CHARACTERIZATION OF FACTORS INFLUENCING THE REGULATION OF DIETARY FOLIC ACID DEPOSITION IN THE EGGS

A Thesis

Submitted to the Faculty

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by

Glenmer Bathan Tactacan

In Partial Fulfillment of the

Requirements for the Degree

Of

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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

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DOCTOR OF PHILOSOPHY

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FOREWORD

This thesis was prepared following a manuscript format. There are three manuscripts. Manuscripts I and II are published in Poultry Science. Manuscript III is under preparation. Manuscript I and II had been presented in Poultry Science Meeting in 2009 and 2010, respectively. The authors in Manuscript I are G. B. Tactacan, M. Jing, S. Thiessen, J. C. Rodriguez-Lecompte, D. L. O'Connor, W. Guenter, and J. D. House. The authors in Manuscript II are G. B. Tactacan, J. C. Rodriguez-Lecompte, K. O., and J. D. House. All manuscripts are formatted to meet the guidelines of Poultry Science.

ABSTRACT

The enrichment of eggs with folate is a viable option for supplying the general population of a food product rich in natural folates. However, attempts to increase the concentration of folate in egg beyond the achieved level of enrichment had been unsuccessful because egg folate reached a maximum plateau when folic acid (FA) was increased in the diet. Thus, experiments were conducted to determine the factors regulating the deposition of dietary FA into the eggs. In the first study, the effect of feeding equimolar intakes of FA or 5-methyltetrahydrofolate (5-methylTHF), the biologically active form of folate on egg folate concentrations, indices of folate status, and activities of folate-dependent enzymes were evaluated. Folic acid and 5-methylTHF demonstrated equivalent effects in enhancing the egg folate concentrations and improving the indices of folate status in the laying hen. The activities of folate-dependent enzymes were similar between the two forms of folate except for hepatic dihydrofolate reductase (DHFR) activity which increased in FA-fed birds compared to 5-methylTHF-fed birds. However, this demonstrated the inherent ability of the laying hen to metabolically convert FA into its biologically active forms. Therefore, the influence of intestinal FA absorption in the regulation of dietary FA deposition in the egg was subsequently evaluated using the *in vitro* intestinal everted sac technique. Folic acid was absorbed in all regions of the intestine. Absorption was maximum at acidic pH 6.0, and was increased in the duodenum and jejunum compared to the ileum and cecum. The rate of FA absorption in the jejunum diminished at higher FA concentrations, resembling a pattern similar to egg folate concentration when dietary FA supplementation was increased. Therefore, further study was conducted to determine the regulation of FA absorption when levels of FA in the

laying hen diet were increased. Supplementation of increased dietary FA levels resulted in a down-regulation of FA absorption in the duodenum, but not in the jejunum of the laying hen. This down-regulation was not associated with a decreased mRNA gene expression of the duodenal folate transporters (reduced folate carrier, RFC and proton-coupled folate transporter, PFCT). Overall, decreased intestinal rate of FA absorption possibly due to a post-transcriptional or translational regulation of specific folate transporters in the intestine of the laying hen, may contribute to the saturation in the egg folate concentration. These novel findings may prove essential for the development of new strategies geared towards increasing the level of folate in eggs.

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10-formylTHF	10-formyltetrahydrofolate
5, 10-methenylTHF	5, 10-methenyltetrahydrofolate
5, 10-methyleneTHF	5, 10-methylenetetrahydrofolate
5, 10-MTHFR	5, 10-methylenetetrahydrofolate reductase
5-methylTHF	5-methyltetrahydrofolate
AICAR	5-amino-4-imidazolecarboxamide ribonucleotide
BH_4	Tetrahydrobiopterin
cDNA	complementary deoxyribonucleic acid
CFD	cerebral folate deficiency
cRNA	complementary ribonucleic acid
CSF	cerebrospinal fluid
CV	coefficient of variation
DFE	Dietary Folate Equivalent
DHFR	dihydrofolate reductase
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dTMP	deoxythymidine monophosphate
dUMP	deoxyuridine monophosphate
EDTA	ethylenediaminetetra-acetic acid
eNOS	endothelial nitric acid synthase
FA	folic acid

FCCP	carbonyl cyanide-4-triflouromethoxy phenylhydrazone
FDA	Food and Drug Administration
FPGS	folylpolyglutamyl synthetase
FR	folate receptor
GAR	glycinamide ribonucleotide
GFP-hPCFT	green fluorescent protein-human proton-coupled folate transporter
GPI	glycosylphospho-inositol
HCP-1	heme carrier protein-1
Hcy	homocysteine
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HFM	hereditary folate malabsorption
HPLC	High Performance Liquid Chromatography
hRFC	human reduced folate carrier
LDL	low density lipoprotein
MAT	methionine adenosyltransferase
MDCKII	Madin Darby canine kidney II
MES	2-(*N*-morpholino)ethanesulfonic acid
MS	methionine synthase
NK cell	natural killer cell
NO	nitric oxide
NRC	Nutrient Requirement Council
NSP	non-starch polysaccharide

NTDs	neural tube defects
PABA	para-aminobenzoic acid
PCFT	proton-coupled folate transporter
PEG	polyethylene glycol
PLP	pyridoxal phosphate
RDA	Recommended Dietary Allowance
RFC	reduced folate carrier
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SEM	standard error of the mean
SHMT	serine hydroxymethyltransferase
SPE	Solid Phase Extraction
TCEP	tris (2-carboxyethyl)-phosphine hydrochloride
THF	tetrahydrofolate
TS	thymidylate synthesis
UPLC-MS/MS	Ultra-High Performance Liquid Chromatography-Tandem Mass Spectrometry

CHAPTER ONE

GENERAL INTRODUCTION

Folate is an essential water soluble B vitamin that is involved in a wide spectrum of biochemical reactions, including serving as cofactors and cosubstrates for different biological methylations such as those involved in amino acid and nucleic acid synthesis (Selhub and Rosenberg, 1996). Adequate folate status in humans is reported to lower the risk of neural tube defects (NTD) in babies (House et al., 2006; De Wals et al., 2007), cardiovascular disease (Yang et al., 2006), certain cancers (Kim, 1999), and inflammatory diseases (Wang et al., 2001). However, humans are devoid of folate biosynthesis, therefore, they are completely dependent on dietary sources to meet their daily folate requirements.

Folic acid (FA) is a synthetic derivative of folate and is the major supplemental form used in food fortification. In the late 1990s, the governments of Canada and the USA along with several others introduced the mandatory fortification of FA in cereal grain products to reduce the number of NTD occurrences in newborn babies (Food and Drug Administration, 1996; Health Canada, 1997). However, while cases of NTDs were reduced significantly (approximately 46%) (House et al., 2006; De Wals et al., 2007), an emerging body of literature suggests that consuming high levels of FA may have several negative consequences. Recently, concern has been expressed that the presence of unmetabolized FA may have detrimental health effects (Mason et al., 2007; Kim, 2007; Smith et al., 2008; Lucock and Yates, 2009). Compelling evidences showed that high circulating FA levels are associated with increased risk of developing anaemia

(Dickinson, 1995; Food and Nutrition Board, 1998), increased risk of cognitive impairment (Reynolds, 2006), decreased activity of natural killer cells (NK cells) (Troen et al., 2006), and increased rate of cancer progression (Cole et al., 2007; Figueiredo et al., 2009). As a result, attention has been directed into other strategies that may help to increase the population's intake of natural folates.

The enrichment of egg with folate is a viable means of supplying the general population with natural folate. Since egg is widely consumed in the human diet, it can serve as an ideal food vehicle to supply the nutritional requirement for natural folates. Folates in egg exist predominantly in the form of 5-methyltetrahydrofolate (5-methylTHF), the most biologically active form of natural folate. Because 5-methylTHF is a natural folate, it is unlikely to pose any potential risk on human health like FA.

It has been previously demonstrated that the folate concentration in chicken eggs can be increased significantly through the dietary supplementation of crystalline FA (Sherwood et al., 1993; House et al., 2002; Hebert et al., 2005; Roth-Maier and Böhmer, 2007; Bunchasak and Kachana, 2009; Hoey et al., 2009, Dickson et al., 2010). Our lab in particular has carried out several studies wherein we demonstrated that eggs could be fortified by approximately 2 to 2.5 times with folate through supplementation of FA at levels equal to or greater than 4 mg/kg diet (House et al., 2002; Hebert et al., 2005; Dickson et al., 2010). However, attempts to further increase the level of folate in eggs by increasing FA supplementation (above 4 mg/kg of the diet) have proven unsuccessful. Along with blood folate, egg folate concentrations demonstrated saturation at increased levels of FA supplementation. Because circulating folate in the blood serves as the precursor pool for egg folate deposition, the observed saturation in blood folate

concentration may regulate the saturation in egg folate concentration. Given the interest in folate-enriched eggs, the examination of factors regulating the transfer of dietary FA into the egg will provide insights on ways to optimize, or possibly further increase the level of folate deposition in the egg. The research studies presented in this thesis address the factors regulating the transfer of FA into the egg.

CHAPTER TWO

LITERATURE REVIEW

2.1 Folate

2.1.1 Biochemistry and forms

Folate is used as a generic term for a family of chemically and functionally related compounds that exhibit a common vitamin activity based on the parent structure of folic acid (FA) (Selhub and Rosenberg, 1996) (**Figure 2.1**). Folic acid (molecular weight 441.4), also known as pteroylglutamate, is the most oxidised and stable form of folate. It consists of an aromatic pteridine ring linked through a methylene bridge to *para*-aminobenzoic acid (PABA), and then to one L-glutamic acid residue (monoglutamate) (Wagner, 1984). Folic acid is the synthetic form of the vitamin and can be generated from natural folates by chemical oxidation (Wagner, 1984). Due to its chemical stability and greater commercial availability compared to other derivatives of folate, FA is the most commonly used form in supplements and in food fortification (Bagley and Shane, 2005).

Naturally occurring folates such as those found in body tissues and foods, exist in different forms than FA. They contain a fully-reduced pteridine ring together with additional glutamic acid molecules (polyglutamate) linked by γ-peptide bonds (Wagner, 1984). They are also usually substituted by different one-carbon units at the N-5 or N-10 positions, or have a single-carbon bridge spanning these positions (Scott and Weir, 1994). These one-carbon units can be at the oxidation level of methanol (5-methyltetrahydrofolate) (5-methylTHF), or formaldehyde (5, 10-methylenetetrahydrofolate) (5, 10-methyleneTHF), or formate (5- or 10-

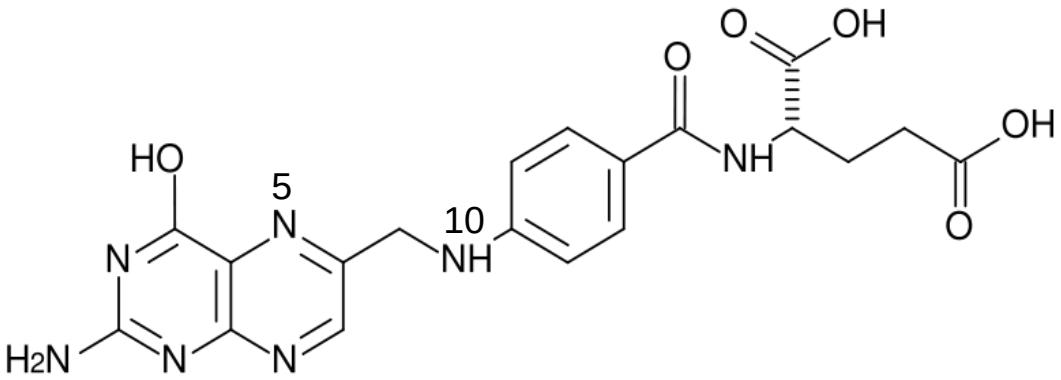

Figure 2.1. Structure of folic acid (FA).

formyltetrahydrofolate or 5, 10-methenyltetrahydrofolate) (5- or 10-formylTHF or 5, 10-methenylTHF) (Scott and Weir, 1994) (**Table 2.1**). Consequently, natural forms of folate exist as a mixture of polyglutamyl tetrahydrofolates (THF), containing various one-carbon forms of THF, depending on which carbon group is attached to them.

The predominant natural form of folate in fruits and vegetables is 5-methylTHF (Vahteristo et al., 1997). In animal products, 5-methylTHF and THF predominate (Vahteristo et al., 1997), while cereal products contain 5-methylTHF, 5-formylTHF, and 10-formlyTHF (Pfeiffer et al., 1997). Liver, mushrooms and green leafy vegetables such as asparagus, brussel sprouts and spinach are excellent sources of natural folates (Perloff and Butrum, 1977).

2.1.2 Absorption and transport

As previously mentioned, naturally occurring folates exist mainly in their polyglutamated form linked by γ-peptide bonds to multiple glutamate side chains. Polyglutamate molecules cannot easily traverse the intestinal cell membranes, and therefore, prior to absorption, are cleaved by γ-glutamylcarboxy conjugase to their monoglutamyl forms at the brush border membrane of the duodenum and jejunum. There are two types of conjugase enzyme. The conjugase of the duodenal and jejunal brush border membrane is a lysosomal exopeptidase, which cleaves the terminal γ-glutamate residue (Chandler et al., 1986). A second distinct enzyme from jejunal mucosa is located intracellularly and is reported to have endopeptidase activity (Wang et al., 1986).

After deconjugation, the resulting monoglutamates are then easily absorbed in the proximal small intestine (Devlin et al., 2000). Chandler et al. (1986) noted that the process of removing the polyglutamate chain from natural folates by intestinal

Table 2.1. Metabolically active forms of folate[a].

Unsubstituted folates	
7,8-Dihydrofolate (reduced at positions 7 and 8)	
5,6,7,8-Tetrahydrofolate	
Substituted folates	**One-carbon group**
5-Methyltetrahydrofolate	$-CH_3$
5-Formyltetrahydrofolate	-CHO
5-Formiminotetrahydrofolic acid	-CHNH
10-Formyltetrahydrofolate	-CHO
5,10-Methylenetetrahydrofolate	$-CH_2-$
5,10-Methenyltetrahydrofolate	-CH-

[a]Active forms are polyglutamate derivatives.

conjugase is not totally complete; therefore, a resultant reduction in the bioavailability of natural folates as compared to FA exists. Since FA occurs only in the monoglutamate form, its bioavailability after ingestion is not limited by the intestinal deconjugation of the polyglutamyl chain. The Dietary Folate Equivalent (DFE) which defines the recommended dietary allowance (RDA) for folate ascribes a constant bioavailability percentage of 50% for all naturally occurring food folates relative to the bioavailability of crystalline FA when taken on an empty stomach (Food and Nutrition Board, 1998). Dietary Folate Equivalent was developed to help account for the differences in absorption of naturally occurring dietary folate and the more bioavailable synthetic FA.

The proximal jejunum has the highest absorptive capacity for folates (Devlin et al., 2000). The absorption of the monoglutamated folate is facilitated by a pH-dependent folate transporter which transports both oxidized and reduced folates across the intestinal cell membrane (Qiu et al., 2006). Because folate transport is concentration driven (Qiu et al., 2006), the high folate levels in enterocytes facilitate the folate efflux across the basolateral membrane into the periserosal space, and from there, folate enters the vascular system (Shin et al., 1995). Absorbed folates are then delivered via the hepatic portal system to the liver where they are taken up by specialized transporters (Shin et al., 1995).

Folates that enter the liver have three potential destinations. (1) Folates can be converted to polyglutamate storage forms; (2) they can be secreted into the bile at the hepatic canalicular membrane, (Masuda et al., 1997), whereby they return to the duodenum and jejunum and are subsequently reabsorbed, thus completing the cycle of enterohepatic circulation. Steinberg et al. (1984) reported that the recycling of folates via the enterohepatic pathway may account for as much as 50% of the circulating blood

folate that ultimately reaches the peripheral tissues. (3) Folate monoglutamates formed by the hydrolysis of stored polyglutamates in the hepatocytes can enter the hepatic vein and ultimately reach the systemic circulation where they can accumulate and meet the one-carbon requirements of peripheral tissues (Steinberg et al., 1984).

Folic acid metabolism after intestinal absorption is different from the natural folates (Bagley and Shane, 2005). After entry into the enterocyte, FA has to be reduced and methylated into 5-methylTHF before it can enter the circulation (Selhub et al., 1973). Other folate monoglutamates aside from FA that are not in the 5-methylTHF form are also transformed to 5-methylTHF during their passage through the liver (Scott and Weir, 1994). Therefore, monoglutamated 5-methylTHF constitutes the only folate derivative appearing in the circulation after the ingestion of normal food. When high doses of FA or other folate forms are consumed, a part is absorbed by passive diffusion, and appears in the peripheral circulation unchanged (Bagley and Shane, 2005). Thirty to forty percent of 5-methylTHF in the blood is associated with low affinity binding proteins (Lucock et al., 1989). In most cases, circulating 5-methylTHF is associated with albumin, while less frequently it can attach to other proteins like α2 macroglobulin and transferrin (Lucock et al., 1989). Blood also contains a less abundant, high-affinity folate binding protein homologous to the cellular folate binding protein also known as the folate receptor (FR) (Selhub and Franklin, 1984; Antony et al., 1985).

