementation to the wheat/rye based diet decreased bacterial counts, especially in the small intestine (Hübener et al., 2002). In the case of water soluble NSP-rich feed stuffs, which are poorly digestible by the hen, bacterial flora is believed to play a role in the negative effect observed on feedstuff digestion (Maisonnier et al., 2003). Bacteria may impair lipid digestion and may modify carbohydrate and protein digestion (Gabriel et al., 2006), and have a negative effect on vitamin nutrition (Gabriel et al., 2006). As reviewed by De Smet et al., (1994), lactic acid bacteria with the active bile salt hydrolase are suggested to cause a lowering of serum cholesterol levels through an interaction with the host's bile salt metabolism (De Smet et al., 1998).

Yolk folate content significantly increased as dietary folic acid supplementation increased. Generally, yolk folate content from the five dietary folic acid supplementation levels remained constant throughout the 6-week trial. Yolk folate content of eggs from hens supplemented at the 4 mg folic acid/kg diet was significantly higher at peak production. İnal et al. (2001) conducted a study using Hisex Brown laying hens to look at the effects of withdrawal of vitamin and trace mineral supplements from layer diets on egg yield and trace mineral composition. İnal et al., (2001) found that the birds that received the unsupplemented diet, had a lower feed intake, lower egg production, produced significantly lighter eggs, and poorer feed efficiency than the birds fed the standard commercial layer diet. Further research trials comparing feed efficiency to vitamin/mineral incorporation into the egg are required to verify the reasoning behind the finding of more yolk folate at peak production during the laying hens' production cycle. Perhaps the intestine of the hen is the most efficient at absorbing nutrients during peak

production, which may result in the most efficient time of transferring folate into the yolk, within the ovary.

Yolk and egg folate content were parameters that had significant ration by period interactions. The two-way interaction for the yolk and egg folate content can be explained by the fact that the yolk folate content of eggs from each dietary folic acid supplementation level generally increased in period three from period one, and decreased again in period five.

The rye/wheat-based basal rations were used to create a viscous gut environment, to evaluate the small intestine as one of the possible control points of folate supply for egg deposition. Rye has not been commonly used in commercial laying hen diets due to the presence of antinutritional factors such as pentosans, which reduce its nutritive value (Fengler and Marquardt, 1988). The antinutritional components in cereals have been identified as nonstarch polysaccharides (NSPs), which are components of the endosperm cell wall. The β-glucan in barley and oats, and pentosans (arabinoxylans) in rye and wheat are the NSPs responsible for the antinutritional effects of these cereals (Guenter, 1993). The water soluble fraction is responsible for increased viscosity of gut contents (Guenter, 1993). Altered viscosity slows rate of passage of ingested materials, impairs interactions between nutrients and digestive components, and restricts nutrient diffusion and transport by gelling chyme, and interfering with the unstirred water layer (Guenter, 1993). The water-soluble pentosans fed to young chicks are not only indigestible, but they also interfere with the digestion of other nutrients, particularly fat (Ward and Marquardt, 1988) and energy (Friesen et al., 1992). The viscous properties of rye pentosans in the digestive tract may impair the rate at which nutrients interact with the

digestive enzymes through reduced rates of diffusion (Ward and Marquardt, 1983). Results from trials performed by Edwards et al., (1988) suggested that increased gut viscosity impeded conditions for the emulsification of lipids and micelle formation, processes which are dependent on the presence and action of adequate amounts of the substrates, lipase, co-lipase, and bile salts. Mathlouthi et al.,(2002) found that feeding a rye-based diet reduced fat digestibility, thus also reducing absorption of fat soluble vitamins.

It was hypothesized that the uptake of folate from the gut would be impaired due to the increased viscosity of the rye/wheat-based rations; and this hypothesis was then tested by determining the difference in yolk folate content in eggs from hens consuming rations with and without enzyme. Sherwood et al., (1993) found that plasma and yolk folates are saturable and proportionate across a range of dietary folic acid levels. Therefore if nutrient and vitamin absorption was impaired due to an increased gut viscosity, plasma and consequently yolk folate content would be decreased (Sherwood et al., 1993)

4.6.2 The Effect of Folic Acid-Supplemented Rye/Wheat-Based Rations on Egg Folate Deposition

Considering the folate content in the basal diet (1.01 mg/kg), the actual folate concentrations: 1.01, 1.51, 2.01, 3.01, and 5.01 mg/kg diet for the respective levels of crystalline folic acid supplementation: 0, 0.5, 1.0, 2.0, and 4.0 mg/kg were used to calculate efficiency of folate deposition.

Efficiency of folate deposition decreased with increasing folic acid supplementation. For example, when no folic acid was supplemented, 1.20 μg/g was

deposited in egg yolk, however when 2 mg/kg crystalline folic acid was supplemented 2.41 μg/g folate was deposited in the egg yolk, which is a 201% increase in egg folate content. In comparison when 4 mg/kg crystalline folic acid was supplemented in the diet, 2.79 μg folate/g yolk was deposited in the egg which is only a 116% (2.79/2.41) increase in egg folate. Therefore, after doubling the folic acid supplementation rate, the increase in egg folate content was half what it had been at the 2.0 mg/kg folic acid supplementation level. The efficiencies of folate deposition for the 0, 0.5, 1.0, 2.0, and 4.0 mg/kg folic acid levels were 16.7, 16.29, 15.73, 11.24, and 7.79% respectively.

4.6.3 The Effect of the Addition of Dietary Enzymes to Rye/Wheat-Based Diets on Egg Folate Content

Yolk and egg folate content had significant period by ration by enzyme interactions. Yolk folate content was not significantly affected by enzyme except at the 4 mg folic acid/kg diet in period one, where enzyme significantly increased yolk folate content. Yolk folate content significantly ($P<0.0001$) increased as the dietary folic acid supplementation level increased. Yolk folate content significantly increased in half of the rations, in period three. Though enzyme did not significantly affect hen performance, or egg folate content, enzyme did significantly ($P<0.0001$) decrease the viscosity of *in vivo* laying hen jejunal digesta. Enzyme did not affect production, for reasons similar to that of why it did not affect egg folate content. Perhaps viscosity of the diets without enzyme was not high enough to adversely affect nutrient absorption, leading to adverse production effects. Rye was only included as 40% of the grain in the diet, and production effects do not usually occur until the percentage of rye in the diet is higher (depending on the variety of rye). In a preliminary trial (data not shown), rye/wheat diets were fed to

laying hens with rye at 50% and 60% of grain in the diet. *In vivo* digesta viscosity readings were often too high to measure. Another reason why the enzyme had no effect on production is due to the short duration of the trial. It is quite possible that after consuming a highly viscous diet over several months, enzyme would affect nutrient absorption, and an effect on production would be observed.