2.1.3 Folate transporter

The hydrophilic and charged nature (anionic) of the folate molecule precludes their passive diffusion across the cell membranes (Matherly and Goldman, 2003). Instead, absorption in the intestine and transport of folate into the systemic tissues is

facilitated by highly specific folate transporters (Matherly and Goldman, 2003). Overall, there are three specialized transport systems that can accommodate the transport of folates across the biological membranes (Matherly and Goldman, 2003). They are the FR, the reduced folate carrier (RFC), and the proton-coupled folate transporter (PCFT).

2.1.3.1 Folate receptors (FRs)

The first route of folate uptake into cells involves the small family of FRs. Often referred to as the high affinity folate binding protein, FRs are cell surface glycosyl phosphatidylinositol (GPI)-anchored glycopolypeptide that characteristically bind FA as well as 5-methylTHF with affinity constant in the nanomolar range. This group of receptors is encoded by three distinct genes known as FRα, FRβ, and FRγ (Lacey et al., 1989; Ratnam et al., 1989; Shen et al., 1994). FRα and FRβ are both glycosylphosphatidylinositol (GPI)-anchored receptors, whereas, FRγ contains no GPI anchor due to lack of an efficient signal sequence for GPI modification (Shen et al., 1995). Each one displays a high affinity for FA and 5-methylTHF (K_m = 0.1-10 nM), but lower affinity (K_m = 10-300 nM) for other reduced folates (Wang et al., 1992; Westerhof et al., 1995).

Folate uptake by membrane-associated FRs involves receptor-mediated endocytosis (Kamen et al., 1988; Kamen and Smith, 2004). The process is initiated when a folate molecule binds to a FR that is associated in the surface of the cell. The binding of the FR results in the invagination of the plasma membrane and the formation of a vesicle (endosome). The vesicle then migrates along the microtubules of the cytoplasm to the perinuclear endosomal compartment (Kamen et al., 1988; Kamen and Smith, 2004). Following migration, the vesicles acidify (pH of approximately 6.0 - 6.5), resulting in the

dissociation of the folate-FR complex (Yang et al., 2007). Folate ligand is then exported into the cytoplasm by a process that requires a transendosomal pH gradient (Kamen et al., 1988; Rothberg et al., 1990). The folate transporter PCFT has been proposed as a mechanism by which folate is exported from endosomes (Qiu et al., 2006; Umapathy et al., 2007).

At present, the physiological roles of FRs are still unclear. Although FRα and FRβ can transport folate into cells, this transport process is considered to be inefficient compared with the transport of folate via RFC and PCFT (Sierra et al., 1995; Spinella et al., 1995; Qiu et al., 2006). Whereas RFC and PCFT exhibit a relatively wide pattern of tissue expression, the expression of FRα, the most abundant FR isoform, is restricted to few tissues including the epithelial cells of the kidney, retina, uterus, placenta, and choroid plexus (Kamen and Smith, 2004; Salazar and Ratnam, 2007). Recently, a novel clinical condition described as cerebral folate deficiency (CFD) was characterized as a result of specific pathological state of loss of FRα function (Ramaekers et al., 2002). Cerebral folate deficiency is any neurological syndrome associated with low cerebrospinal fluid (CSF) concentration of 5-methylTHF in the presence of normal peripheral folate metabolism (Ramaekers and Blau, 2004). This condition has been attributed to the presence of circulating serum auto-antibodies against the membrane-attached FRα of the choroid plexus epithelial cells, preventing the passage of 5-methylTHF into the spinal fluid compartment (Ramaekers et al., 2002). Recently, genetic defects of FRα gene have been found in patients with CFD (Cario et al., 2009). Cerebral folate deficiency has a wide clinical presentation, with reported signs and symptoms generally beginning at around 4 months of age with irritability and sleep disturbances.

These can be followed by psychomotor retardation, dyskinesia, cerebellar ataxia and spastic diplegia (Hyland et al., 2010).

2.1.3.2 Reduced folate carrier (RFC)

The second route of folate uptake into cells, and by far the best characterized folate transporter is the reduced folate carrier (RFC). First cloned in 1994 (Dixon et al., 1994), RFC is localized to chromosome 21 in humans (Moscow et al., 1995) and chromosome 10 in the mouse (Roy et al., 1998). The gene consists of five coding exons and preceeded upstream by multiple noncoding exons (Matherly and Goldman, 2003). In chickens, the RFC protein contains 496 amino acids and 12 putative transmembrane domains with an intracellular localization of the N-terminus (Jing et al., 2009).

The RFC operates as a bidirectional anion exchanger (Goldman, 1971; Henderson and Zevely, 1981). Functioning optimally at neutral pH (7.4), it has high affinity (Km = 1 μM) for reduced folates but low affinity (Km = 200-400 μM) for FA (Goldman, 1971; Sirotnak, 1985; Sirotnak, 1987). Compared to other folate transporters, RFC can neither bind nor hydrolyze ATP in order to drive folate substrate translocation across the cell membrane (Henderson and Zevely, 1983). It is also neither Na^+- nor H^+-dependent (Goldman, 1981). Rather, the RFC-dependent uphill influx of folates involves a physical translocation of anions down a large concentration gradient (Zhao et al., 2002). For instance, the RFC-mediated transport of folates is coupled with the downhill efflux of organic phosphates like thiamine monophosphate and thiamine pyrophosphate (Zhao et al., 2002). Organic phosphates are synthesized in high concentrations within cells for use in ATP-dependent reactions. They are largely retained in the cell because of their minimal passive diffusion across the cell membrane (Zhao et al., 2002). Their resulting

asymmetrical distribution across cell membranes provides the driving force for RFC-mediated transport of folates into cells (Zhao et al., 2002).

The RFC is widely expressed in tissues and is considered to be the major transport system for folates in the mammalian cells (Matherly and Goldman, 2003). When Whetstine et al. (2002) probed a commercial array of mRNAs prepared from 68 human tissues and 8 human tumor cell lines with ^{32}P labeled full-length human RFC cDNA, the highest RFC transcript levels were measured in the placenta and the liver. This was followed by leukocytes, kidney, lung, bone marrow, intestine, portions of the central nervous system, and the brain. Low but detectable RFC was also seen in the heart and skeletal muscle (Whetstine et al., 2002). In chickens, a broad tissue expression profile of RFC transcripts was also observed. The expression of RFC was detected in the brain, heart, liver, kidney, spleen, lung, pancreas, intestine, skeletal muscle, thymus, Bursa of Fabricius, ovary, and testis (Jing et al., 2009) **(Figure 2.2)**.

The distribution pattern of RFC can be localized to specific cell segments. For instance, RFC is expressed in the apical brush border membranes of the small intestine (e.g., duodenum, jejunum, ileum, and colon), and in the basolateral membranes of cortical and medulary renal tubular epithelial cells (Wang et al., 2001). Reduced folate carrier was also detected in plasma membranes on the apical surface of the choroid plexus, in axons and dendrites, and on the apical membrane of cells lining the spinal canal (Wang et al., 2001).

2.1.3.3 Proton-coupled folate transporter (PCFT)

The third route of folate uptake into cells is facilitated by the recently discovered

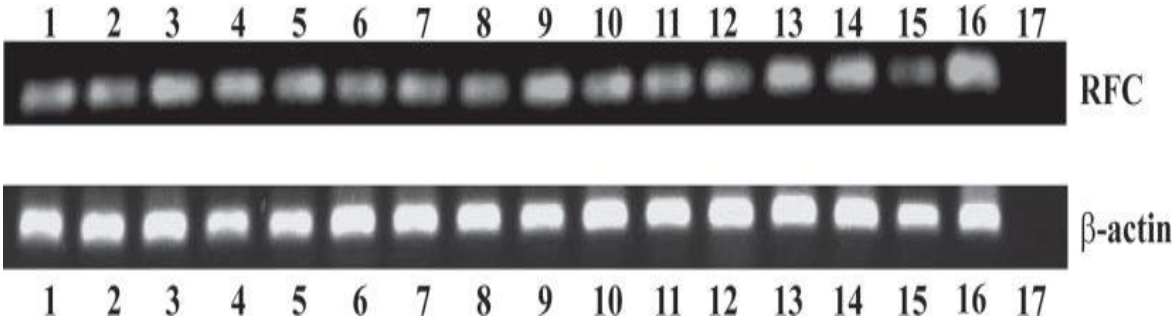

Figure 2.2. Tissue distributions of the reduced folate carrier (RFC) transcripts in the chicken (Jing et al., 2009). The bands of β-actin were also shown as references. 1 = brain; 2 = aorta; 3 = thymus; 4 = lung; 5 = gizzard; **6 = small intestine; 7 = cecum**; 8 = pancreas; 9 = liver; 10 = spleen; 11 = kidney; 12 = testis; 13 = ovary; 14 = bursa of Fabricius; 15 = skeletal muscle; 16 = plasmid; 17 = no template control. (Used with permission of Poultry Science, February 21, 2011).

folate transporter called proton-coupled folate transporter (PCFT) (Qiu et al., 2006). Although initially deposited into the GenBank as a heme carrier protein (HCP1) (Shayeghi et al., 2005), subsequent studies on its kinetic characteristics revealed that the predominant transport function of PCFT involved a high-affinity transport of FA as well as that of 5-methylTHF (Km = 0.5-2 μM) (Qiu et al., 2006). Its identification finally provided explanation on the molecular basis of the long recognized low pH transport activities in several mammalian cells which was originally thought to be mediated by RFC.

Proton-coupled folate transporter gene is highly conserved in mammals. In particular, the PCFT's of human, rat, and mouse all consist of 459 amino acids, and rat PCFT and mouse PCFT are both 87% identical with human PCFT (Qiu et al., 2007; Inoue et al., 2008). In chickens, the PCFT gene shared 70%, 71%, and 71% homology with the sequences of PCFT from human, rat, and mouse, respectively (Jing et al., 2010). They are each predicted to have a structure harboring 11 or 12 transmembrane domains with both the N- and C-termini located intracellularly (Qiu et al., 2007; Inoue et al., 2008; Jing et al., 2010).

Folate transport via PCFT is characterized by a pH-dependent transport activity. In the everted intestinal sac of rats, PCFT transport was greatest at around pH 4.5-5.5 and was negligibly small at neutral pH and above (Inoue et al., 2008). Accordingly, folate-induced currents in Xenopus oocytes microinjected with PCFT cRNA increased as the pH were decreased (Qiu et al., 2006; Qiu et al., 2007). In contrast, the transport of folate via RFC falls with decreasing pH from pH 7.4, and becomes negligible when pH falls below 6.0-6.5 (Wang et al., 2004). Early studies with rodent jejunal apical brush-border

membrane vesicles provided insight into the energetics of PCFT-mediated folate transport (Schron et al., 1985). A transvesicular pH gradient resulted in increased unidirectional transport of folate, while substantial transmembrane folate concentration gradients resulted when vesicles were transferred from a low-pH to a high-pH compartment (Schron et al., 1985). Dependence on pH was further confirmed when PCFT-mediated transport at pH 5.5 was reduced extensively by carbonyl cyanide-4-trifluoromethoxy phenylhydrazone (FCCP), an ionophore that is known to dissipate cellular membrane H^+ gradient (Qiu et al., 2006).

There may be some species differences in the tissue distribution of PCFT. In humans, PCFT is abundantly expressed in the small intestine, and to a lesser extent in the kidney, liver, placenta, spleen, colon, and testis (Qiu et al., 2006). In rats, PCFT is also expressed in a more small intestine-specific manner, being most abundant in the small intestine and far less in all the other tissues including kidney and liver (Nakai et al., 2007). In chickens, PCFT transcripts have a broader tissue expression pattern, being detected in the brain, heart, liver, kidney, spleen, lung, pancreas, intestine, skeletal muscle, thymus, Bursa of Fabricius, ovary, and testis (Jing et al., 2010) **(Figure 2.3)**.

The cellular localization of PCFT is mainly concentrated in the apical membrane of the cell (Zhao et al., 2009). Proton-coupled folate transporter in mice was immunohistologically demonstrated to be localized at the brush border (apical) membrane in the duodenum (Shayeghi et al., 2005). Similarly, human PCFT fused with green fluorescent protein (GFP-hPCFT) was demonstrated to be mainly localized at the apical membrane when expressed in polarized Madin-Darby canine kidney II (MDCKII)

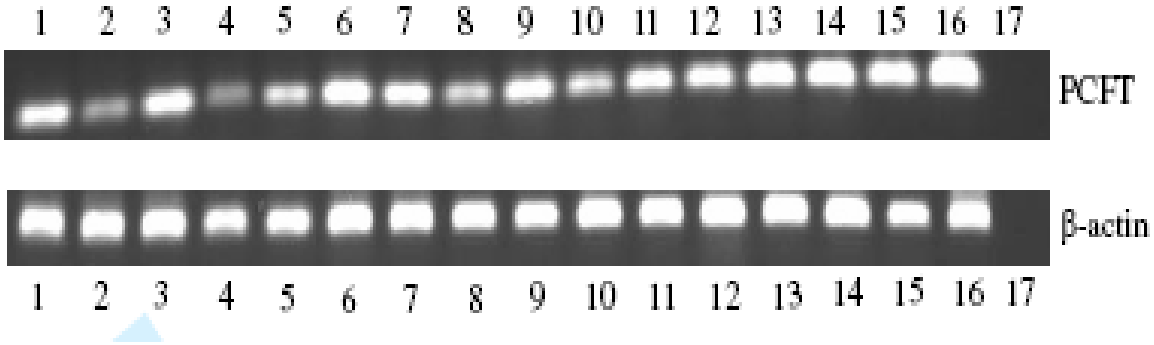

Figure 2.3. Tissue distributions of the proton-coupled folate transporter (PCFT) transcripts in the chicken (Jing et al., 2010). The bands of β-actin were also shown as references. 1 = brain; 2 = aorta; 3 = thymus; 4 = lung; 5 = gizzard; **6 = small intestine; 7 = cecum**; 8 = pancreas; 9 = liver; 10 = spleen; 11 = kidney; 12 = testis; 13 = ovary; 14 = bursa of Fabricius; 15 = skeletal muscle; 16 = plasmid; 17 = no template control. (Used with permission of Taylor & Francis, February 21, 2011).

cells (Nakai et al., 2007). Accordingly, GFP-hPCFT- mediated folate transport occurs in a polarized manner only through the apical membrane, indicating its functional localization at that site (Nakai et al., 2007). Therefore, PCFT is an apical protein and, in the small intestine, it should be expressed at the brush border membrane of epithelial cells. Since RFC is expressed in the basolateral membrane of epithelial cells, PCFT may work in concert with RFC in the absorption of folates.

Hereditary folate malabsorption (HFM) is a rare autosomal recessive familial condition that was identified to be caused by mutations in the human PCFT gene (Qiu et al., 2006). The disorder which is symptomatic within months after birth is characterized by severe folate deficiency in serum, erythrocytes, and CSF, and signs may include megaloblastic anemia, diarrhea, seizures, and mental retardation (Qiu et al., 2006). A change between G to A nucleotide at position 5882 was shown to be a null function mutation and the cause of HFM (Zhao et al., 2007). This mutation causes a deletion of exon 3 and defective trafficking of a shorter variant protein to the cell surface (Zhao et al., 2007). Subsequent studies in patients with HFM have resulted in the identification of seven additional mutations in PCFT gene, underscoring the crucial role that PCFT plays in the intestinal absorption of dietary FA (Zhao et al., 2007; Min et al., 2008).

2.1.4 Metabolism

2.1.4.1 Folate cycle

The process of converting FA into the metabolically active forms of folate is relatively complex. Because FA is not a natural folate metabolite, it has to undergo a series of biochemical reactions before it can enter the cycle of folate metabolism (Bagley and Shane, 2005) **(Figure 2.4)**. After absorption, FA is reduced to dihydrofolate (DHF)

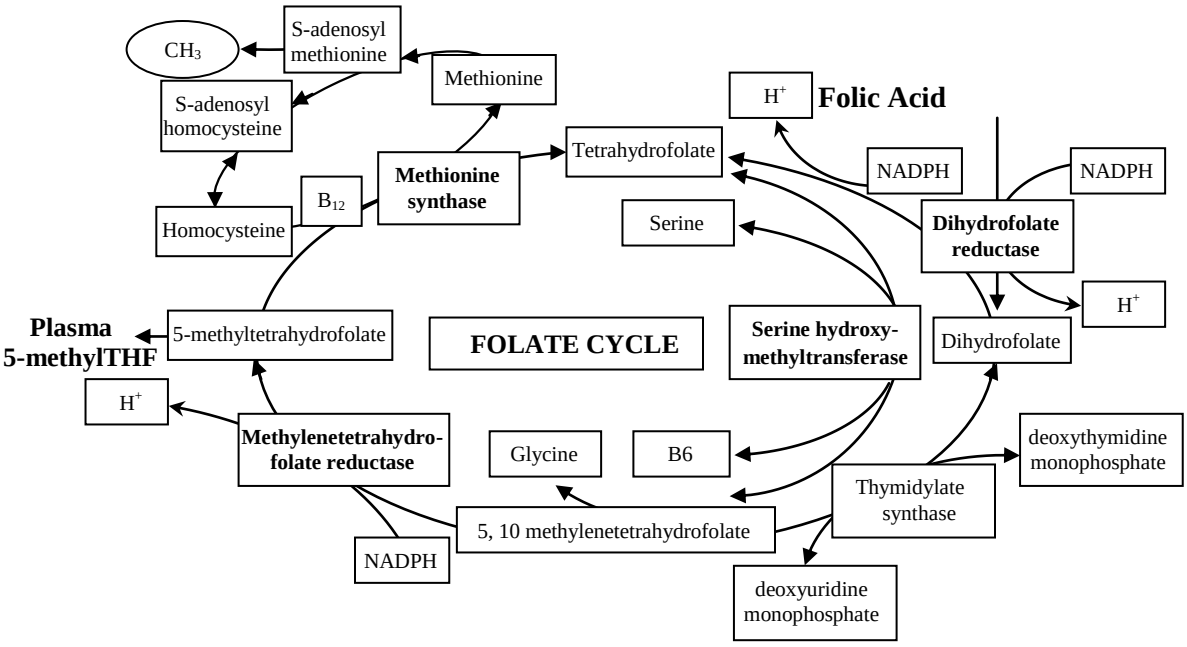

Figure 2.4. The folate cycle. 5-methylTHF = 5-methyltetrahydrofolate

and then to THF in the upper small intestine and to a much larger extent in the liver by dihydrofolate reductase (DHFR) (EC 1.5.1.3) (Whitehead et al., 1987). Dihydrofolate reductase facilitates the reduction of FA into DHF and THF by catalyzing the transfer of hydride molecules from NADPH to the C6 position of the pteridine ring (Benkovic and Hammes-Schiffer, 2003). In humans, the low expression of DHFR coupled by the increased intake of FA in recent years has been attributed as the primary reason for the presence of unmetabolized FA in the systemic circulation (Bailey et al., 2003; Wright et al., 2007; Bailey and Ayling, 2009).

The next step in the activation of the folate coenzyme is catalyzed by serine hydroxymethyltransferase (SHMT) (EC 2.1.2.1). This enzyme requires the amino acid serine to combine with pyridoxal phosphate (PLP) or vitamin B_6 in order to transfer a hydroxymethyl group to THF to generate 5, 10-methyleneTHF and glycine (Geller and Kotb, 1988). Because this reaction is reversible, SHMT is also known to play a role in gluconeogenesis, through the reconversion of glycine to serine, a glucogenic amino acid (Nijhout et al., 2006).

The generated 5, 10-methyleneTHF from the SHMT reaction is a central compound in the folate cycle (Bagley and Shane, 2005). It either provides the supply of one-carbon groups for the formation of thymidylate for pyrimidine synthesis, or it can be reduced to 5-methylTHF for use in the remethylation of homocysteine (Hcy) to methionine. In the synthesis of pyrimidine nucleotides, thymidylate synthase (TS) (EC 2.1.1.45) catalyzes the transfer of the one-carbon group (methylene) from 5, 10-methyleneTHF to the 5'-position of deoxyuridine monophosphate (dUMP) and its reduction to a methyl group to generate deoxythymidine monophosphate (dTMP) (Fox

and Stover, 2008). In the formation of 5-methylTHF, 5, 10 methylenetetrahydrofolate reductase (5, 10-MTHFR) (EC 1.5.1.20) catalyzes the NADPH-dependent reduction of 5, 10-methyleneTHF to 5-methylTHF (Bagley and Shane, 2005). The 5-methylTHF then provides methyl groups for the formation of methionine from Hcy in the methionine synthase (MS) (EC 2.1.1.13) catalyzed reaction. This reaction links folate metabolism to Hcy metabolism and finally allows THF to re-enter the pool of reduced folates (Bagley and Shane, 2005). In 1991, Kang et al. (1991) first described a point mutation in the gene which codes for 5, 10-MTHFR. The mutation due to a C → T substitution at base pair 677 results in a thermolabile variant of the 5, 10-MTHFR which has a significantly reduced specific activity as well as reduced residual activity (Kang et al., 1991). Homozygosity for the variant is associated with higher than normal plasma Hcy levels and low folate status (Frosst et al., 1995; Bailey and Gregory, 1999). Carriers of the gene variant have been reported to have increased risk of stroke (Casas et al., 2005), depression (Lewis et al., 2006; Gilbody et al., 2007), schizophrenia (Muntjewerff et al., 2006), bipolar disorder (Gilbody et al., 2007), male infertility (Bezold et al., 2001), neural tube defects (Christensen et al., 1999), some but not all cancers (Kim, 2005) and possibly Down syndrome (Hobbs et al., 2000).

The unsubstituted THF released from the methionine synthase catalyzed reaction is either utilized for purine nucleotide synthesis or is converted to polyglutamated forms of folates. In the synthesis of purine nucleotides, THF reacts with formate (via 10-formylTHF synthetase) (EC 6.3.4.3) to produce 10-formylTHF (Tan et al., 1997). This molecule donates two carbons to the synthesis of the purine ring in the reaction mediated first by β-glycinamide ribonucleotide (GAR) transformylase (EC 2.1.2.2.) and then by 5-

amino-4-imidazolecarboxamide ribonucleotide (AICAR) transformylase (EC 2.1.2.3) (Tan et al., 1977). The C2 and C8 positions of the purine ring are both derived from 10-formylTHF. In the synthesis of the polyglutamated form of folate, the monoglutamated THF is converted into polyglutamate derivatives by folylpolyglutamyl synthetase (FPGS) (EC 6.3.2.17) (Steinberg, 1984). The metabolism of folates into the polyglutamated form is required for the biological activity of folate as the polyglutamates are much more effective substrates for folate-dependent enzymes than are the monoglutamate derivatives (Shane, 1989). Additionally, the conversion of folates to polyglutamates of chain length greater than 3 is also required for effective retention of folate by tissues (Osborne et al., 1993).

2.1.4.2 Methylation cycle

Folate serves as substrate in the remethylation cycle which involves the conversion of Hcy to methionine. As mentioned earlier, 5, 10-methyleneTHF is reduced to 5-methylTHF by 5, 10-MTHFR. The vitamin B_{12}-dependent MS enzyme then remethylates Hcy into methionine using 5-methylTHF as a source of methyl groups. *De novo* synthesized methionine is then activated by ATP through the reaction catalyzed by methionine adenosyl transferase (MAT) (EC 2.5.1.6) to yield the methyl donor S-adenosylmethionine (SAM) (Bagley and Shane, 2005). The high-energy sulfonium compound SAM then serves as a methyl donor in a multitude of methylation reactions initiated by a variety of methyltransferase enzymes (Stipanuk, 2004).

SAM-dependent methylations are essential for biosynthesis of different cellular components including creatine, epinephrine, carnitine, phospholipids, proteins, DNA, and RNA (Chiang et al., 1996; Stipanuk, 2004). Overall, SAM serves as the methyl donor for

essentially all known biological methylation reactions, with the notable exception of those involved in the methylation of Hcy (Stipanuk, 2004). During the process of methylation, SAM is converted to S-adenosylhomocysteine (SAH), which is hydrolyzed back to Hcy. The Hcy then recommence a new remethylation cycle or condense with serine to form cystathionine (Finkelstein, 1990). In vertebrates, this is the only route for Hcy production (Finkelstein, 1990).

2.1.5 Functions of folate

The vital functions of folate can be ascribed to its participation in different biochemical reactions. As previously discussed, folate plays a pivotal role in the biosynthesis of thymine and purine nucleotides. Reduced availability of folate will impair adequate nucleic acid synthesis and diminish the normal rate of cell division and cell proliferation (Blount et al., 1997). Thymine and purine nucleotides maintain the structural integrity of DNA and RNA. Strand breaks due to defective cycling of the cell may occur if uracil is misincorporated into DNA in place of thymine (Blount et al., 1997). A potentially mutagenic lesion requiring repair through excision by specialized enzymes (glycosylases) ensues if excessive DNA misincorporation of uracil continues (Blount et al., 1997). Even the repair mechanisms may become defective during periods of folate deficiency (Duthie and Hawdon, 1998). Simultaneous excision of multiple opposing strands will result in double strand breaks with further risk of increase DNA instability. If double strand breaks remain unrepaired, there is cellular transformation and degeneration. Both excessive uracil incorporation and chromosomal strand breaks are potentially reversible through folate supplementation (Blount et al., 1997).

As earlier mentioned, another key role of folate is in the provision of methyl groups for the conversion of Hcy to methionine. Folate-dependent methionine synthesis contributes methyl groups to the methylome. These methyl groups are utilized for the methylation of cytosine residues in DNA and of arginine and lysine residues in histones, both of which are involved in regulating gene expression (Fuks, 2005). Furthermore, they can also be used in the methylation of proteins, neurotransmitters, hormones, and phospholipids (Chiang et al., 1996; Stipanuk, 2004).

2.1.6 Folate and health

Folate is essential for many aspects of human health. Originally, interest in the health benefits of folate was mainly due to its role in the prevention of NTDs in babies (MRC, 1991); however, further interest arose when several studies found a clear association between low folate intake and the risk of cardiovascular disease (Perry et al., 1995; Selhub et al., 1995; Rimm et al., 1998). This is in connection to the ability of folate to lower the blood concentration of the potentially atherogenic thiol, Hcy, which has been implicated in the etiology of cardiovascular disease (Perry et al., 1995; Selhub et al., 1995). At present, knowledge of the health benefits of folate extends well beyond these two important conditions. The major disorders now known to be under the influence of either folate status or due to variation in genes coding for folate transporters and folate-dependent enzymes include ailments such as cancer (cervical, colon, and breast) (Butterworth, 1993; Giovannucci et al., 1998; Zhang et al., 1999; Kim, 2005; Cole et al., 2007; Figueiredo et al., 2009), stroke (Casas et al., 2005), Alzheimers disease (Clarke et al., 1998), depression (Lewis et al., 2006; Gilbody et al., 2007), schizophrenia (Muntjewerff et al., 2006), bipolar disorder (Gilbody et al., 2007), Down’s syndrome

(James et al., 1999; Hobbs et al., 2000), male infertility (Bezold et al., 2001), and inflammatory bowel disease (Lashner et al., 1997).

Most of these disorders can be explained within the context of folate-dependent one-carbon transfer reactions involving methionine, Hcy, purine, and pyrimidine biosynthesis. However, the precise underlying cause is most probably linked to low intakes of dietary folate, polymorphisms of genes involved in folate transport and metabolism, and impairement in DNA elaboration and gene expression linked to folate metabolism (Bagley and Shane, 2005). It is also highly possible that a combination of these factors may come into play and precipitate the disease.

2.1.6.1 Neural tube defects

Neural tube defects are among the most common of all birth defects (MRC, 1991). They occur when the neural tube fails to close properly leaving the developing brain and spinal cord exposed to amniotic fluid. Neural tube defects develop as a result of a disturbance in the embryonic process of neurulation (first organogenetic process) (Wagner, 1995). This begins at approximately 21 days post-fertilization, and is complete by 28 days. Thus, neurulation is ongoing before a woman may first realize her pregnancy.

The biochemical and genetic mechanisms that underlie the association between FA and NTDs have yet to be fully established. It has been assumed that NTDs result from impaired maternal and fetal folate supply and metabolism, with affected pathways being either dTMP or methionine syntheses (Molloy and Scott, 2001; Mathers, 2005). Proposed mechanisms include the accumulation of Hcy, decreased rates of DNA synthesis due to impaired dTMP synthesis, and hypomethylation of genomic DNA (Scott, 2001; Mathers, 2005). Evidence from human studies indicated that impairments in the Hcy remethylation

pathway and in SAM-dependent methylation reactions can impair normal embryonic development. Shields et al. (1999) and Rozen (2001) demonstrated that moderate maternal hyperhomocysteinemia increases the risks for NTDs. Many genes coding for folate-dependent enzymes have also been studied for mutations that might account for NTDs. However of these enzymes, only 677 C →T - 5, 10-MTHFR (Whitehead et al., 1995) and a second mutation (A to C substitution at bp 1298) (Van der Put et al., 1998; Stegmann et al., 1999) currently represent increased risk for NTDs.

2.1.6.2 Cardiovascular disease

Several observational studies found a clear association between a low folate intake and the risk of cardiovascular disease (Perry et al., 1995; Selhub et al., 1995; Rimm et al., 1998). However, because numerous mechanisms of Hcy-mediated vascular damage have been suggested, almost all interventional studies focused primarily on the relationship between Hcy levels and the risk of cardiovascular disease. Folate was only seen as a means of reducing Hcy. It was only a few years after that a beneficial effect on vessels independent of Hcy has been attributed to folate itself (Wilmink et al., 2000; Doshi et al., 2001; Stanger et al., 2001).

Vascular endothelial dysfunction is a recognized key event in the etiology of atherosclerosis and is an accepted surrogate end point for clinical investigations in cardiovascular disease (Perry et al., 1995). Folic acid supplementation was demonstrated to improve endothelial dysfunction in asymptomatic subjects with hyperhomocystenemia (Bellamy et al., 1999), as well as in hyperhomocysteinemic patients with established coronary heart disease (Title et al., 2000). Interestingly, this beneficial effect was also observed in subjects without elevated Hcy concentrations (Wilmink et al., 2000; Doshi et

al., 2001), suggesting a distinctive effect of folates on the endothelium independent of Hcy. In interventional studies, it is rather difficult to distinguish between the effects of folate administration and the Hcy-lowering effect related to the vitamin increase. This is particularly the case when a study group is compared with a placebo group without any changes. In an attempt to separate the effects, Stanger et al. (2001) designed a study of patients with coronary heart disease without a placebo group, in which the patients served as their own controls. In subjects with no significant changes in Hcy levels, they found that folate supplementation reversed endothelial dysfunction through stimulation of nitric oxide (NO) production and inhibition of lipoprotein oxidation by FA. Protection against oxidative modifications of human low density lipoprotein (LDL) by FA was also demonstrated by Nakano et al. (2001), providing more evidence that folate exerts direct and indirect antioxidant effects on the vascular endothelium. The NO system offers further potential mechanisms through which folates may effectively interact. Nitric oxide formation from L-arginine by endothelial nitric oxide synthase (eNOS) (EC 1.14.13.39) requires cofactors such as tetrahydrobiopterin (BH_4) (Marletta, 1994). It was suggested that folates may stimulate regeneration of BH_4 from the inactive oxidized quinoid dihydrobiopterin (Kaufman, 1991; Hyndman et al., 2002; Das, 2003) thus increasing NO bioavailability *in vivo* with a protective effect on vascular function (Tiefenbacher et al., 1996; Antoniades et al., 2006).

2.1.6.3 Cancer

Both folate deficiency and polymorphisms in folate-dependent enzymes modify risk for colon and other cancers (Zhang et al., 1999; Kim, 2005; Cole et al., 2007; Figueiredo et al., 2009). The biochemical mechanisms that account for associations

between folate deficiency and cancer risk are not yet established, but two mechanisms have been proposed: (1) increased rates of uracil misincorporation into DNA resulting from impaired dTMP synthesis (Blount et al., 1997) and (2) decreased DNA methylation resulting from depressed SAM synthesis (Kim, 2007).

Low cellular dTMP due to folate deficiency retards conversion of dUMP to dTMP, resulting in thymidine depletion and elevated uracil concentrations (Blount et al., 1997). As uracil and thymidine differ only by a single methyl group, uracil is misincorporated into DNA in place of thymidine. However, during DNA synthesis, uracil is quickly removed by DNA repair enzymes, leaving a single-strand break in the DNA molecule. If folate is persistently limited, this cycle of DNA breakage and repair continues, leading to DNA double-strand breaks, chromosomal aberrations and malignant transformation (Blount et al., 1997). Uracil misincorporation, genome-wide DNA strand breakage and chromosomal instability in response to experimental folate deficiency have been observed in various cell culture models, including human lymphocytes, human colonocytes and Chinese hamster ovary cells (Duthie and McMillan 1997; Zhang et al., 1999; Melnyk et al. 1999; Beetstra et al. 2005). Moreover, human lymphocytes grown under conditions of low folate display increased gene-specific DNA strand breaks in the p53 tumour suppressor gene and a three fold increase in micronuclei frequency, a marker of chromosomal instability (Crott et al. 2007).