Due to the fact that viscosity interferes with the digestion and absorption of nutrients in the ration (Friesen et al., 1991), it was hypothesized that gut viscosity would therefore impair the uptake of folic acid by the enterocyte's membrane-bound transport system. The impaired absorption of folic acid would result in a lower yolk folate content. To further develop the concept that the small intestine is a control point for folate uptake, the addition of enzyme would result in improved feed utilization, more effective uptake of folic acid by the membrane-bound transport system, and a higher yolk folate content. This however, was not the case, enzyme supplementation did not significantly ($P>0.05$) affect yolk folate content, although *in vivo* viscosity was significantly reduced.

Both Sherwood et al., (1993) and House et al., (2002) supported the fact that plasma and egg folate content saturate simultaneously. House et al., (2002) found that the response of egg folate to dietary folic acid supplementation was saturable, with 90% of maximal egg folate levels established at approximately 4 mg folic acid/kg diet. Both authors agree that the saturation of egg folate content is not due to limitations in the transport processes from plasma to the egg yolk. Sherwood et al., (1993) concluded that regulatory processes controlling plasma folate levels might be imposed by the systems that supply or remove plasma folates: assimilation from the intestine, exchange with tissues, and excretion by the kidney. Through the use of the brush-border membrane

vesicles derived from rat jejunum, folate transport has been shown to be a saturable process (Said et al., 2000). The intestine may therefore, play a role in the regulation of egg yolk folate concentrations. It may be speculated that viscosity of jejunal digesta, of the diet with enzyme added, was not low enough to facilitate an increase in intestinal absorption, and consequently egg folate deposition.

The average viscosity of the jejunal digesta from the rye/wheat diets without enzyme was 15.2 centipoise, and the addition of enzyme brought the average viscosity of jejunal digesta down to 6.9 centipoise. Choct et al., (1996) reported that viscosities of jejunal digesta from birds fed an NSP-rich wheat milling by-product were decreased from 78.4 to 4.4 centipoise. Choct et al., (1996) was studying the effect of increased fermentation caused by the addition of viscous NSPs, on performance and well-being of poultry. Pan et al., (1998) were able to decrease digesta viscosity to approximately 1-3 centipoise after adding enzyme to laying hen wheat/rye diets. They also reported that the lowered digesta viscosity improved hen performance. Both Choct et al. (1996) and Pan et al., (1998) reported lower digesta viscosity readings than 6.9 centipoise, to facilitate better hen production performance. Perhaps additional dietary enzyme would have lowered viscosity to a further extent, facilitating better nutrient utilization, and an increase in egg folate deposition.

Enzymes such as pentosanase, have been developed to reduce the negative effects of NSPs and improve the feeding value of cereal-based diets (Guenter, 1993). It is believed that the enzyme breaks the pentosans into smaller subunits (Annison and Choct, 1991), and therefore alters the ability of these polysaccharides to form highly viscous solutions that inhibit nutrient diffusion and transport. The application of pentosanases to

rye and wheat based diets, has improved bird performance and increased nutrient digestibility in numerous studies (Friesen et al., 1991; Friesen et al., 1992; Lázaro et al., 2003). In a short term trial when a barley-based ration was supplemented with β-glucanase, Hebert (2005) also found that the enzyme did not significantly ($P>0.05$) increase egg folate content.

It is possible that there is another folate control point along the folate metabolism pathway. Following uptake of folic acid by the enterocyte's membrane-bound transport system, folic acid is then reduced to THF via dihydrofolate reductase within the enterocyte (Henderson, 1990). THF is then transported to the liver. Folate binding proteins are synthesized in the liver. Within the liver, THF is converted to 5-MTHF and attached to folate binding proteins and secreted into the bloodstream (Henderson, 1990). Due to the fact that enzyme did not improve yolk folate content, it may be suggested that the absorption of folate via the enterocytes may not be a control point for folate uptake. Absorption of folate via the enterocytes would have been less efficient in the diets without enzyme; however, with or without enzyme supplementation, the same amount of folate was deposited in the egg. As previously stated, there are additional potential reasons for enzyme not improving yolk folate content, supporting the possibility that enterocytes may control folate uptake. The *in vivo* jejunal digesta had a high viscosity even after enzyme supplementation, which could be a reason for poor enterocyte folate absorption.

There are a number of possible steps within folate metabolism that could be responsible for limiting folate uptake. Folate uptake could be limited by dihydrofolate reductase. There could be a limited amount of dihydrofolate reductase, which would

limit the amount of folic acid reduced to THF. Folate uptake could be limited by the transportation system of THF to the liver. Perhaps only a certain amount of THF is transported to the liver. It is possible that a limited amount of THF is converted to 5-MTHF, however since 5-MTHF can be formed by a variety of pathways, that suggestion is not likely. Folate uptake could be limited by the number of folate binding proteins that are available. For example if there are a limited number of folate binding proteins, only a limited amount of 5-MTHF could be secreted into the bloodstream. Riboflavin binding proteins determine the upper limit of riboflavin that can be delivered to the embryo, and dietary riboflavin in excess of the amount required to saturate the system is not available to the embryo (White and Merrill, 1988). Riboflavin binding proteins and other vitamin binding proteins are the products of specific genes (White and Merrill, 1988). Therefore, the genes would determine the production of folate binding proteins, which would in turn limit folate deposition in the avian egg. These maximum levels of vitamins that may be deposited in the egg yolk correspond to a fitness optimum because both the overproduction and underproduction of particular binding proteins could reduce the fitness of an animal.