In addition to inducing DNA damage and compromising DNA repair, folate deficiency also modulates the epigenome. DNA methylation is an important determinant of genomic stability, mutagenesis and gene expression (Fuks, 2005). Generally, genes methylated at specific sites (e.g. upstream of a promoter) are either not transcribed or are

transcribed at a reduced rate, and its translation into the protein for which the gene codes is decreased. Therefore, epigenetic site-specific DNA methylation contributes to the control of gene, and ultimately, protein expression (Costello and Plass 2001). Folate deficiency, by attenuating remethylation of SAH to SAM in the methionine cycle, may disrupt this function. Under conditions of low dietary folate, SAM is reduced whereas SAH is elevated, resulting in hypomethylation of newly synthesised DNA and, potentially, increased proto-oncogene expression (Kim, 2007). Surprisingly, folate deficiency is also associated with hypermethylation in specific gene regions, notably in tumour suppressor genes. Folate deficiency may induce both gene-specific DNA hypermethylation and global DNA hypomethylation by its DNA-damaging effect. DNA methyltransferase (DNMT) (EC 2.1.1.72), an ancestral DNA repair protein, is sequestered away from the DNA replication fork to regions of folate-deficiency-induced DNA damage, resulting in both genomic hypomethylation and site-specific hypermethylation in gene promoter regions (James et al., 2003). Global and gene-specific DNA hypomethylation and site-specific hypermethylation are common features in tumorigenesis (Arasaradam et al. 2008).

Whereas high folate status is positively associated with biomarkers of genomic stability, the effect of intervention with FA to prevent or reduce cancer recurrence in large-scale human trials is contradicting (Kim, 2007; Cole et al., 2007; Figueiredo et al., 2009). There is now an overwhelming concern over the potential harmful effects of long-term intervention with high doses of synthetic FA. Data from human intervention trials and analyses of cancer incidence data suggest that supplementation with synthetic FA may promote growth of initiated cancer cells (Cole et al., 2007; Figueiredo et al., 2009).

Rodent studies on the other hand, report a reduction in early markers of colon cancer, such as aberrant crypt foci, when FA is given prior to initiation of lesions (Song et al., 2000; Kim, 2007). However, carcinogenesis is accelerated if FA is given after the emergence of lesions, presumably through provision of DNA precursors for cancer cell growth (Song et al., 2000). Therefore, it appears that folate plays a dual role; it may protect against the initiation of cancer, but facilitate the growth of cancer cells.

Polymorphisms in the 5, 10-MTHFR gene was also found to modulate the risk of human cancer. Homozygosity (TT) for the variant is associated with changes in the distribution of blood folates. Red cells from homozygous individuals have low 5-methylTHF but high formylTHF levels (Frosst et al., 1995; Bailey and Gregory, 1999). Mechanistically, impaired 5, 10-MTHFR activity would be expected to increase cancer risk due to low blood 5-methyl THF, DNA hypomethylation and proto-oncogene activation. Surprisingly, the homozygous variant is in fact associated with reduced colorectal cancer risk (Ma et al., 1997; Sharp and Little 2004), although this effect is profoundly dependent on folate status. It is hypothesised that low 5, 10-MTHFR activity may reduce colorectal cancer risk by increasing the availability of 5,10-methyleneTHF (and subsequently formyl THF) for thymidine and purine production, thereby providing nucleoside precursors for normal DNA synthesis and repair, and preventing uracil misincorporation and chromosomal breakage (Sharp and Little, 2004).

2.2 Mandatory folic acid fortification

In view of the evidence linking increased folate intake with the prevention of NTDs in babies (MRC, 1991; Czeizel and Dudas, 1992; Werler et al., 1993), health authorities in the early 1990s recommended that all women capable of becoming

pregnant should increase their dietary folate intake and take a daily supplement of 400 µg of FA (Department of Health Expert Advisory Group, 1992; Centers for Disease Control; 1992; National Health and Medical Research Council, 1993). However, because compliance of those who are most at risk has been poor (Clark and Fisk, 1994; Knudsen et al., 2004), and since the recognition of as much as 50% of all pregnancies are unplanned (Grimes, 1986), the governments of the USA and Canada, along with several other countries, introduced the mandatory fortification of grain products with FA (Food and Drug Administration, 1996; Health Canada, 1997; Mills and Signore, 2004; Hertrampf and Cortés, 2008). In Canada, the mandatory FA fortification program was introduced in 1998 (Canada Gazette Part II, 1998). The program mandates that different products like flour, corn, and rice be fortified with FA from 95 to 309 µg/100 g of cereal product (Ray et al., 2002). The goal is to ensure a target level of 140 µg FA/100 g cereal product and a 100 µg/day projected average increase in FA intake in the general population due to fortified cereal grain products (Choumenkovitch et al., 2002).

Reflecting its success in Canada, Ray et al. (2002) reported a significant rise in the blood folate concentrations (41%) among women of reproductive age following FA fortification. In Manitoba in particular, none among the 95 young women participants (aged 18 to 25 years) in a cross-sectional study was found to have a serum folate value indicative of compromised folate status (Shuaibi et al., 2008). Consequently, the birth prevalence of NTDs in newborn babies declined (approximately 46%) markedly (House et al., 2006; De Wals et al., 2007). Also, there has been a significant fall in stroke-related mortality in Canada (from -1% per year from 1990 to 1997 to -5.4% per year from 1998 to 2002) (Yang et al., 2006); while a report from Quebec revealed a significant decreased

(6% per year from 1998 to 2005) in the birth prevalence of severe congenital heart defects among babies (Ionescu-Ittu et al., 2009). A recent evaluation of folate intakes in Canada confirmed the positive folate status for Canadians after FA fortification. Using the national dietary and supplement folate intake data collected from the Canadian Community Health Survey, Shakur et al. (2010) reported that except for women aged more than 70 y, the prevalence of folate inadequacy (POFI) among Canadians is low. The POFI for young children (less than 14 y) and men subgroups even approached zero without FA supplementation. In the US, similar positive outcomes were also reported. The number of cases of NTDs in the US reportedly fell by approximately 20-50% (Honein et al., 2001; Williams et al., 2002; Mills and Signore, 2004), while stroke-related mortality dropped substantially (from -0.3% per year from 1990 to 1997 to -2.9% per year from 1998 to 2002) in the general population (Yang et al., 2006). Thus, more than a decade after the mandatory FA fortification, the data on folate status and aspects of human health confirmed that the fortification of FA in cereal grain products effectively optimized the folate intake and improved the health status of Canadians and Americans.

2.2.1 Is folic acid good for everyone?

While definite evidence of the health benefits from FA fortification have been documented, a growing body of literature also suggests that consuming high levels of FA may have several negative consequences (Mason et al., 2007; Kim, 2007; Smith et al., 2008; Lucock and Yates, 2009). To prevent NTDs, Health Canada (1997) and Food and Drug Administration (FDA) (1996), set a fortification level of 140 μg FA/100 g of ceral product to supply approximately 100 μg FA daily. This amount was selected on the basis that it is unlikely to result in intake in excess of 1 mg (upper safe daily limit) by any

population group. However, doubt has been cast on the actual level of fortification as recent calculations have shown that the level of FA is likely to have been over twice the amount mandated with average intakes ranging from 215 to 240 μg FA/day (Quinlivan and Gregory, 2003). The primary concern is that certain segments of the population will be exposed to amounts of FA greater than 1 mg/day and that this increased level of FA may have some adverse effects.

As already known, FA is different from the predominantly occurring natural forms of folate as it is in the oxidized state and contains only one conjugated glutamate residue (Bagley and Shane, 2005). On the other hand, the folates that are used as co-enzymes and regulatory molecules in the body are all in the reduced form and are mainly polyglutamated (Bagley and Shane, 2005). Bailey et al. (2003), Wright et al. (2007), and Bailey and Ayling (2009) reported that the activity of the DHFR enzyme which catalyzes the reduction of FA to DHF and to THF prior to its disposition in the folate cycle is much lower in humans than in other animals. Therefore, it is possible that repeated consumption of supraphysiological amounts of FA may lead to the accumulation of unmetabolized circulating FA in the blood (and perhaps of DHF). It has been reported that FA may potentially interfere with the metabolism, cellular transport, and regulatory functions of the naturally occurring folates (Troen et al., 2006; Smith et al., 2008). This may be through competition for binding sites within enzymes, carrier proteins, and binding proteins between FA and natural folates (Smith et al., 2008).

Folic acid has the potential to mask the early haematological manifestations of anaemia due to vitamin B_{12} deficiency (Dickinson, 1995; Food and Nutrition Board, 1998; Reynolds, 2006). If deficiency in vitamin B_{12} continues, more supply of FA will

continue to masks signs of anaemia and allow the neurologic complications associated with the deficiency to progress (Dickinson, 1995; Food and Nutrition Board, 1998; Reynolds, 2006). Theoretically, excess FA in persons with low vitamin B_{12} status could bypass the metabolic block in nucleic acid synthesis allowing cell division in bone marrow to continue and thus mask anaemia. The consequence however, will be an increased demand for methyl groups by the growing cells and further depletion of the methylation potential, particularly in the non-dividing cells of the nervous system (Scott and Weir, 1998). Recently, a study revealed that combination of high folate levels and low vitamin B_{12} status has been associated with increased cognitive impairment in seniors (Morris et al., 2007).

Some other safety considerations of high FA consumption ($\geq$ 1 mg/d) are the possible exacerbation of pre-existing cancers or progression of pre-cancerous lesions such as in the colon (Cole et al., 2007) or prostate (Figueiredo et al., 2009) (discussed in section 2.1.6.3), and the potential reduction in the activity of natural killer cells (NK cells) in post-menopausal women due to high levels of unmetabolized FA in the blood (Troen et al., 2006). With respect to NK cell activity, Troen et al. (2006) found an inverse U-shaped relation between total folate intake and NK cell cytotoxicity in post-menopausal women. Although there was no relation between total plasma folates and NK cell cytotoxicity, there was a highly significant inverse linear association between the amounts of unmetabolized FA in plasma and NK cell cytotoxicity. However, further studies are needed to confirm whether excess FA consumption could indeed impair the normal immune function.

Overall, the complexity and biological importance of folate nutrition makes it a difficult nutrient to deploy as a simple intervention at a population level. Folate is involved in many biological functions that it holds too many influences on different important biological processes. As a result, it is very complicated to predict the effects of folate in a generalized way. At the moment, the scientific community does not have a 100% consensus on whether mandatory fortification of FA is appropriate as a population measure or not. Therefore, other strategies geared towards improving the population intake of folate should be explored.

2.3 Folate-enriched eggs

On account of the growing concern about the possible health risks of high doses of FA, attention has been directed to other strategies that might help increase the consumption of natural folates. These strategies involve the development of novel foods enriched with natural folates (Buttriss, 2005). Recently, pioneering work has been undertaken to screen for the folate producing capacity of yeast, with a view to enhancing the folate content of fermented foods (Hjortmo et al., 2004). Some others have looked at the potential effects of processing on the folate content of beer (Walker et al., 2004), fruits, and vegetables (Indrawati et al., 2004; Oliveres et al., 2004). However, a proven and effective way of enhancing food with natural folate is through the enrichment of folate in eggs (Sherwood et al., 1993; House et al., 2002; Hebert et al., 2005; Roth-Maier and Böhmer, 2007; Bunchasak and Kachana, 2009; Hoey et al., 2009; Dickson et al., 2010).

It is generally known that chicken eggs are an excellent dietary source of many essential nutrients (Surai and Sparks, 2001) and one of the least expensive animal

products. Our group and several others have shown that the folate content of eggs can be increased significantly through supplementation of the laying hen diet with synthetic crystalline FA (Sherwood et al., 1993; House et al., 2002; Hebert et al., 2005; Roth-Maier and Böhmer, 2007; Bunchasak and Kachana, 2009; Hoey et al., 2009; Dickson et al., 2010). Through supplementation of 4 mg FA/kg of diet, and fed for at least 3 weeks, the level of folate concentration in the egg can be increased by about 2 to 2.5 fold compared to birds fed the basal diet (House et al., 2002; Hebert et al., 2005; Dickson et al., 2010). More importantly, the majority of the supplemented FA is converted to the natural form of folate. Of the total folate composition of the egg, more than 80% is in the form of 5-methylTHF, and less than 10% is FA (Vahteristo et al., 1997; Seyoum and Selhub, 1998; McKillop et al., 2003; Hoey et al., 2009). Regardless of the dietary FA supplementation, the unmetabolized FA remained about 10% of the total egg folate content (Hoey et al., 2008). At 10% FA in a medium size egg (50-60 μg folate/egg; thus 5-6 μg FA/egg), the level of FA ingestion is well below the threshold for humans at which unmetabolised FA starts to appear in the circulation (when the capacity of the small intestine and the liver to reduce FA is exceeded) (Sweeney et al., 2007). Therefore, the consumption of folate-enriched eggs is not likely to expose the general population to the potential risks that are associated with high intakes of FA.

5-methyltetrahydrofolate, unlike FA, does not mask signs of anaemia due to vitamin B_{12} deficiency. Therefore, it averts the neurological complications associated with vitamin B_{12} deficiency to progress (Scott and Weir, 1998). Because 5-methylTHF is the biologically active form of folate, the enzymatic reduction and methylation processes required for the metabolic utilization of FA are not pre-requisites for its utilization. The

Food and Nutrition Board (1998) places an upper limit on synthetic FA in fortified foods, however, it considers "no health risk' for 5-methylTHF. Indeed, folate-enriched eggs can improve the population's intake of natural folates without carrying the same safety concerns associated with synthetic FA.

2.3.1 Bioavailability and stability

The relative bioavailability of folate in eggs is believed to be greater than or equal to 100% compared to FA (House et al., 2003). Using growth, plasma folate, liver folate, and plasma glycine concentrations as response criteria, House et al. (2003) did not find any significant effects due to folate source in a rat bioassay evaluation conducted to determine the bioavailability of egg folate relative to FA. However, they found that egg folate effectively lowered plasma homocysteine concentration more than the crystalline FA; thereby suggesting that folate in eggs may be more bioavailable than crystalline FA (House et al., 2003). Currently, the Dietary Folate Equivalent (DFE) ascribes a constant bioavailability percentage for all naturally occurring food folates, equivalent to 50% of crystalline FA bioavailability taken on an empty stomach (Food and Nutrition Board, 1998). However, it should be noted that folate in egg exists in the monoglutamate form (Mason and Rosenberg, 1994), as opposed to other foods where the polyglutamate form predominates. Because polyglutamated folates must be hydrolyzed prior to absorption, the monoglutamated forms of folate in egg should have an advantage relative to absorption. In addition, the relatively simpler food matrix that an egg possesses (no non-starch polyssacharides, NSP) may confer an advantage relative to its bioavailability compared to other food sources (House et al., 2003).

Folate-enriched egg is also a stable food product and its folate content does not oxidize during refrigerated storage for 4 weeks (House et al., 2002). When stored for 4 weeks at 4^{o}C, folate-enriched eggs experienced no change in folate levels relative to freshly collected eggs. These conditions correspond with the typical storage of commercial eggs and thus indicate that storage losses for folate-enriched egg should not be of any concern. Relative to green leafy vegetables, folates in animal products including eggs are also generally more stable during cooking (McKillop et al., 2002).

2.3.2 Acceptability and cost

The perception of consumers toward dietary lipids in general has changed their attitude towards egg consumption. This is brought about by fear that egg cholesterol may raise their blood cholesterol levels. However, as understanding of the relationship between cholesterol intake and cardiovascular disease risk has increased, it has become clear that dietary cholesterol intake per se does not impart a significant risk for acquiring cardiovascular disease (McNamara, 2000; Kritchevsky; 2000). The latest evidence suggests that egg consumption up to six times per week is not associated with an increased risk of heart disease (Djousse and Gaziano, 2008).

For the last decade, the egg industry has been very responsive in seeking new technology in exploiting eggs beyond their traditional food value. For instance, several specialty eggs are now being marketed routinely. Eggs enriched with omega-3 and selenium, both enhanced through the hen's diet, are now marketed routinely in many parts of the world (Surai and Sparks, 2001). In as much as both were able to find a niche in the specialty egg market, it is likely that folate-enriched eggs will also be able to create a market of its own. Recently developed genetically modified plants capable of producing

high folate containing fruits and vegetables have met consumer reluctance due to its potential unknown effects on human health (Diaz et al., 2007). Therefore, compared to folate-enriched egg, it is less likely to offer a viable means of enhancing folate intakes generally.