4.6.4 The Effect of Diet and Enzyme on Laying Hen Performance

In this study, performance parameters were not significantly affected by a viscous dietary cereal base, or enzyme supplementation. Period did have a significant effect on all of the performance parameters, which would be expected, based on a normal production cycle. Compared to research on broilers, only limited research has been reported on the use of dietary enzymes for laying hens. In many investigations, no effect on the performance of laying hens was reported when β-glucanase or an enzyme complex

(β-glucanase, pentosanase, and cellulase) were added to barley-based diets (Al-Bustany and Elwinger 1988; Benabdeljelil, 1992; Brufau et al., 1994). In some cases, weight gain of the hens and feed intake were positively affected by enzyme supplementation, without influencing egg production and quality (Brenes et al., 1993). Mathlouthi et al., (2003) found that xylanase and β-glucanase supplementation of laying hens given wheat/barley-based diets had no significant ($P>0.05$) effect on egg production (% hen-day), egg weight, egg mass (gram/day), or feed intake (gram/day). Xylanase and β-glucanase, did significantly ($P<0.05$) improve feed conversion efficiency (g/g) and increased body weight of the laying hens. Brenes et al., (1993) supported these results, concluding that the addition of a commercial enzyme preparation containing cellulase, xylanase, and β-glucanase had beneficial effects on weight gain and feed intake but not on egg production in laying hens given wheat-barley, oat- or rye-based diets from 22 to 34 weeks of age.

The lack of response of the hens to the rye/wheat-based rations in the current trial could be attributed to the fact that the level of rye in the diet was only 40% of grain. Perhaps the inclusion of a higher percentage of rye, or a different cultivar of rye, in the diet could have elicited a greater response to the enzyme, due to the fact that the gut conditions would have been more viscous, causing nutrient absorption problems, and a decline in hen performance. Additionally, the short duration of the trial could have attributed to the lack of response of the hens to the two rye/wheat rations (with and without enzyme). The metabolic systems and body stores of the laying hens may not have been challenged during the trial, and therefore performance was not affected by the diet.

Few studies have been reported on enzyme supplementation of layer diets and their findings are not as conclusive as those with broiler chicks (Pan et al., 1998). Pan et al., (1998) investigated the effects of enzyme and inorganic phosphorus supplements in wheat- and rye-based diets on laying hen performance, energy, and phosphorus availability from week 22 to week 42. As was found in the current trial, Pan et al., (1998) reported that with the addition of enzyme there was no significant ($P \geq 0.05$) difference in egg production, feed intake, feed efficiency, or body weight gain. However, Pan and coworkers (1998) did not report gut viscosity data.

Scott et al., (2001) conducted an experiment to determine the feeding value of hull-less barley with and without enzyme fed to male broilers or Leghorn chicks from 4 to 21 days of age. Viscosity of the digesta without enzyme supplementation from the Leghorns was twice as high as that of the broilers, although an equal enzyme response in digesta viscosity was observed (Scott et al., 2001). Viscosity of jejunal digesta of the hens in the current trial was lowered to ~ 6.0 centipoise, which is the same as the digesta viscosity of the broiler and Leghorn chicks (Scott et al., 2001). The lowered enzyme response measured for growth, feed intake, and feed conversion ratio for Leghorns may be a factor of their genetics (Nir et al., 1993; Nitsan 1995; Mahagna and Nir 1996), or a function of lower feed intake. At lower feed intake, even though digesta viscosity was higher, the Leghorn chicks were still able to derive sufficient nutrients to maintain a relatively normal rate of growth. This may relate to decreased rate of passage of viscous digesta and/or development of different gut microflora populations (Choct et al., 1999). Additionally, laying hens have more stable gut microflora than broiler birds, due to the fact that laying hens are older than broilers. In the broiler, for the first 30 days post

hatch, it is likely that composition of the microflora undergoes major changes (Mathlouthi, et al., 2002). The digestive flora differs between fast-growing animals reared according to standard management practices for broilers and animals reared in more extensive conditions such as slow-growing strains (Gabriel et al., 2006); which would explain the difference in mircroflora between laying hens and broiler birds. Differences in gut microflora between laying hens and broilers combined with an NSP-rich diet may cause the difference in viscosity between the two types of birds (Boyd and Edwards, 1967; Maisonnier et al., 2003).

Jaroni et al., (1999) examined the effect of the addition of xylanase plus protease on the performance of two strains of Leghorn laying hens (DeKalb Delta and Hissex White) fed diets with wheat middlings. The addition of xylanase to the hens' diets did not result in any improvement in the intestinal viscosity. Gut viscosity of the hens at 50 and 60 weeks of age with or without enzyme ranged from 3.81-6.97 centipoises. This could be attributed to variations in sampling procedures and grain cultivars used.

There are situations where diets may or may not respond positively to enzyme supplementation. The ingredients may have low levels of soluble NSP, which are known to be dependent on genetics and growing or harvesting conditions of the grain (Jeroch and Dänicke, 1995). The diets may not respond to enzyme depending on the target animal and the age of the target animal (Rotter et al., 1990; Classen et al., 1991; Petersen et al., 1999). It has been reported that the efficacy of enzyme supplementation within poultry diets is variable (Brenes et al., 1993). More recently, it has been reported (Ponte et al., 2004) that xylanase inhibitors (in wheat for example) may inhibit the activity of exogenous enzymes such as xylanase. Consequently the exogenous enzyme will not act

effectively to hydrolyze NSPs, and viscosity of intestinal contents will not be decreased. The presence of enzyme inhibitors in certain varieties of wheat and/or rye (Ponte et al., 2004) may account for variable efficacy of exogenous enzymes in poultry diets.

In summary, yolk folate content significantly increased as the dietary folic acid supplementation level increased. The enzyme cocktail used significantly decreased *in vivo* viscosity of jejunal digesta of laying hens, but did not significantly affect egg folate content, feed efficiency, or other production parameters. This short term trial suggests that intestinal absorption of nutrients was not affected enough to cause any drop in hen performance, or decline in egg folate deposition. Perhaps diets used should have been more viscous, to increase digesta viscosity, facilitating more adverse conditions for nutrient absorption and possibly folate deposition, or more enzyme should have been used to lower viscosity enough to cause a difference in egg folate deposition without and with enzyme.

5.0 General Discussion

The long term trial discussed in Manuscript One of this thesis was performed to determine if a lower supplemental folic acid level, 2 mg/kg diet similarly to a 4 mg/kg diet, could maximize egg folate content and be consistent in deposition, over an eleven month laying hen production cycle. In order to ensure a consistent folate enriched egg, production and age effects were evaluated. Hens consuming the 4 mg folic acid/kg of diet deposited yolk folate at a significantly higher amount throughout the entire eleven periods, except periods three and nine, than the hens consuming the 2 mg folic acid/kg of diet. In period three, during peak hen-day production, the yolk folate content and efficiency of yolk folate deposition also peaked. The simultaneous yolk folate content and hen-day production peaks may be explained by the fact that during this time period (period three), the hens are also the most efficient, therefore, the vitamin transfer is the greatest. Hebert's (2005) yolk folate data for a 9 month production trial also showed that yolk folate content peaked with hen-day production, then declined slightly and remained constant for the remaining periods. Additionally, egg production of hens consuming the ration with 4 mg/kg folic acid, was significantly better than egg production of hens consuming the ration with the 2 mg/kg folic acid in periods four and eleven.

Generally although egg production decreased with age of the hen, egg folate content remained constant. Yolk folate content of eggs from hens consuming the 4 mg folic acid/kg diet remained at 3.40 ± 0.46 µg folic acid/g yolk; CV~13.50% throughout the entire production trial. Therefore, when standards are developed for the production of folate-enriched eggs, farmers can be confident that eggs laid at the beginning or end of the laying hen production cycle will remain consistent in amount of folate. Consistency

of folate deposition is crucial for successful marketing of such eggs. For the folate enriched eggs to be marketed as "folate enriched", a uniform level of folate must be present in each egg available for sale.

Incidences of folate deficiency in humans (Dhur, et al., 1991), and an increased recommended dietary allowance (RDA) of folic acid, to reduce the risk of delivering a baby with a neural tube defect (Czeizel and Dudas, 1992) necessitated methods of increasing intake of folic acid. The development of the folate-fortified egg has introduced an effective method of folate delivery, a vitamin with several human health benefits. The folate in eggs is ≥100% bioavailable compared to crystalline folic acid based on a rat bioassay (House et al., 2003). Furthermore, the egg is a vehicle of folate delivery that is superior to grain products due to the fact that 5-MTHF, the form of folate found in eggs, does not mask the symptoms of cobalamin (vitamin B_{12}) deficiency, associated with crystalline folic acid used to enrich grain products (Institute of Medicine, 1998). House et al., (2002) reported that one large folate enriched egg contains 12.5% of RDA for adult humans (RDA=400μg). Folate plays a role in maintaining homocysteine at a level low enough to lower incidence of neural tube defects in human babies (Czeizel and Dudas, 1992), cardiovascular disease (Boushey et al., 1995), and inflammatory diseases in adults (Seshadri et al., 2002) .

The level of crystalline folic acid supplementation is crucial for the successful production of folate-enriched eggs. Using the lowest possible supplementation level that will consistently deposit the maximum amount of folate in the egg will ensure successful, least cost egg production with optimal human nutritional benefits. The search for the optimal level of folic acid that could be supplemented in laying hen diets began following

the finding that plasma and yolk folates proportionately increase as the dietary supplemental folic acid increases (House, et al., 2002; Sherwood et al., 1993). House et al., (2002) found that a linear increase in egg folate levels was observed when crystalline folic acid was added from 0 to 2 mg/kg to the laying hen diet, after which egg folate levels reached a plateau at 4 mg/kg diet. Additions of folic acid above 2 mg/kg diet yielded no further significant increases in egg folate content until dietary concentrations reached 32 mg/kg suggesting a biphasic response (House et al, 2002). However, in a short term trial, Hebert (2005) fed two strains of laying hens rations supplemented with dietary folic acid at levels ranging from 0 to 128 mg/kg. Plasma folate concentrations in birds consuming 128 mg of folic acid/kg diet did not translate into egg folate concentrations higher than those of hens consuming the 4 mg/kg folic acid ration. This disproved the suggestion of a biphasic response, and raised questions relating to factors regulating the absorption of dietary folic acid and its appearance in the systemic circulation in the form of 5-MTHF. A long term production trial (Hebert, 2005) comparing egg folate content from 0 and 4 mg/kg folic acid supplementation levels, in two different strains of laying hens, showed that egg folate deposition reached a maximum early in production, and remained relatively consistent throughout the lay period.

During a long term production trial involving two stains of birds (W36 and W98), Hebert (2005) considered the benefits of folic acid supplementation on bird health. Hens receiving additional folic acid supplementation had significantly lower plasma homocysteine levels (Hebert et al., 2005). High homocysteine levels have been linked to cardiovascular disease in humans (Boushey et al., 1995); suggesting folic acid depletion

may cause vascular abnormalities in hens. Post mortems were immediately performed on mortalities that occurred throughout the 11-period laying hen trial (Manuscript One) to verify cause of death. Cause of death of each hen was documented, and the results showed that cardiovascular abnormalities did not contribute to any mortalities. Though folic acid supplementation may lower homocysteine levels in laying hens and humans, it remains to be demonstrated that decreasing homocysteine levels reduces cardiovascular morbidity and mortality in humans (Eikelboom, 1999) and poultry.

The long term production trial supports the use of 4 mg folic acid/kg diet, as the optimal level of folic acid supplementation within laying hen diets. The mean egg folate content for the 4mg folic acid/kg diet over the production cycle was 51.33±8.37 μg; CV~16.30% folate/egg. However the mechanism responsible for controlling folate uptake has yet to be elucidated.