On the basis of an optimal inclusion level of 4 mg FA/kg of feed to enrich eggs with folate, the production costs associated with folate-enriched egg can be considered minimal. At this inclusion rate, the cost of FA per 1000 kg of feed is approximately CAN$ 9.14 based on a price of CAN$ 228.50 per 100 gm of crystalline FA (Sigma-Aldrich Canada Ltd.). Therefore, at an average feed efficiency of 1.7 and an average egg weight of 60 gm, the cost of FA supplementation will only be around 0.09 cents per egg. This will be even much lower in a commercial production setting if one will take into consideration the disparity in price used in this calculation and the price that commercial producers will be able to command on the basis of their volume in operation. With a required feeding duration of at least 3 weeks to achieved optimal egg folate concentration, the enrichment of folate in eggs can be considered as a cost-effective option for increasing folate intakes, particularly in nutritionally vulnerable groups, such as those of lower socio-economic status. Indeed, folate-enriched egg can be considered as a viable option from a practical standpoint, given the low production costs and the probable acceptability by both consumers and health professionals.

2.3.3 Effect of management-related factors on egg folate concentration

Assessment of factors that are likely to influence the level of folate concentration in egg is important to determine the optimal strategies for the production of folate-enriched eggs. With respect to factors related to management, several studies have been

conducted to determine the effect of the stage of production, strain of the laying hen, cereal type in the diet, and dietary exogenous enzyme supplementation on egg folate concentration (Dickson et al., 2010; Hebert et al., 2010, in press).

2.3.3.1 Stage of production and strain of the laying hen

One of the critical components in the production of nutrient-enriched eggs is the characterization and determination of the potential changes in the ability of the laying hen to incorporate the nutrient at different stages of production. In a study conducted by Dickson et al. (2010), they reported that the laying hen's stage of production is an important factor which influenced egg folate concentration. In their study, they found that laying hens at 25 week of age achieved optimum egg folate concentration early in production (3 weeks after FA supplementation; 4 mg FA/kg diet), attained maximum egg folate level at peak of production (29 weeks) and maintained egg folate level at fairly stable concentration throughout the entire period of production (44 weeks). Thus, the duration of FA supplementation required to achieve optimal egg folate concentation is relatively short, and feeding of FA during peak of production is the most ideal period for maximizing egg folate concentration. In addition, a long-term consistency in folate deposition can be expected throughout the whole cycle of egg production.

The strain of the laying hen may influence the level of egg folate concentration. However, Dickson et al. (2010) reported that the sensitivity and responsiveness to dietary FA supplementation due to differences in strain did not influence egg folate concentration. When 4 mg FA/kg diet was supplemented to Hyline W36 and Hyline W98 laying hens, the level of egg folate concentrations between the 2 strains were the same (W98 = 45.70 ± 2.34 μg/egg vs W36 = 48.10 ± 2.99 μg/egg) (Dickson et al., 2010).

Hyline W36 is a strain of hen that has been selected for their excellent feed efficiency, while Hyline W98 hen has been selected for their ability to produce larger eggs (Hyline, Inc., 2004)

2.3.3.2 Effect of cereal type and exogenous enzyme

The type of the primary cereal used in the laying hen diet supplemented with 4 mg/kg of FA influenced the level of folate deposition in eggs (Hebert et al., 2010; in press). Laying hens fed corn-based diet (57.3%) had increased total egg folate content compared to hens fed wheat-based diet (67.2%) (37.2 vs 28.1 µg/egg), however, the levels of egg folate content did not differ significantly between hens fed corn and barley-based diets (57.6%) (37.2 vs 30.4 µg/egg). β-glucan is the primary NSP in barley (White et al., 1983; Rotter et al., 1990) while arabinoxylan (pentosan) is the primary NSP in wheat (Andrewartha et al., 1979). Neither NSP is hydrolyzed by the endogenous enzymes of the laying hens. Because barley and wheat are known to contain substantial amount of soluble NSPs, which result in increased digesta viscosity (Andrewartha et al., 1979; White et al., 1983; Rotter et al., 1990), these anti-nutritive factors could have diminished the amount of folate that was transferred into eggs. Because corn contains lower levels of total NSP that are largely insoluble compared to barley and wheat; availability of folate for egg deposition was not as likely to be depressed. Thus, feed constituents like NSP which may affect folate digestion and absorption, may influence the egg folate concentration.

There was no compelling evidence regarding the effect of enzyme supplementation on egg folate content (Hebert et al., 2010; in press). Addition of β-glucanase in the barley-based diet only increased egg folate content slightly but was not

significantly different from an unsupplemented barley-based diet (34.8 vs 30.4 µg/egg) (Hebert et al., 2010; in press). Similarly, the supplementation of β-glucanase and xylanase enzymes in rye/wheat (25/36.8%) based laying hen rations did not show a significant increase in egg folate concentration (Hebert et al., 2010; in press). Since this study did not detect any significant response to enzyme supplementation, it is possible that the gut viscosity created by the NSP component of barley and rye/wheat was not high enough to create a gut environment that interfered with folate bioavailability. It is also possible that the effects of the viscosity reducing enzymes were not adequate to elicit a significant effect. In general, there is a close relationship between the amount of soluble β-glucans and arabinoxylans and the effect of enzyme supplementation on the availability of nutrients for the laying hens. Feed ingredients that create a very high viscous environment in the gut have generally shown greater response to enzyme treatment while those that do not, showed expectedly a lesser response (Choct et al., 1995).

2.3.4 Deposition of folate in egg is a saturable process

In total, more than 95% of folates in egg are located in the yolk (Sherwood et al., 1993). Most of these are derived from dietary FA and those that originate from feed ingredients, including 5-methylTHF or other natural derivatives of folate that are ultimately converted to 5-methylTHF. Circulating blood 5-methylTHF from the diet and from folate stores within various tissues of the laying hen is transferred to specific sites within the egg (Seyoum and Selhub, 1998). Since the yolk constitutes the largest single deposit of folate within the egg, transfer through the ovary is clearly an important route for supplying the egg with its folate stores. Targeted delivery of folate to the ovary and

ultimately to the yolk of the egg is facilitated by a folate binding protein that is produced in the liver of the laying hen (Henderson, 1990).

At the current level of folate enrichment in egg (50-60 μg of folate/egg), an average size egg (60 g) can provide approximately 33-40%, 12.5-15%, and 8-10% of the folate RDA for toddlers (150 μg DFE/d), adults (400 μg DFE/d), and pregnant women (600 μg DFE/d), respectively. However, if a folate-enriched egg can deliver an amount of folate comparable with the RDA, this will be more ideal because a single egg will be able to deliver a substantial amount of the nutrient without carrying as much concern on dietary intake of cholesterol.

Previous attempts to further increase the level of folate in egg have been unsuccessful because egg folate reached a maximum plateau when FA was increased in the diet (Sherwood et al., 1993; House et al., 2002; Hebert et al., 2005; Roth-Maier and Böhmer, 2007; Bunchasak and Kachana, 2009; Hoey et al., 2009; Dickson et al., 2010). In particular, Sherwood et al (1993), House et al. (2002), and Hebert et al. (2005) conducted dose-response studies to determine the optimal dietary FA levels required for maximal egg folate deposition and found a linear increase in egg folate concentrations when crystalline FA was added from 0 - 4 mg/kg in the laying hen diet. However, at higher levels of FA supplementation, the efficiency of FA deposition in eggs dropped, (**Figure 2.5**) and egg folate levels demonstrated features of saturation. Further addition of FA above 4 mg/kg of diet yielded no further significant increases in egg folate concentration. Interestingly, blood folate concentration responded similarly to egg folate concentration (**Figure 2.6 and 2.7**). Depicting the same pattern of response as egg folate concentration, blood folate concentration showed saturation when hens were fed diets

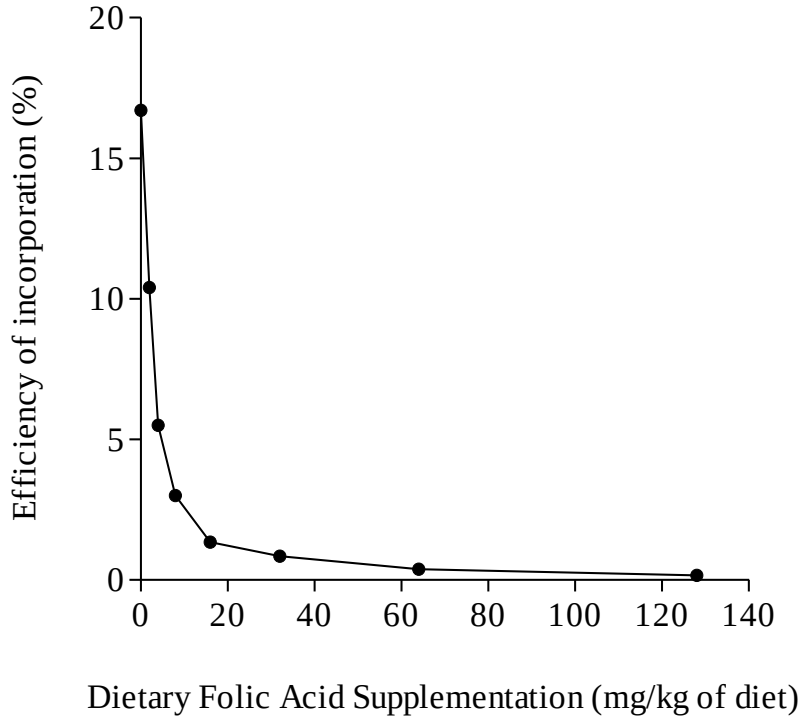

Figure 2.5. Efficiency of incorporation into eggs of supplemental folic acid (FA) in a barley-based basal diet. Efficiency was calculated as the difference in the average folate content of eggs from FA supplemented and the basal diet (baseline folate content), divided by the average FA intake per treatment and expressed as percentage. (Data were adapted from Hebert et al., 2005).

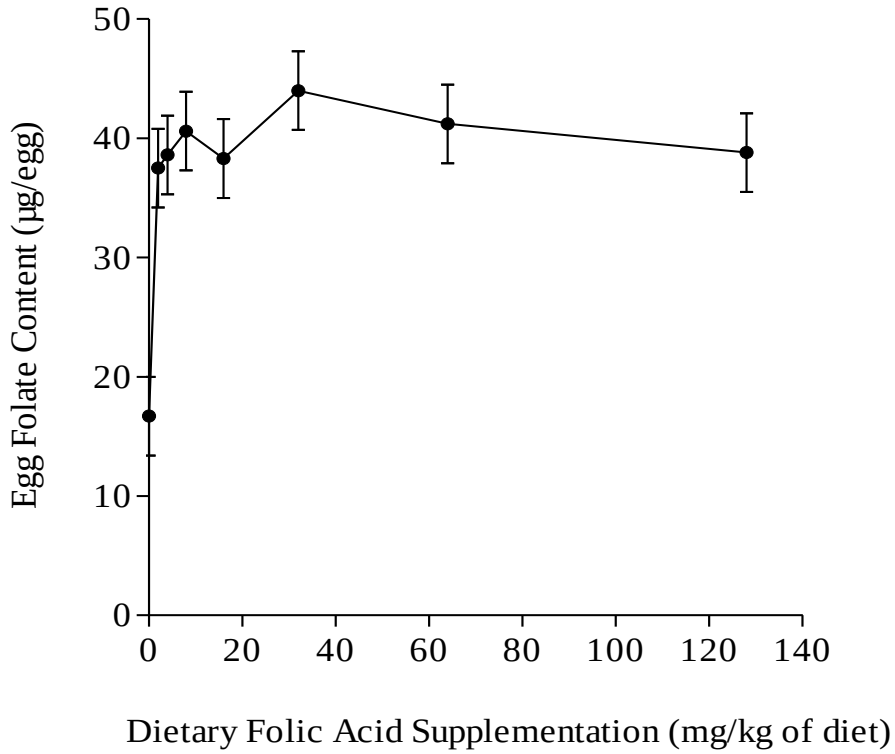

Figure 2.6. Dose response relationship between dietary folic acid (FA) supplementation and the folate content of egg (Data were adapted from Hebert et al., 2005).

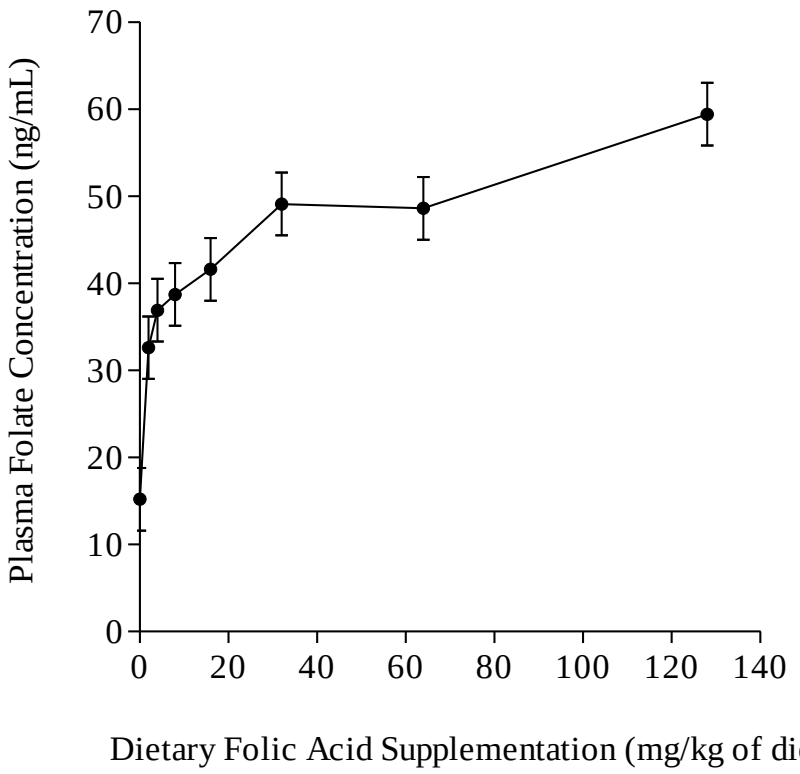

Figure 2.7. Dose response relationship between dietary folic acid (FA) supplementation and plasma folate concentration (Data were adapted from Hebert et al., 2005).

high in FA (Sherwood et al., 1993; House et al., 2002; Hebert et al., 2005). Because circulating folate in the blood serves as the precursor pool for egg folate deposition, the observed saturation in blood folate concentration may be regulating the saturation in egg folate concentration. Therefore, by indentifying the control points and understanding the mechanisms at which they regulate the blood plasma folate levels, better insights on ways to further increase the level of egg folate concentration may be elucidated.

2.3.4.1 Absorption of dietary folic acid in the laying hen

The rate of absorption in the epithelial cells of the intestine determines the availability of nutrients derived from the diet. In studies with rats and *in vitro* intestinal cell model systems, FA has been shown to be absorbed from the gut via a membrane-bound folate transport system (Said et al., 2000; Said, 2004; Inoue et al., 2008) that accepts both oxidized and reduced forms of the monoglutamated forms of folate. This process has been shown to be saturable in a number of model systems (Said, 2004), and thus, represents a potential control point for blood folate concentration.

Two folate transporters, the RFC and PCFT have been reported to facilitate the intestinal absorption of dietary FA in mammals (Matherly and Goldman, 2003). Before the discovery of PCFT, RFC had long been regarded as the molecular entity of the carrier-mediated intestinal folate transport system. However, doubts have been cast on its role in intestinal folate absorption, as RFC-mediated transport is optimal at near-neutral pH, whereas the intestinal folate transport system functions optimally at acid pH (Wang et al., 2004). Recently, PCFT was identified as a proton-dependent, high-affinity FA transporter, with properties that are similar to the intestinal folate transport system (Qiu et al., 2006; Inoue et al., 2008). Our group has investigated the mRNA expression of RFC

and PCFT in the digestive tract of the laying hen and found that both transporters are widely expressed in the entire region of the small and large intestine (Jing et al., 2009; 2010). However, there is limited available information regarding the functional characteristic and regulation of these transporters in the avian model system.

2.3.4.2 Metabolism of dietary folic acid in the laying hen

Folic acid is the major form of supplemental folate that is use for folate enrichment in eggs. As it is not a natural folate metabolite, a chain of biochemical reaction ensues as FA crosses the mucosal cells of the intestine. This is required to convert the dietary FA into its biologically active forms. The group of enzymes involved in this bioconversion are called the folate-dependent enzymes.