Sherwood et al., (1993) stated that saturation of egg folate content is not due to limitations in the transport processes from plasma to the egg yolk. They also concluded that regulatory processes controlling plasma folate levels might be the point of metabolic control. In studies with rats and *in vitro* intestinal cell model systems, folic acid has been shown to be absorbed from the gut via a membrane-bound folate transport system (Said, 2004). Since this process has been shown to be saturable in a number of model systems, Said (2004), also believed that the gut would represent a potential control point for plasma folate. To explore the relationship between egg folate deposition and intestinal folate absorption, a short term trial involving rye/wheat-based, rations with and without enzyme supplementation was performed. When a rye/wheat-based ration was fed to the laying hens, yolk folate content increased significantly with dietary folic acid

supplementation. In a short term trial, Hebert (2005) found that dietary ingredients appeared to affect total egg folate content. For example, a corn-based diet resulted in eggs with significantly higher total folate content than the wheat based diet.

In the rye/wheat viscosity trial referred to in Manuscript Two, the addition of enzyme significantly decreased laying hen *in vivo* viscosity; however the enzyme had no effect on yolk folate content. This finding is in agreement with Hebert (2005) who observed no significant difference in yolk folate content, when the barely-based ration was supplemented with β-glucanase. Numerous studies (Friesen, et al., 1992; Marquardt et al., 1994) have demonstrated that enzyme supplementation improves bird performance and increases nutrient digestibility. For example, Mathlouthi et al., (2003) found that xylanase and β-glucanase, significantly ($P<0.05$) improved the feed conversion ratio (g/g) and increased body weight of the laying hens. Due to the fact that enzyme supplementation did not improve intestinal folate uptake, it may be suggested that the intestine is not a control point for folate uptake, and deposition in the egg. However, it may have been more difficult to elicit a yolk folate response upon the addition of a water-soluble vitamin such as folic acid. Viscosity has a significant effect on fat and fat soluble vitamins but much less on water soluble vitamins. It is possible that the regulatory mechanism for egg folate saturation exists within the enterocyte during folate reduction, at the time of folate transport to the liver, or during attachment to binding proteins within hepatocytes, prior to transport via circulation to the yolk for deposition. Multiple control points exist to regulate plasma folate levels, and further research is required to elucidate the key control point(s) within the avian system.

After comparing Manuscripts 1 and 2, it is evident that the null hypothesis in Manuscript 2 stating that egg folate deposition will not be affected by increased gut viscosity, can be accepted. Furthermore, the limitations of the experiment in Manuscript 2 should be addressed. As previously stated it is possible that the viscosity of the *in vivo* jejunal digesta was still too high after enzyme had been added to facilitate an increase in enterocyte folate absorption and consequently an increase in egg folate deposition. Perhaps a future trial could be performed using more enzymes, to decrease *in vivo* jejunal viscosity to 2 or 3 centipoise, rather than 6.9 centipoise, to facilitate absorption of nutrients and folate within the small intestine. Due to the fact that after comparing egg folate levels from Manuscript 1 and 2 it was found that egg folate content was higher in eggs from hens consuming the less viscous barley-based diet than the more viscous rye/wheat diet, it is suggested that absorption may affect egg folate levels. Therefore, a future trial using a variety of feedstuffs including rye, wheat, barley, and hulless barley (feedstuffs listed in order of decreasing viscosity, although highly variable depending on variety) may be useful in providing more information on egg folate levels in viscous diets. For example as the viscosity of the feedstuff increases, perhaps a decrease in egg folate content will be reported. However, the two future trials that have been suggested are only addressing one possible folate control point along the pathway of folic acid metabolism and egg folate deposition.

There are many potential control points as crystalline dietary folic acid is metabolized, absorbed, and deposited in the egg yolk. In general, metabolic control points are saturable, therefore, only a limited amount of folate will exist at a given control point. Saturable steps often occur when the chemical structure under investigation is

being converted from one form to another, which is usually enzyme dependent (for example when dihydrofolate is converted to tetrahydrofolate by dihydrofolate reductase) or when the chemical structure is moving from one site to another. For example, when folate is transported from the plasma to the egg yolk, both the plasma and the egg yolk are sites that are saturable with folate. Due to the fact that egg folate saturates at the same crystalline supplementation level as plasma folate, it is evident that one possible control point for folic acid occurs before folate is in the plasma. Perhaps a limited amount of folate is able to be absorbed within the intestinal cell, which limits the plasma and consequently egg folate. In Manuscript 2, the intestine was evaluated as a potential folate control point. Results from the trial performed showed that enzyme did not improve egg folate deposition. Perhaps viscosity was not sufficiently decreased to facilitate a significant increase in egg folate deposition, or else the rye/wheat diets may have not been viscous enough to adversely affect egg folate deposition. It is also possible that intestinal absorption is not a control point for egg folate deposition. Short term metabolic trials may be performed to test other potential folate control points. Referring to figure 5, the first potential folate control point is when the polyglutamic folic acid is converted to monoglutamic folic acid by folyl conjugase. If the enzyme folyl conjugase is limiting, polyglutamic folic acid cannot be converted to monoglutamic folic acid, and a limited amount of folic acid may be absorbed through the intestinal lumen. Another possible folate control point is the transportation of folic acid from the intestinal lumen to inside the enterocytes. This control point was investigated in Manuscript 2, when viscous conditions were created in the intestine, and folate absorption was indirectly evaluated by determining egg folate content. It was hypothesized that increased viscosity in the small

intestine would decrease folate absorption (decreased carriers), and furthermore decrease egg folate deposition. The next possible folate control point is the conversion of folic acid to dihydrofolate via dihydrofolate reductase, then the conversion of dihydrofolate to tetrahydrofolate. If the enzyme dihydrofolate reductase is limiting (Jeong and Gready, 1995), folic acid may not be able to be converted to dihydrofolate and tetrahydrofolate. The folate cycle within the liver is another potential folate control point. If the serine hydroxymethyl transferase (SHMT) is limiting, tetrahydrofolate cannot be converted to 5,10 methylenetetrahydrofolate (CH_2THF). If the methyltetrahydrofolate reductase enzyme is limiting, 5,10 methylenetetrahydrofolate cannot be converted into 5-methyltetrahydrofolate (CH_3THF). Another possible folate control point exists when folate binding proteins are synthesized. If a limited number of folate binding proteins are synthesized, a limited amount of folate can be transported into the egg yolk. Folate binding proteins are synthesized in response to estrogen produced by the laying hen. Folate binding proteins may be limited by the amount of estrogen produced by the hen, or by the genes which transcribe the binding proteins. The final potential folate control point exists when 5-methyltetrahydrofolate attached to the folate binding proteins is transported into the plasma from the liver. It is possible that only a limited number of folate binding proteins (with 5-MTHF) are transported from the liver into circulation.

When comparing yolk folate content between eggs from the trial described in Manuscript 1, and the eggs from the trial described in Manuscript 2, it is evident that yolk folate content is higher in eggs from Manuscript 1. Hens on the barley-based diet (Manuscript 1) laid eggs with a higher yolk folate content, than hens on the rye/wheat-based diet (Manuscript 2). For example, over an eleven-month production cycle, hens on

the barley-based ration consuming 0, 2, and 4 mg folic acid/ kg diet, deposited means of 1.42, 2.93, and 3.40 μg folate/g yolk respectively. Hens on the rye/wheat-based ration consuming 0, 2, and 4 mg folic acid/kg diet without enzyme, deposited means of 1.3, 2.5, and 2.6 μg folate/g yolk respectively over a five week period. It is evident that consumption of the barley basal diet resulted in a greater egg folate content (1.42 μg folate/g yolk) than consumption of the rye/wheat basal diet (1.2 μg folate/g yolk). Dietary analysis of folate content in basal diets revealed that there was slightly more folate in the rye/wheat-based diet than the barley-based diet (1.01 versus 0.91 mg/kg diet).

Egg folate deposition efficiencies were also higher for the barley-based diet than the rye/wheat based diet. Efficiencies of egg folate deposition for the basal diet, 2 and 4 mg folic acid/kg diet for the barley-based rations compared to the rye/wheat based rations were 22.3 versus 16.8 %, 14.13 versus 11.29%, and 9.74 versus 7.8 % respectively. There was 9.23, 17.20, and 30.77% more folate in eggs from the barley-based ration than the rye/wheat ration for the 0, 2, and 4 mg folic acid/kg diet respectively. After calculating egg folate deposition efficiencies, it is evident that mean egg folate deposition was the factor that differed the most when comparing efficiency of deposition between rye/wheat rations and barley based rations. More folate was deposited in the eggs from laying hens fed barley-based rations than from hens fed the rye/wheat based diet, which resulted in higher deposition efficiency in eggs from barley based ration hens. This finding suggests that there is a diet related response with respect to type of diet fed and amount of egg folate deposition. Due to the fact that the diets used were chosen specifically to evaluate how egg folate deposition is affected by increased gut viscosity;

it is suggested that the more viscous rye/wheat diet does affect egg folate absorption, possibly by increasing gut viscosity, and thus interfering with enterocyte folate absorption.

Perhaps the viscosity of the diets and the fact that the rye/wheat-based diet was more viscous than the barley-based diet interfered with intestinal folate absorption. B-glucans are the primary NSP in barley (Rotter et al., 1990; White et al., 1983) and arabinoxylans (pentosans) are the primary NSP in wheat (Andrewartha et al., 1979).

Since feed efficiency is determined by the formula: feed consumption/egg weight x henday production there are three ways that laying hens may improve their feed efficiency. The feed consumption may be decreased, egg weight may be increased, and henday production may also be increased. The mean overall feed consumption from Manuscript One was higher than the mean overall feed consumption of Manuscript Two (94.03 versus 90.88 grams/hen/day). It is possible that the feed consumption of laying hens was lower for Manuscript Two because the trial in Manuscript Two was run from August 12-September 23, during which some hot weather in August (Environment Canada, 2006) caused the laying hens to eat less. Egg weight and henday production were relatively similar in Manuscript One and Two (59.63 versus 59.22 and 87.63% versus 86.99% respectively).

NSPs cannot be hydrolysed by the enzymes that are naturally produced by the bird. NSPs have an antinutritional effect, which prevent the access of endogenous enzymes to the nutrients contained within the grain. The uptake of folate from the gut appears to be impaired due to the increased viscosity of barley-based rations (Brufau et al., 1994; Iji, 1999) and wheat-based rations (Andrewartha et al., 1979). Hebert (2005)

also found that dietary ingredients appeared to affect total egg folate content. Hebert (2005) found that a corn-based diet contained a higher endogenous folate content than the barley and wheat-based diets. The corn-based diet had significantly higher total egg folate content than the wheat-based diet. The author explained the differences in egg folate content between corn and wheat-based diets to be due to the fact that antinutritional factors in the wheat-based decreased folate absorption at the intestinal level, resulting in less egg folate deposition. It is possible that the folate levels in the feed could affect the egg folate levels, however it has yet to be determined to what extent different amounts of endogenous folate in hen feed (for example amount of folate in barley versus rye/wheat rations) effect the concentration of yolk folate deposition (Hebert, 2005).

Research has confirmed that exogenous enzymes increase the utilization and absorption of nutrients. Therefore the addition of enzyme to the rye/wheat-based diet should have improved the use of the diet, decreasing antinutritional effects of the grains, and resulting in better absorption of the diet. It is suggested that xylanase breaks arabinoxylans and pentosans in wheat and rye down into smaller subunits (Annison and Choct, 1991), which alters the ability of these polysaccharides to form highly viscous solutions that inhibit nutrient diffusion and transport. Since the enzyme had no effect on the egg folate content; it may be suggested that absorption of nutrients via the intestinal membrane does not control the amount of folate deposited in the egg. Perhaps another folate control point exists within the metabolism of folic acid that limits the amount of folate deposited in the egg. However, it is quite possible that the absorption of intestinal contents was not affected; therefore egg folate deposition was not affected. It is also possible that the laying hen diet was still too viscous even after the addition of enzyme,

and the jejunal digesta was too viscous to facilitate improved folate absorption within the enterocytes.