In the avian system, absorbed FA is reduced subsequently to DHF and THF in the intestinal cells and hepatocytes by DHFR enzyme (Whitehead et al., 1987). Consequently, THF combine with a hydroxymethyl group from serine to produce 5, 10-methyleneTHF and glycine in a reaction catalyzed by SHMT, a PLP-dependent enzyme. The generated 5, 10-methyleneTHF is then reduced even more by 5, 10-MTHFR to 5-methylTHF, the physiologically transportable form of folate which is secreted and reabsorbed into the small intestine with the bile (enterohepatic cycle) before being transferred by a folate binding protein to the yolk (Henderson, 1990). Therefore, the efficient conversion of dietary supplied FA into its biologically active form, in particular the 5-methylTHF, is a prerequisite for its eventual transfer and deposition into eggs.

In humans, the metabolic capacity for the biotransformation of FA to 5-methylTHF appeared to be limited (Lucock et al., 1989; Priest et al., 1999; Bailey et al., 2003; Wright et al., 2005). This is reflected by the lower DHFR activity detected in

human tissue compared to corresponding animal tissue (Bailey et al., 2003; Wright et al., 2005). Lucock et al. (1989) and Priest et al. (1999) reported that the metabolism of FA to 5, 10 methyleneTHF and THF as well as 5-methylTHF manifested a saturable behaviour. Little is currently known regarding the metabolic capacity of the laying hen to convert FA into 5-methylTHF at a higher level of dietary FA supplementation.

CHAPTER THREE

HYPOTHESES AND OBJECTIVES

3.1 Hypotheses

Null hypothesis: Regulatory factors involve in the deposition of dietary FA into egg do not limit the egg folate concentration.

Alternative hypothesis: Regulatory factors involve in the deposition of dietary FA into egg limit the egg folate concentration.

3.2 Objectives

The main purpose of this research was to determine the different factors that regulate the deposition of dietary FA into egg. This will be achieved through the following objectives:

i) To characterize the biochemical changes in egg folate concentration, indices of folate status, and activities of different folate-dependent enzymes in laying hens supplemented with dietary FA and 5-methylTHF.

ii) To functionally characterize the absorption of FA in the intestine of the laying hen using the *in vitro* intestinal everted sac model.

iii) To examine the functional and molecular regulation of intestinal folate absorption in laying hens supplemented with increased levels of dietary FA.

CHAPTER FOUR

MANUSCRIPT 1

Characterization of folate-dependent enzymes and indices of folate status in laying hens supplemented with folic acid or 5-methyltetrahydrofolate

4.1 ABSTRACT

The conversion of folic acid (FA) to the biologically active 5-methyltetrahydrofolate (5-methylTHF) is necessary for the deposition of folate in the egg. In order to investigate potential differences between dietary FA and 5-methylTHF in the laying hens, a study was conducted to compare the production performance, egg folate concentration, indices of folate status, and activities of folate-dependent enzymes in response to equimolar intake of either folate compound. Forty-eight laying hens, 24 weeks of age, from 2 different strains (Shaver White and Shaver Brown) were randomly assigned to receive 1 of 3 (n = 8 per strain) dietary treatments: 1) basal diet with no supplemental folate, 2) basal diet + 10 mg/kg FA, or 3) basal diet + 11.3 mg/kg 5-methylTHF for 3 wk. A completely randomized design with 3 dietary treatments and 2 laying hen strains in a 3 x 2 factorial arrangement was used in this experiment. Data were subjected to ANOVA, using the PROC GLM procedure of SAS. Production performance was not affected by the form of folate supplementation except for feed efficiency which improved ($P < 0.05$) in 5-methylTHF-fed birds relative to FA-fed birds. Plasma homocysteine, serum, and egg folate concentrations, as well as hepatic serine hydroxymethyltransferase, (SHMT) and methionine synthase (MS) activity were affected by dietary folate supplementation but not by its form (FA or 5-methylTHF). Activities of duodenal dihydrofolate reductase (DHRF) and hepatic 5, 10-methylene tetrahydrofolate reductase (5, 10-MTHFR) were also not affected by the supplemental form of folate. Relative to the control hens, plasma homocysteine was decreased ($P < 0.05$) by 14.2%, whereas, serum and egg folate concentrations were increased ($P < 0.001$) by 78.3 and ($P < 0.001$) 61.8%, respectively, in hens fed either folate compound. Hepatic SHMT and MS activity were increased ($P <$

0.001) and decreased ($P < 0.018$) respectively, in folate-fed birds compared with control-fed birds. Hepatic DHFR was influenced by both the addition and form of dietary folate, being higher ($P < 0.034$) in FA-fed birds than in 5-methylTHF and control-fed birds. Strain of hen influenced serum folate and plasma homocysteine concentrations but did not affect the other indices of folate metabolism. Overall, the supplementation of 5-methylTHF compared to FA may improve the production performance of the laying hens. However, FA and 5-methylTHF supplementation have equivalent effects in enhancing egg folate concentrations, improving folate status, and the overall activity of the different folate-dependent enzymes.

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Keywords: folic acid, 5-methyltetrahydrofolate, folate-dependent enzyme, egg, laying hen

4.2 INTRODUCTION

The link between folate nutrition and human health is well recognized (Czeizel and Dudas, 1992; Kim, 1999; Wang et al., 2001; Yang et al., 2006; De Wals et al., 2007). Folate is a vitamin that is involved in a wide spectrum of biochemical reactions, including serving as a cofactor and cosubstrate for biological methylation reactions such as those involved in amino acid and nucleic acid synthesis (Selhub and Rosenberg, 1996). Adequate folate status in humans is documented to reduce the risk of neural tube defects in babies (Czeizel and Dudas, 1992; De Wals et al., 2007), stroke (Yang et al., 2006), certain cancers, (Kim, 1999) and inflammatory diseases in adults (Wang et al., 2001). In particular, the strong link between adequate maternal folate stores and the reduced risk for neural tube defects prompted Canadian and US lawmakers to mandate the fortification of cereal grain products with this vitamin (MRC, 1991).

The type of folate solely used in food enrichment and fortification is the synthetic form of the vitamin known as folic acid (FA). Folic acid is different from the predominantly occurring natural forms of folate because it is in the oxidized state and contains only one conjugated glutamate residue (Bagley and Shane, 2005). However, the folates that are used as coenzymes and regulatory molecules in the body are all in the reduced form and are mainly polyglutamated (Bagley and Shane, 2005). Recently, concerns about excessive intake of FA have emerged (Weir and Scott, 1999; Reynolds, 2006; Troen et al., 2006; Smith et al., 2008; Lucock and Yates, 2009), due potentially to its interference with metabolism, cellular transport, and regulatory functions of the naturally occurring folates. Competition between FA and the reduced forms of folate for

binding sites within enzymes, carrier proteins, and binding proteins may be at the root of the concerns (Smith et al., 2008).

The enrichment of eggs with folate has been explored as an additional vehicle to provide folate into the human diet. Eggs contain 5-methyltetrahydrofolate (5-methylTHF) as the predominant source of folate, representing more than 80% of the total folate composition of eggs (Seyoum and Selhub, 1998). Unlike the synthetically derived FA, 5-methylTHF is a naturally occurring derivative of folate, and therefore unlikely to interfere with normal folate metabolism. Previous studies have shown that dietary FA addition can lead to increased egg folate concentrations, with one enriched egg containing 50-60 µg dietary folate equivalents or approximately 12.5-15% of the recommended dietary allowance for adults (Food and Nutrition Board, 1998). However, attempts to further increase the level of folate in eggs have proven unsuccessful, because folate levels reach a maximum plateau, likely attributable to the presence of saturable processes during intestinal folate absorption (Said et al., 2000; Said, 2004; Inoue et al., 2008) and the potential limited supply of folate-dependent enzymes involved in catalyzing the biochemical processes leading to the conversion of dietary FA to 5-methylTHF (Lucock et al., 1989; Priest et al., 1999; Bailey et al., 2003; Wright et al., 2005) (**Figure 2.4**). To our knowledge, studies in laying hens comparing the influence of dietary FA and 5-methlyTHF supplementation on folate deposition in eggs are completely lacking. Therefore, this study was conducted to determine the effect of equimolar supplementation of FA and 5-methylTHF on egg folate concentration. The indices of folate status and activity of folate-dependent enzymes were also investigated.

4.3 MATERIALS AND METHODS

4.3.1 General

Shaver White and Shaver Brown laying hens (Manitoba Perfect Pullets, Rosenort, Manitoba) were kept in confinement housing under semi-controlled environmental conditions and exposed to a 16-h photoperiod. Forty-eight birds were housed individually; the cage dimensions were 25.4 cm by 40.6 cm, providing 1,032 cm^2 per bird. Feed and water were available to permit *ad libitum* consumption. 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).

4.3.2 Diets

The wheat-based basal diet was formulated to meet the recommendations for laying hens consuming 100 g of feed per day (NRC, 1994) (**Table 4.1**). The diet was analyzed in duplicate to determine the CP, total P, and Ca concentrations. Nitrogen for CP analysis was measured using a N analyzer (Model NS-2000; LECO Corporation, St. Joseph, MI, USA) while samples for Ca and total P analyses were prepared using the AOAC (1990) procedures (method 990.08) and were analyzed using an inductively coupled plasma mass spectrometer (Varian Inc, Palo Alto, CA, USA). The National Research Council (1994) reports 0.25 mg FA/kg of diet as the requirement for laying hens. The basal diet included no crystalline FA or commercially produced 5-methylTHF, a practice consistent with industry standards (BASF, 2000). Diets were offered daily, and the test diets were stored in the dark at 4^oC during the course of the trial.

4.3.3 Experimental approach

Table 4.1. Composition of the basal wheat-based laying hen diet

Item	Amount
Ingredients, %	
Wheat (13.5% CP)	54.50
Soybean meal (45.8% CP)	25.70
Limestone (38% Ca)	10.00
Vegetable Oil (8800 kcal/kg ME)	6.50
Monocalcium phosphate	1.60
Vitamin Premix[1]	1.00
Mineral Premix[2]	0.50
DL-Methionine	0.18
Antioxidant	0.02
Nutrient composition	
Crude Protein, % (Calculated)	19.00
Crude Protein, % (Analyzed)	19.24
ME, kcal/kg	2950
Calcium, % (Calculated)	4.20
Calcium, % (Analyzed)	4.36
Available P, % (Calculated)	0.45
Total P, % (Analyzed)	0.67
Lysine, % (Calculated)	0.91
Methionine, % (Calculated)	0.45
Methionine+Cysteine, (Calculated)	0.79
Folate, mg/kg (Analyzed)	1.49

[1]Provided per kilogram of diet: 11000 IU of vitamin A; 3000 IU of vitamin D_3; 20 IU of vitamin E; 3 mg of vitamin K_3 (as menadione); 0.02 mg of vitamin B_{12}; 6.5 mg of riboflavin; 10 mg of calcium pantothenate; 40.1 mg of niacin; 0.2 mg of biotin; 2.2 mg of thiamine; 4.5 mg of pyridoxine; 1000 mg of choline; 125 mg of ethoxyquin (anti-oxidant).

[2]Provided per kilogram of diet: 66 mg of Mn (as manganese oxide); 70 mg of Zn (as zinc oxide); 80 mg of Fe (as ferrous sulfate); 10 mg of Cu (as copper sulfate); 0.3 mg of Se (as sodium selenite); 0.4 mg of I (as calcium iodate); 0.67 mg of iodized salt.

For 2 wk before the commencement of the study, 96 healthy hens of each strain were monitored for egg production, and the 24 highest-producing hens of each strain (percentage hen-day egg production of 93.8 ± 1.4) were selected for the experiment. Percentage hen-day egg production of the selected hens was not significantly different before the start of the feeding period. At 24 wks of age, the selected hens were placed individually into battery cages and were randomly assigned to receive 1 of 3 dietary treatments: 1) basal diet with no supplemental folate (n = 16), 8 of each strain and 2) basal diet + 10 mg/kg crystalline FA (Shircks Laboratories, Jona, Switzerland) (n = 16), 8 of each strain and 3) basal diet + 11.3 mg/kg 5-methylTHF (equimolar concentration of 10 mg/kg FA) (Shircks Laboratories, Jona, Switzerland) (n = 16), 8 of each strain. The diets were fed for a 2 wk adjustment period followed by a 7 d collection period. All birds were weighed individually at the start and the end of the 3 wk experiment and feed consumption for each cage unit was measured for average daily feed intake and feed efficiency calculations. Feed efficiency was calculated as gram of feed consumed per gram of egg mass produced. Egg production was recorded daily and calculated as percentage hen-day egg production. All eggs laid during the 7 d collection period were weighed to give an average egg weight for the treatment period and were processed for egg folate determination.

At the end of the 3-wk experiment, a 2 mL blood sample was collected from all 48 birds via wing venipuncture using a 3 mL syringe with a 23-gauge needle. Blood samples were divided into 2 aliquots (1 mL each) and transferred to a 2 mL sterile syringe containing 50 µL of a porcine heparin saline solution (68.6 USP units) and a 2 mL serum tube. The tubes containing the heparinized blood were cooled on ice while the

blood in the serum tubes was clotted at room temperature (approximately 25ºC) for 2 hr. Both plasma and serum were separated by centrifugation at 12,000 x *g* for 5 min. After the blood collection, all the birds were killed by cervical dislocation and the liver and duodenal tissues were removed. Liver and duodenal tissue samples were weighed, rinsed with ice cold PBS and frozen as aliquots in liquid nitrogen. The entire duodenal segment was removed from the pancreatic loop section of the small intestine. Plasma, serum, liver and duodenal tissue samples were stored at -80ºC until analysis.

4.3.4 Extraction and analysis of dietary folate content

The extraction of the basal and folate supplemented diets were performed as described by Tamura et al. (1997) and Wilson and Horne (1984). In brief, feed samples were homogenized with a 50mM CHES-HEPES buffer with 2% ascorbic acid and 0.2 M 2-mercaptoethanol (pH 7.8) and stored at -80ºC until analysis. Aliquots of the thawed homogenate were treated to liberate folates from food matrices and binding proteins and convert folates to their microbiologically assayable form using the tri-enzyme digestion method (Tamura et al., 1997). The total folate concentration of the resultant supernatants were measured by microbiological assay, as described by Molloy and Scott (1997), modified to use the test organism *Lactobacillus rhamnosus* (ATCC 7469; American Type Tissue Culture Collection, Manassas, VA, USA), a strain that responds to FA and its reduced, metabolically active derivatives. The accuracy and reproducibility of these assays were assessed using lyophilized liver with a certified value (13.3 mg folate/kg, Pig Liver BCR 487, IRMM, Geel, Belgium). Our analysis yielded a folate concentration of 13.4 ± 1.12 mg/kg, with an overall coefficient of variation (CV) of 8.4%.

4.3.5 Extraction and analysis of egg yolk folate content

The extraction and analysis of the egg yolk folate content was performed as described previously (House et al., 2002). In brief, eggs were weighed, placed in boiling water for 10 min, cooled, and the yolks separated, weighed and retained for analysis by storing at -80°C. Previous research has documented that more than 95% of the folate in egg is located in the yolk (Sherwood et al., 1993), and we have confirmed this from our previous study (House et al., 2002). Egg folate in the form of 5-methylTHF, the major form of folate in eggs (Seyoum and Selhub, 1998), was extracted into an ascorbate buffer (pH 7.8). The extracts were analyzed for 5-methylTHF via reverse-phase HPLC with fluorescence detection, using the method of Vahteristo et al. (1997). An external standard curve with purified 5-methylTHF was used to quantify egg folate concentrations. The inter and intra assay CV for determinations was less than 2%, and recovery of 5-methylTHF added to egg yolk was 99%.

4.3.6 Analysis of serum folate and plasma homocysteine

Serum folate concentrations were measured by the same microbiological assay of Molloy and Scott (1997), as described in the analysis of dietary folate content. Accuracy and interassay variability were assessed by using a whole-blood standard with a certified value of 29.5 nmol/L (whole blood 95/528; National Institute of Biological Standards and Control, Hertfordshire, United Kingdom). Our analysis yielded a folate content of 30.6 ± 1.0 nmol/L with an interassay CV of 3.4%. Plasma homocysteine was determined by reverse-phase HPLC with fluorescence detection, using the method of Araki and Sako (1987), as modified by Gilfix et al. (1997).

4.3.7 Analysis of liver folate

Liver folates were extracted according to the method of Abad and Gregory (1987). In brief, approximately 1 g of each liver sample was measured into centrifuge tubes in which 10 mL of 0.05 M sodium acetate buffer (pH 4.9) containing 57 mM ascorbate was added. The samples were homogenized, topped with nitrogen gas, and placed in a 41ºC water bath for 90 min prior to centrifugation at 12,000 x g at 4ºC for 30 min (Beckman Coulter Canada Inc., Mississauga, ON, Canada). The resulting supernatants were analyzed for 5-methylTHF via reverse-phase HPLC with fluorescence detection, using the method of Vahteristo et al. (1997). An external standard curve with purified 5-methylTHF was used to quantify liver folate concentrations, following the same procedure used in determining egg yolk folate concentration.