Dänicke and colleagues (1997) found that significantly higher vitamin A contents were detected in the livers of broiler birds receiving a rye and soya protein diet, after enzyme supplementation. Results from Dänicke et al., (1997) suggested that impeded conditions for the emulsification of lipids and micelle formation, processes were dependent on the presence and action of adequate amounts of the substrates, lipase, co-lipase and bile salts. The enzyme, xylanase, improved fatty acid digestibility by causing a decrease in the viscosity in the small intestine, resulting in chyme conditions more suitable for lipid digestion and absorption (Dänicke et al., 1997). Although the mechanism of metabolism may differ, the idea of increased egg folate content after the addition of enzyme to viscous laying hen rye/wheat diets is the same as that of the work done with vitamin A and Dänicke and collegues (1997). Like vitamin A, folate is also concentrated in the liver prior to folate binding proteins transporting folate in the blood to be deposited in the egg yolk. However unlike vitamin A, folic acid is a water soluble vitamin, and since water soluble vitamins are metabolized differently than fat soluble vitamins, it is possible that the increased viscosity did not have the detrimental effects it does on fat soluble vitamins, and egg folate deposition was thus not affected.

Overall, the 4 mg folic acid/kg diet supplementation level appears to be the optimal level of folic acid supplementation within laying hen diets to maximize consistency and quantity of egg folate deposition over a hen's production cycle. When barley-based and rye/wheat based diets were used, 4 mg folic acid/kg diet resulted in 51.33 ± 8.37 µg folate/egg and 39.01 ± 9.37 µg folate/egg respectively and 2 mg folic

acid/kg diet resulted in 44.09± 8.65 μg folate/egg and 36.92±10.10 μg folate/egg. The fact that a uniform amount of folate can be predicted in an egg at any time throughout the production cycle from a folic acid supplemented hen offers a promising marketing future for folate-fortified eggs.

6.0 Summary and Conclusions

The overall objective of this thesis was to evaluate egg folate deposition throughout the production cycle of laying hens. The research aimed to determine the optimal level of folic acid supplementation within laying hen diets to maximize egg folate content over time by evaluating the effects of age and rate of production on egg folate content, and determine the effects of folic acid supplementation on consistency of egg folate deposition and mortality of laying hens. Additionally to better understand egg folate saturation, the relationship between egg folate deposition and intestinal folate absorption was explored by determining the effect of a rye/wheat-based diet with and without enzymes on egg folate content.

The following conclusions can be drawn:

1. Hens consuming the 4 mg folic acid/kg diet deposited yolk folate at a higher (3.4 ± 0.46 µg folate/g yolk), amount throughout the production cycle (except in periods three and nine) than hens consuming the 2 mg folic acid/kg diet (2.9 ± 0.49 µg folate/g yolk).
2. In rations containing 0, 2, and 4 mg folic acid/kg diet, egg folate content peaked at the same time that hen-day production peaked. However, after peak egg production, when production was declining, egg folate content was consistent for the remainder of the production cycle. Overall, hen age and rate of lay did not affect total egg folate content; however at peak hen-day production egg folate content peaked.
3. Overall, performance including laying hen mortality is not affected by folic acid supplementation.

4. Although the addition of dietary enzymes to a rye/wheat-based laying hen diet significantly decreased the *in vivo* jejunal viscosity it did not affect production.

5. The addition of dietary enzymes to a rye/wheat-based diet did not affect yolk folate content. However, yolk folate content was greater in eggs from hens fed the barley-based diet (4 mg folic acid/kg diet: 3.4±0.46 μg folate/g yolk) than eggs from hens fed rye/wheat based diets (4 mg folic acid/kg diet without enzyme: 2.63±0.59 μg folate/g yolk).

7.0 Future Work

In order to further progress the area of folate research in laying hens, it is recommended that future research focus on elucidating the mechanisms responsible for the regulation of egg folate saturation.

The short term viscosity trials (Hebert, 2005; trial discussed in Manuscript Two) have suggested that intestinal folate absorption may be responsible for limiting folate uptake, and consequently egg folate deposition. The level of folate in the egg is directly related to the plasma folate concentrations, and both appear to be governed by a saturable process. Therefore, the possible regulatory folate mechanisms within folate metabolism between activity within the enterocyte, and attachment of 5-MTHF to binding proteins in the hepatocytes should be explored.

Due to the fact that cereal grains including barley are main energy sources in poultry diets, and increased gut viscosity associated with rye and barley results in decreased digestion and absorption of nutrients, further work may be done to evaluate the effect of enzyme supplementation on egg folate content. A short term trial, involving the feeding of a folic acid supplemented rye/wheat diet with a higher percentage of rye in the diet, or more enzymes to lower gut viscosity, may verify the involvement of intestinal viscosity in folate uptake and subsequent yolk folate deposition. Long term production trials could be beneficial in evaluating whether or not enzyme supplementation of rye/wheat diets supplemented with folic acid, has an effect on egg folate content over time.

Metabolism research is warranted to further understand the mechanisms limiting yolk folate deposition. If the intestinal absorption step could be bypassed, the conversion

of folic acid to THF within the enterocyte could be examined to determine if the enzyme involved in the conversion step, dihydrofolate reductase, is limiting, which would limit the amount of THF that is formed. Alternatively, molecular biology work may be required to evaluate if genes responsible for synthesizing folate binding proteins are limiting, thus limiting the THF uptake within the yolk.

Further mechanistic work could explain yolk folate saturation, and may be beneficial in explaining why yolk folate content peaked at the same time as peak hen-day production.

8.0 References

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9.0 Appendix

Solution Preparation

Ascorbate Buffer (pH 7.8) (Buffer A)

1. Add 12.11 g trizma base to ~ 800 mL of deionized water and stir.
2. Add 20.0 g ascorbic acid to the trizma base solution.
3. Adjust pH to 7.8 and bring to a final volume of 1 L using deionized water.
4. Make final slight pH adjustments.

0.03 M Potassium Phosphate Buffer (pH 2.2) (Buffer B)

1. Add 4.08 g potassium phosphate monobasic (KH_2PO_4) to ~ 900 mL of deionized water and stir.
2. Adjust pH to 2.2 using phosphoric acid (85%).
3. Bring to a final volume of 1 L using deionized water.
4. Filter.

Causes of Laying Hen Mortalities

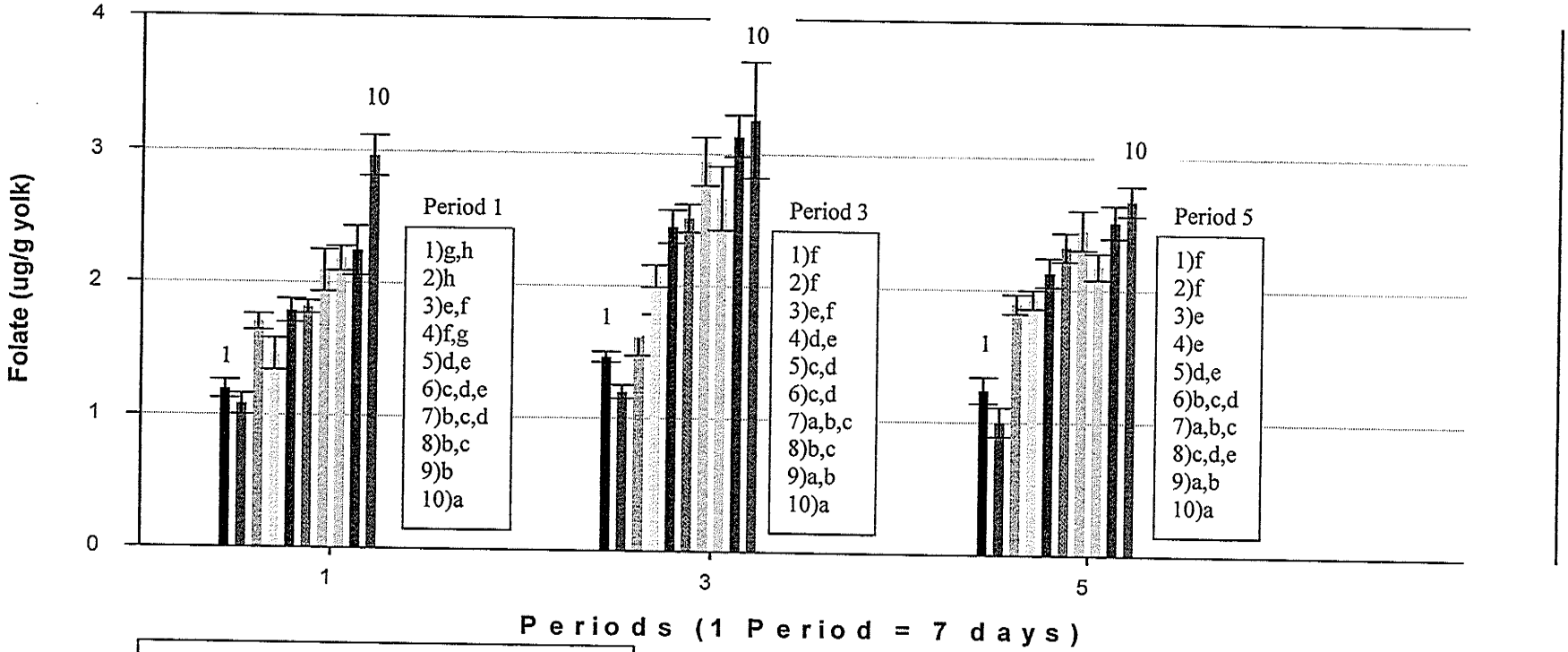
Dietary Folic Acid Supplementation (mg/kg diet)	% of Ration Mortalities	Cause of Mortality
0	53.80% 30.80% 7.70% 7.70%	urolithiasis egg related sick cage related
2	46.20% 19.20% 30.80% 3.80%	urolithiasis egg related sick cage related
4	42.90% 21.40% 21.40% 14.30%	urolithiasis sick cage related egg related

Upon finding any dead laying hens in their cages, hens were immediately weighed. Hen weight, row, and cage number, the date and time that the hen was found dead, and details related to death (ex: hen looked emaciated, dried up comb, etc) were recorded. Post mortems were promptly performed, and if needed a veterinary pathologist was called in to make a diagnosis. A series of diagnostic tests were performed on the first several laying hens that died from urolithiasis to confirm the diagnosis by a veterinary pathologist. The veterinary pathology noted the hens had urates (white, chalky deposits) within the heart, liver, and ureters. Chalky deposits were found on the epicardium, kidneys were swollen and pale and contained urates on sections, and the ureters were dilated with firm urates. The heart section had pale, feathery urate deposits on the epicardium. Kidney sections had dilated tublules and ureter branches and ureters were dilated with mineralized deposits. Bacteriology tests were performed on kidney, bone marrow, ovum and eggs, virology tests were done the on kidney, and the hens were tested for Infectious Bronchitis Virus (IBV). Histopathology tests were done on brain,

lung, liver, spleen, proventriculus, ventriculus, duodenum, pancreas, intestine, and caecal sections. Bacteriology, virology, and histopathology tests came out negative for causes of death other than urolithiasis. The final diagnosis was urolithiasis, also known as visceral gout.

In general for laying hens that died not due to urolithiasis, cause of death could be determined by using poultry production expertise and past experience by looking at the dead laying hen. For example, if a hen died of calcium shock, the hen was in the act of laying the egg and the egg was often protruding from the vent of the laying hen.

Figure 12. Yolk folate content (µg/g yolk) of Hy-Line CV20 laying hens throughout periods 1, 3, and 5-Variable with significant (P<0.05) period*ration*enzyme interactions[1] within period.



NOTE: Due to limited space, superscripts for bars on the graph are listed for respective periods. Bar numbers follow in order from one to ten. For example number one corresponds to the black bar on the graph, 0 mg/kg, and number ten corresponds to the dark green bar, 4 mg/kg + enzyme. The same numbers correspond to the same bars for each period.

[1]Data are reported as least squares means +/- standard error
a,b,c means within each period with differing letters are significantly different (P<0.05)
All means are composed of 9 replicates