4.3.8 Enzyme activity assays

Dihydrofolate reductase (DHFR) activity was measured using the DHFR Assay Kit (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada). The assay kit is a kinetic spectrophotometric assay based on a slightly modified procedure of Hillcoat et al. (1967). The principle is based upon marked change in absorption at 340 nm due to oxidation of NADPH as the dihydrofolate (DHF) is reduced to tetrahydrofolate (THF). The reaction mixture in total volume of 1 mL contained 48 mmole potassium phosphate buffer (pH 6.5), 50 μmole DHF, 60 μmole NADPH, and 40 μL of tissue extract. DHFR activity is defined as equivalent to the amount of enzyme required to transform 1 mmole of DHF to THF per hour per gram liver.

Serine hydroxymethyltranferase (SHMT) activity was measured using a binding assay modified from the method of Geller and Kotb (1989). The assay measures the transfer of L-[3-^{14}C]serine (Amersham Pharmacia Biotech, Bucks, UK) to THF to form

^{14}C-methylenetetrahydrofolate (methyleneTHF). The reaction mixture in total volume of 100 µL contained 50 mM Tris buffer (pH 8.0), 2.0 mM THF, 2.5 mM EDTA, 1.0 mM 2-mercaptoethanol, 0.25 mM pyridoxal 5' phosphate, 0.4 mM serine, 90,000 cpm/100 µL reaction L-[3-^{14}C]serine, and 25 µL tissue extract. Aliquots of the mixture were incubated for 10 min at 41ºC and spotted onto DE-81 cellulose paper (Whatman, Maidstone, UK). The paper was dried and the counts in each square were determined by liquid scintillation counter.

The 5, 10-methylenetetrahydrofolate reductase (5, 10-MTHFR) activity was determined radiochemically in its physiological reverse direction following the procedure by Engbersen et al. (1995), with slight modifications. The reaction mixture in total volume of 600 µL contained 0.18 M potassium phosphate (pH 6.8), 1.15 mM EDTA (pH 7.0), 11.5 mM ascorbic acid, 54 µM FAD, 20 µM [^{14}C]CH_3-THF, 3.5 mM menadione, and 200 µL tissue extract. Aliquots of the mixture were incubated for 20 min in the dark at 41ºC and were subsequently terminated by the addition of formaldehyde, dimedone, and potassium acetate (pH 4.5). [Me-^{14}C] Methyl-THF served as substrate in the presence of menadione as electron acceptor and the 5, 10-methylene- tetrahydrofolate (5, 10-methyleneTHF) formed dissociates easily to yield labelled formaldehyde. Formaldehyde is extracted as formaldehyde dimedone and is counted for radioactivity. Enzyme activity is expressed as nmole of formaldehyde formed per hour per gram liver.

Methionine synthase (MS) activity was measured using an endpoint assay in which the [5-^{14}C]methionine produced was separated by anion-exchange chromatography and counted in a beta-counter. The method was based on the procedure of Koblin et al.

(1981) with slight modifications. The entire reaction mixture in total volume of 200 μL contained 20 μM cyanocobalamin, 58 mM D,L-dithiothreitol, 0.5 mM S-adenosyl-L-methionine, 15 mM D,L-homocysteine, 14 mM β-mercaptoethanol, 1 mM methylTHF with 0.25 μCi of [5-^{14}C]-methylTHF, 175 mM phosphate buffer (pH 7.5), and 100 μL of tissue extract. The reaction mixture was initially topped with nitrogen, capped and incubated under foil in a 41ºC heating block for 15 min. Each reaction was stopped with ice cold deionized water and samples were added to the top of a drained AG1-X8 (200-400 mesh) chromatography column (Bio-Rad, Hercules, CA, USA) and the effluent was collected in a scintillation vial. Sample radioactivity was expressed as nmole of methionine per hour per gram liver.

Optimization of assay conditions, specifically for protein concentration and incubation length, for DHFR, SHMT, 5, 10-MTHFR, and MS was performed prior to tissue enzyme analysis. Optimal condition was established such that measurement of enzyme activity occurred along the linear portion of the curve when plotted against protein concentration and incubation length.

4.3.9 Statistical analysis

A completely randomized design with 3 dietary treatments and 2 laying hen strains in a 3 x 2 factorial arrangement was used. Data were subjected to ANOVA, using the PROC GLM procedure of SAS software (SAS Institute, Cary, NC). When evidence of heterogeneity of variance was present, data were log transformed prior to analysis. To account for the differences ($P < 0.001$) in bodyweight between strains of hen, feed consumption, egg weight, and feed efficiency were subjected to analysis of covariance using initial bodyweight as covariate, while liver weight and duodenum weight were

subjected to the same analysis using final bodyweight as covariate. Data are presented as least-square means plus standard errors, with differences between means determined using Tukey's Honestly Significance Difference. Differences with an α level of $P < 0.05$ were considered to be statistically significant.

4.4 RESULTS

The results from the feed folate analysis confirm that the two folate treatments were equimolar (FA = 8.82 mg/kg vs. 5-methylTHF = 9.05 mg/kg) in folate content. While the actual values are less than the predicted values, the levels are substantially greater than the 4 mg/kg dietary FA at which saturation in egg folate has been observed (House et al., 2002; Hebert et al., 2005). Our intention was to provide a level of dietary 5-methylTHF supplementation above and beyond the point of egg folate saturation so we can have a much better assessment as to the extent at which 5-methylTHF may potentially enhance egg folate concentration. There was no significant supplemental folate by strain interaction observed in any measured response, therefore only the main effects are reported. Percentage hen-day egg production, feed consumption, initial bodyweight, final bodyweight, liver weight, duodenum weight, liver folate concentration, and hepatic 5, 10-MTHFR activity did not differ among folate treatments (**Table 4.2.**). Supplementation of 5-methylTHF/kg diet resulted in the production of heavier ($P < 0.015$) eggs compared to birds fed the control diet. Also, 5-methylTHF supplementation led to a significant improvement ($P < 0.047$) in feed efficiency compared to birds consuming equimolar concentration of FA and birds fed the control diet. Egg and serum folate concentrations increased ($P < 0.001$) in birds consuming folate diets (FA and 5-methylTHF) than birds consuming the control diet, while plasma homocysteine of birds

Table 4.2. Performance, tissue weight, egg folate content, indices of folate status, and selected tissue activity (g of tissue basis) of dihydrofolate reductase (DHFR), serine hydroxymethyltransferase (SHMT), 5-10, methylenetetrahydrofolate reductase (5, 10-MTHFR), and methionine synthase (MS) enzymes in laying hens fed diets containing FA or 5-MTHF – Main effects of diet

	Diet[2]				
Response[1]	Control	FA	5-methylTHF	SEM[3]	*P*-value[4]
Hen-day egg production, %	94.1	94.1	97.0	2.1	0.523
Feed consumption, g/bird/day	107.1	109.5	106.4	2.8	0.721
Feed efficiency, g of feed/g of egg	1.98[a]	1.96[a]	1.77[b]	0.06	0.047
Egg weight, g	58.3[b]	59.8[ab]	61.8[a]	0.8	0.015
Initial bodyweight, kg	1.72	1.84	1.72	0.04	0.061
Final bodyweight, kg	1.73	1.86	1.73	0.05	0.167
Liver weight, g	34.6	37.7	34.5	1.2	0.117
Duodenum weight, g	8.9	9.6	8.8	0.3	0.125
Egg folate, µg/egg	28.2[b]	43.9[a]	47.3[a]	1.5	<0.001
Liver folate, nmol/g	14.27	16.07	15.81	0.61	0.094
Serum folate, ng/mL	35.6[b]	63.8[a]	63.2[a]	3.0	<0.001
Plasma homocysteine, µmol/L	14.34[a]	12.06[b]	13.08[b]	0.54	0.017
Duodenum DHFR, mmol x hr^{-1} x g^{-1} duo	4.65[a]	4.05[b]	4.25[ab]	0.13	0.009
Liver DHFR, mmol x hr^{-1} x g^{-1} liver	15.58[b]	17.89[a]	15.60[b]	0.69	0.034
Liver SHMT, µmol x hr^{-1} x g^{-1} liver	4.65[b]	5.50[a]	5.78[a]	0.20	0.001
Liver 5, 10-MTHFR, nmol x hr^{-1} x g^{-1} liver	187	189	197	3	0.093
Liver MS, nmol x hr^{-1} x g^{-1} liver	728[a]	600[b]	633[b]	32	0.018

[a, b]Data within a row that do not share superscripts are significantly different ($P < 0.05$).
[1]Data are presented as least square means.
[2]Total measured folate activity (expressed as mg FA equivalents/kg of diet): control = 1.49; FA = 8.82; 5-methylTHF = 9.05.
[3]n = 16/treatment.
[4]*P*-value for main effect of diet.

consuming the control diet was increased ($P < 0.017$) compared to birds consuming FA or 5-methylTHF containing diets. Duodenal activity of DHFR was decreased ($P < 0.009$) in birds fed dietary FA than birds fed the control diet, but not with birds supplemented with 5-methylTHF. However, hepatic DHFR activity was increased ($P < 0.034$) in FA supplemented birds than birds fed the control diet and birds supplemented with 5-methylTHF. Hepatic activity of SHMT was increased ($P < 0.001$) in hens fed FA and 5-methylTHF supplemented diets than birds fed the control diet, while neither influenced the hepatic activity of 5, 10-MTHFR. Methionine synthase activity in the liver was increased ($P < 0.018$) in birds fed the control diet than birds fed FA and 5-methylTHF supplemented diets.

The strain of the laying hen did not influence percentage hen-day egg production, liver folate, and egg folate concentrations (**Table 4.3.**). When initial bodyweight was used as a covariate, feed consumption and feed efficiency were the not different, but weight of egg was increased ($P < 0.011$) in Shaver Brown hens than Shaver White hens. Similarly, liver weight was increased ($P < 0.012$) in Shaver Brown hens but duodenum weight was the same when final bodyweight was used as a covariate. Serum folate and plasma homocysteine was decreased and increased respectively, ($P < 0.05$) in Shaver Brown hens than in Shaver White hens. With respect to the folate-dependent enzyme activity, strain did not influence the hepatic activity of DHFR, SHMT, 5, 10-MTHFR, and MS enzymes; however, duodenal DHFR activity was significantly increased ($P < 0.004$) in Shaver White hens than Shaver Brown hens.

4.5 DISCUSSION

Table 4.3. Performance, tissue weight, egg folate content, indices of folate status, and selected tissue activity (g of tissue basis) of dihydrofolate reductase (DHFR), serine hydroxymethyltransferase (SHMT), 5, 10-methylenetetrahydrofolate reductase (5, 10-MTHFR), and methionine synthase (MS) enzymes in laying hens fed diets containing FA or 5-MTHF – main effect of strain

	Strain			
Response[1]	Shaver White	Shaver Brown	SEM[2]	*P*-value[3]
Hen-day egg production, %	96.2	93.9	1.7	0.336
Feed consumption,[4] g/bird/day	104.1	111.2	2.5	0.083
Feed efficiency,[4] g feed/g egg	1.88	1.93	0.06	0.655
Egg weight,[4] g	58.2[b]	61.7[a]	0.8	0.011
Initial bodyweight, kg	1.62[b]	1.90[a]	0.03	<0.001
Final bodyweight, kg	1.60[b]	1.94[a]	0.04	<0.001
Liver weight,[5] g	33.3[b]	37.9[a]	1.1	0.012
Duodenum weight,[5] g	8.9	9.3	0.3	0.308
Egg folate, µg/egg	39.2	40.4	1.2	0.462
Liver folate, nmol/g	14.96	15.81	0.50	0.235
Serum folate, ng/mL	61.6[a]	46.8[b]	2.5	<0.001
Plasma homocysteine, µmol/L	11.87[b]	14.44[a]	0.44	<0.001
Duodenum DHFR, mmol x hr^{-1} x g^{-1} duo	4.54[a]	4.08[b]	0.11	0.004
Liver DHFR, mmol x hr^{-1} x g^{-1} liver	16.90	15.81	0.57	0.181
Liver SHMT, µmol x hr^{-1} x g^{-1} liver	5.13	5.49	0.17	0.132
Liver 5, 10-MTHFR, nmol x hr^{-1} x g^{-1} liver	194.00	188.00	3.00	0.130
Liver MS, nmol x hr^{-1} x g^{-1} liver	666.00	641.00	26.00	0.511

[a, b]Data within a row that do not share superscripts are significantly different ($P < 0.05$).
[1]Data are presented as least square means.
[2]n = 24/strain.
[3]*P*-value for main effect of strain.
[4]Subjected to analysis of covariance using initial BW as covariate
[5]Subjected to analysis of covariance using final BW as covariate

This study was conducted to compare the egg folate concentration, indices of folate status, and activity of different folate-dependent enzymes in response to equimolar supplementation of dietary FA and 5-methylTHF. We have recently determined the presence and expression profiles of both the reduced folate carrier (RFC; Jing et al., 2009) and the proton-coupled folate transporter (PCFT; Jing et al., 20010), and have found that both transporters are expressed in the avian intestine. However, functional studies have yet to be conducted. In the present study, the egg folate concentration of birds supplemented with FA and 5-methylTHF increased significantly compared to birds fed diets without dietary folate supplementation. However, the increased egg folate concentration between the FA and 5-methylTHF supplemented birds did not differ significantly. This study was designed based on the premise that dietary supplementation of 5-methylTHF in laying hens will result in a higher egg folate concentration than dietary FA supplementation. This is because 5-methylTHF is the biologically active form of folate which can be directly incorporated into the blood and immediately deposited into the egg (Henderson, 1990). In contrast, FA must undergo a series of reduction and methylation reactions before being incorporated into the circulating pool of 5-methylTHF. Because a similar level of enrichment in egg folate concentration was achieved between the two supplemental forms of folate, it indicated that FA is equally effective as 5-methylTHF in terms of enhancing the folate concentration in egg. This also means that absorbed dietary FA is efficiently metabolized by the laying hen to provide the same degree of folate enrichment in egg as the dietary supplemented 5-methylTHF.

The folate status of the laying hen can dictate the level of folate concentration in the egg. In the present study, the serum folate concentrations of FA and 5-methylTHF

supplemented birds increased proportionately with egg folate concentrations. Serum folate is used primarily as an important and sensitive indicator to measure folate adequacy (Jacques et al., 1993). Previous studies (Sherwood et al., 1993; House et al., 2002) observed a similar increasing parallel response between blood folate and egg folate levels when FA was supplemented in laying hens (1-32 mg/kg). In contrast, plasma homocysteine in FA and 5-methylTHF supplemented birds decreased with increasing egg folate concentration. Plasma homocysteine concentration is an accurate inverse indicator of folate status (House et al., 2003). Hebert et al. (2005) reported that increasing supplemental FA level from 0 mg/kg in the basal diet resulted in a significant reduction of plasma homocysteine in Hy-Line W-98 hens. The mechanism by which dietary folate supplementation can decrease plasma homocysteine concentration is through its participation in the remethylation pathway. In this pathway, the intracellular 5-methylTHF donates a methyl group to homocysteine to yield methionine (Bagley and Shane, 2005). Because serum folate and plasma homocysteine concentrations were similar in FA and 5-methylTHF supplemented birds, this indicated that FA is not only as effective as 5-methylTHF in increasing egg folate concentration, but also, it is as effective as 5-methylTHF in increasing and lowering the serum folate and plasma homocysteine of the laying hens, respectively.

Liver folate concentration was not affected by dietary folate supplementation. A study by Han et al. (1999) looking at the kinetic profile of the overall biliary excretion of 5-methylTHF in rats, reported a similar observation. In their study, they demonstrated that the clearance process of 5-methylTHF in the liver occurred at a constant rate and that uptake and excretion of liver folate did not reach a saturation point even during periods

when 5-methylTHF was supplemented at higher rates (3 nmol/h). Shin et al. (1995) further explained that although liver is the main storage organ for folates, stored folates in the liver are readily mobilized to different tissue systems in the body to satisfy tissue requirements.

The activities of folate-dependent enzymes which catalyzed the conversion of FA to 5-methylTHF may influence the availability of dietary FA for deposition in the egg. Previous studies suggested that the capacity of folate-dependent enzymes to convert diet-derived FA into 5-methylTHF is limited (Lucock et al., 1989; Priest et al., 1999; Bailey et al., 2003; Wright et al., 2005). Although there have not been any similar studies conducted in the laying hens, it is logical to assume that any limitation in the activity of these enzymes may negatively influence the extent at which FA is transferred into the egg. In the present study, the activities of all folate-dependent enzymes, except for hepatic DHFR were not affected by the form of folate, but were generally affected by the level of dietary folate supplementation. Dihydrofolate reductase activity in the duodenum was increased in control-fed birds compared to FA-fed birds, but hepatic DHFR activity was increased in FA-fed birds compared to the control and 5-methylTHF fed birds. Since hepatic activity of DHFR was observed to be quantitatively larger than the DHFR activity in the duodenal tissue of laying hens, the liver must play a more significant role than the duodenum in the biochemical conversion of FA to DHF. Wright et al. (2005) reported that, at least in humans, there is evidence to suggest that the capacity to reduce oxidized folate is limiting in the intestine. During periods when FA is adequately supplemented in the diet, increased hepatic DHFR activity makes physiological relevance. As 5-methylTHF is not a substrate for DHFR catalyzed reduction reaction, the activity of

DHFR in 5-methylTHF supplemented birds was not increased. The same result holds true with laying hens that were not supplemented with FA. This is because FA is not present in natural feed ingredients. Bagley and Shane (2005) reported that folates occur naturally in reduced and polyglutamated form, predominantly as 5-methylTHF or 10-formyltetrahydrofolate (10-formylTHF).

The activity of SHMT in the liver was also affected by the supplementation of dietary folate. When laying hens were supplemented with FA and 5-methylTHF, their hepatic SHMT activity increased compared to folate-unsupplemented birds. In concert with the amino acid serine, THF initiates the SHMT catalyzed reaction to yield 5, 10-methyleneTHF and glycine. Tetrahydrofolate is either synthesized from the reduction of FA by DHFR or by the transfer of a methyl group from 5-methylTHF to homocysteine during methionine synthesis (Geller and Kotb, 1989). Therefore, consistent with hepatic DHFR activity, hepatic SHMT activity appeared to be similarly influenced by the presence of its targeted substrate.

Hepatic 5, 10-MTHFR activity was not influenced by the supplementation and form of dietary folate. 5, 10-methylenetetrahydrofolate reductase is involved in catalyzing the transformation of 5, 10-methyleneTHF to 5-methylTHF, the first step in the *de novo* biosynthesis of methyl groups (Bagley and Shane, 2005). In condition where blood homocysteine concentration is high, 5, 10-MTHFR activity is elevated (Bagley and Shane, 2005). This is because the conversion of intracellular folate to 5-methylTHF immediately becomes a favoured reaction to support the increasing cellular demand for homocysteine methylation. In the current study, the observed similarity in terms of hepatic 5, 10-MTHFR activity among treatments was not totally clear. However, it

should be noted that the liver 5-methylTHF concentrations were also similar between the folate supplemented and the control fed birds. This indicated that birds fed the control diet have sufficient level of 5, 10-MTHFR activity in the liver to meet the increased demand for 5-methylTHF which is required to methylate the increased homocysteine level in the blood. On a similar note, blood homocysteine also represents a critical regulatory factor for MS activity (Scott and Weir, 1994). In this study, hepatic MS activity was increased in birds fed the control diet as compared to birds supplemented with dietary folate. This may serve to regulate the higher plasma homocysteine concentration in control diet fed birds and at the same time maintain a continuous production of methionine. Scott and Weir (1994) reported that the level of homocysteine, both in cells and in plasma, is controlled by MS through its reconversion back to methionine. Methionine synthase catalyzes the transfer of a methyl group from 5-methylTHF to homocysteine leading to the formation of methionine and THF.

Production performance was similar between FA and 5-methylTHF supplemented birds, except for feed efficiency. Hebert et al. (2005) noted that it is the lack of demonstrable impacts on performance that has generally limited the inclusion of FA in the layer diets, a fact supported by previous surveys conducted in commercial feedmills (BASF, 2000). Because the supplemented amount of folate is much higher than the folate requirement of the laying hens (NRC, 1994), perhaps blood and tissue indices might have plateaued, however, the presence of different forms of folate may be affecting the efficiency of usage. This could explain the improvement in feed efficiency in 5-methylTHF supplemented birds and therefore may indicate a more important role for 5-

methylTHF in laying hen nutrition. However, this remains to be determined in larger production studies.

Hebert et al. (2005) reported previously that strain can contribute differences in plasma homocysteine concentration after dietary FA supplementation. In the present study, Shaver White hens appeared to be more sensitive to folate supplementation than Shaver Brown hens as reflected by their increased serum folate and decreased plasma homocysteine concentration. This was in spite of their lower feed intake than Shaver Brown hens which have consumed more FA and 5-methylTHF than Shaver White hens. However, the increased serum folate concentration in the Shaver White hens did not translate to increased egg folate concentration. A longer term study is needed to further validate the significance of this observation.

In summary, dietary supplementation of FA and 5-methylTHF at equimolar concentration exerted similar influence on egg folate concentration and other biochemical indices of folate status in laying hens. The supplementation of 5-methylTHF may improve production performance, but this remains to be determined in a larger scale production. The strain of laying hens influenced serum folate and plasma homocysteine concentrations, but demonstrated no effects on egg folate concentration. The supplementation and form of dietary folate may modulate the activity of different folate-dependent enzymes.

CHAPTER FIVE

MANUSCRIPT 2

Functional characterization of folic acid transport in the intestine of the laying hen using the everted intestinal sac model

5.1 ABSTRACT

Absorption at the level of the intestine is likely a primary regulatory mechanism for the deposition of dietary supplemented folic acid (FA) into the chicken egg. Therefore, factors affecting the intestinal transport of FA in the laying hen may influence the level of egg folate concentrations. To this end, a series of experiments using intestinal everted sacs were conducted to characterize intestinal FA absorption processes in laying hens. Effects of naturally occurring folate derivatives 5-methyltetrahydrofolate (5-methylTHF) and 10-formyltetrahydrofolate (10-formylTHF) as well as heme on FA absorption were also investigated. Folic acid absorption was measured based on the rate of uptake of ^{3}H-labeled FA in the everted sac from various segments of the intestine. Folic acid concentration, incubation length, and pH condition were optimized prior to the performance of uptake experiments. The distribution profile of FA transport along the intestine was highest in the upper half of the small intestine. Maximum uptake rate (nmol•100 g tissue^{-1}•min^{-1}) was observed in the duodenum (20.6 ± 1.9) and jejunum (22.3 ± 2.0) and decreased significantly in the ileum (15.3 ± 1.1) and cecum (9.3 ± 0.9). Transport increased proportionately *(*$P < 0.05$*)* between 0.0001 and 0.1 μM FA. Above 0.1 μM, the slope of the regression line was not significantly different from zero ($P < 0.137$). FA uptake in the jejunum showed maximum rate of transport at pH 6.0, but was lowest at pH 7.5. Presence of 5-methylTHF and 10-formylTHF as well as heme impeded FA uptake, reducing intestinal FA absorption when added at concentrations ranging from 0-100 μM. Overall, these data indicated the presence of a FA transport system in the entire intestine of the laying hen. Uptake of FA in the cecum raises the likelihood of absorption of bacterial-derived folate.

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Keywords: folic acid, 5-methyltetrahydrofolate, heme, everted sac, intestine, egg, laying hen

5.2 INTRODUCTION

The supplementation of FA at 2-4 mg/kg in the laying hen diet leads to the production of folate-enriched eggs with an approximately two-fold increase in egg folate concentrations relative to regular commercial eggs (House et al., 2002; Hebert et al., 2000). However, increases in egg folate concentration do not go beyond an achieved maximum level (45-50 μg folate/egg) when FA is further increased in the diet (above 4 mg FA/kg diet), demonstrating a pattern of saturation (Sherwood et al., 1993; House et al., 2002; Hebert et al., 2005; Roth-Maier and Böhmer, 2007; Bunchasak and Kachana, 2009; Hoey et al., 2009; Dickson et al., 2010). The observations led to the hypothesis that factors limiting the absorption of dietary FA, its biochemical conversion, and its eventual transfer into the blood circulation explain the observed saturation profile. In previous studies (Sherwood et al., 1993; House et al., 2002; Hebert et al., 2005), a pattern of parallel responses between blood and egg folate concentrations when FA was supplemented in the diet was established, indicating an efficient transport mechanism of absorbed FA in the blood and into the egg. It was also demonstrated that supplemental FA in the laying hen's diet is equally effective as dietary 5-methyltetrahydrofolate (5-methylTHF) in terms of enhancing egg folate concentrations (Tactacan et al., 2010), thereby dismissing any views of inadequate conversion of absorbed FA to 5-methylTHF, the major form of folate in the egg. However, the role of the intestine as a potential control point in regulating egg folate concentration has yet to be determined.

It is generally accepted that the rate of absorption in the intestine determines the availability of nutrients derived from the diet. With respect to folate absorption, its transportation in the intestine is believed to be mediated by a highly specific folate

transporter which transports folate optimally at acidic pH condition (Steinberg, 1984; Sirotnak and Tolner, 1999). Overall, there are three major folate transporters that may facilitate folate transport across the intestinal cell membrane. They are the folate receptor (FR), the reduced folate carrier (RFC), and the proton-coupled folate transporter (PCFT). An earlier study reported that FR is negligibly expressed in the small intestine (Steinberg et al., 1994), therefore its contribution to folate absorption in the intestine has never been considered in subsequent studies. Our group has investigated the mRNA expression of RFC and PCFT in the digestive tract of the laying hen and found that both transporters are widely expressed in the entire region of the small and large intestines (Jing et al., 2009; 2010). These studies were the first to confirm that both folate transporters are present in the intestine of the laying hen. However, whether their expression implies functional capability for absorption of dietary folate remains to be known. Because FA is the most commonly used supplemental dietary source of folate for egg fortification, an understanding of the underlying mechanisms by which it is absorbed may provide insights on how the intestine plays part in regulating egg folate concentrations. Therefore, a series of experiments was conducted to characterize the absorption of FA in the intestine of the laying hen using an *in vitro* absorption model of everted intestinal sacs developed by Inoue et al. (2008) in rats. The effects of naturally occurring folate derivatives (5 methylTHF and 10-formyltetrahydrofolate; 10-formylTHF) and heme on FA absorption were also determined.

5.3 MATERIALS AND METHODS

5.3.1 General

Shaver White laying hens (Manitoba Perfect Pullets, Rosenort, Manitoba) confined under semi-controlled environmental conditions were used in 6 experiments. A laying hen diet based on wheat and soybean meal (2900 kcal/kg, 19.0% CP, 4.0% calcium, 0.4% available phosphorus) and water were made available to provide *ad libitum* consumption. As basal dietary ingredients provided sufficient folate to meet the needs of the laying hen, no additional FA was included in the diets. Representative birds at 40-50 wks of age were selected for intestinal tissue isolation. The laying hens weighed (means ± SD) 1,498 ± 128.0, 1,545 ± 151.5, 1,706 ± 132.9, 1,655 ± 121.9, 1,602 ± 171.7, and 1,640 ± 132.1 g in Experiments 1, 2, 3, 4, 5, and 6, respectively.

5.3.2 Folic acid uptake in everted intestinal sacs

Folic acid uptake experiments were conducted using everted sacs from the small intestine and cecum of laying hens following the procedure of Inoue et al. (2008) with slight modifications. The experiments were conducted with the approval of the University of Manitoba's Animal Care Protocol Review Committee and in accordance with the Canadian Council on Animal Care (1984). In brief, hens were fasted overnight and were killed by cervical dislocation. From their abdominal cavity, duodenal, jejunal, ileal, and cecal segments were immediately isolated and rinsed free of digestive contents using oxygenated physiological saline. Intestinal segments of approximately 4 cm in length from each region were cut and everted by a glass rod and infused with previously oxygenated 1 mL Krebs-Ringer-bicarbonate buffer (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$) and added with 20 mM HEPES (pH 6.0), 10 mM glucose, and 15 mM sodium ascorbate using a tuberculin syringe. Each length of the everted segment was tied off in both ends by a thread ligature to form the intestinal sac.

Sacs were transferred to 50 mL conical flask and incubated in 20 mL Krebs-Ringer-bicarbonate buffer solution (mucosal medium) at 41^{o}C and a shaking rate of 100 strokes per min. Throughout the incubation period, the intestinal sac was continuously oxygenated with 95% O_2 - 5% CO_2 gas. Trace amounts of 100,000 cpm/50 µL of [^{3}H] FA (37 MBq per mL) (Moravek Biochemicals Inc., Brea, CA), 20,000 cpm/50 µL of [1,2-^{14}C]polyethylene glycol (PEG) 4000 (1.850 MBq per mL) (American Radiolabeled Chemicals, St. Louis, MO) as a non-absorbable marker, and also unlabeled FA (to adjust FA concentration) (Shircks Laboratories, Jona, Switzerland) were added to the mucosal medium. An intestinal sac was incubated without adding labeled FA in the mucosal medium for each measurement to deduct background FA uptake. At the end of incubation, sacs were removed in the conical flask and briefly washed with ice-cold saline to stop uptake. Sac content (serosal fluid) was drained into individual scintillation vials and counted for radioactivity. Uptake in the mucosal tissue of the sac was evaluated by determining the radioactivity after solubilisation of the tissue sample using 2 mL of Soluene-350 (Perkin Elmer Inc., Waltham, MA) as a tissue solubilizer. Mucosal to serosal uptake of FA which constitutes the overall transport processes (apical transport, intracellular processing, basolateral transport) of FA across the intestinal wall was calculated by adding uptake in the mucosal tissue and the serosal transfer (Smyth, 1974) and expressed per 100 g of wet tissue weight of intestinal segment.

5.3.3 Experimental approach

Experiment 1 was conducted to determine an optimum incubation time of everted gut sacs according to the change of FA uptake when time varied. The incubation time of 5 treatments were 1, 2, 5, 10, and 20 min, respectively. A FA concentration of 0.1 µM

and an acidic medium of pH 6.0 were used. The optimum incubation time (5 min) determined in Experiment 1 was adopted and used in the succeeding experiments. For Experiment 2, FA uptake at pH 6.0 was evaluated with increasing FA concentration (0.0001, 0.001, 0.01, 0.1, 1, 10, and 100 μM). To investigate the effect of pH on FA uptake at 0.1 μM FA, pH conditions of 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, and 7.5 were used as treatments in Experiment 3. For treatments with pH 5.5 and below, 20 mM 2-(*N*-morpholino)ethanesulfonic acid **(**MES) was used, while 20 mM HEPES was used for treatments with pH 6.0 and above. 1 N HCl or 1 N NaOH were used to adjust pH levels for each treatment. Experiment 4 was conducted to investigate FA uptake in the different segments of the intestine. Duodenal, jejunal, ileal, and cecal sections of the intestine were used to measure uptake at 0.1 μM FA concentration, pH 6.0, and 5 min incubation. Because the rate of FA uptake in Experiment 3 was found to be at maximum at acidic pH of 6.0, a transport mechanism requiring proton-gradient may facilitate movement of FA across the intestinal cell membrane (Qiu et al., 2007; Inoue et al., 2008). Therefore, to determine the effect of eliminating transmembrane proton-gradient on intestinal transport of FA, the presence of carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) (Sigma-Aldrich, Oakville, ON) an ionophore that dissipates H^+ gradient across the cell membrane was used in Experiment 5. FA uptake in the jejunal everted sac containing 0.1 μM FA was measured after incubation for 5 min at pH 6.0 in the presence of serial FCCP concentrations (0, 10, 20, 30, 40, and 50 μM). Lastly, Experiment 6 was designed to determine the effect of 5-methylTHF (6S-5-methyl-5, 6, 7, 8-tetrahydrofolic acid) (calcium salt) (Shircks Laboratories, Jona, Switzerland), 10-formylTHF (Shircks

Laboratories, Jona, Switzerland), and heme (Sigma-Aldrich, Oakville, ON) on FA transport in the jejunum. Uptake of FA at 0.1 μM was measured in the jejunal everted sac after incubation for 5 min at pH 6.0 and in the absence and presence of varied concentrations of 5-methylTHF, 10-formylTHF, and heme (0, 0.01, 0.1, 1, 10, 100 μM).

Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone and heme were pre-dissolved in dimethylsulfoxide (DMSO) (Sigma-Aldrich, Oakville, ON) as both compounds are poorly soluble in aqueous solutions. The final concentration of DMSO in the mucosal medium was 1%. To account for the amount of DMSO added, controls for FCCP and heme were run in the presence of the same concentration of DMSO without added inhibitors.

The jejunum was used in all uptake studies except when FA uptake in the other segments of the intestine was evaluated (Experiment 4). Each bird was used as a replicate of each treatment.

5.3.4 Cell viability

Samples of the intestinal sac used in the FA uptake study were subjected to a cell viability test using the trypan blue exclusion assay (Strober, 1997). The effect of incubation time, region of the intestine, and FCCP concentration on percentage cell viability were assessed. In brief, preparations of intestinal everted sacs were scraped with a glass slide to harvest mucosal cells. Fifty microliters of harvested mucosal cells were suspended in 1 mL Dulbecco Ca^{2+} and Mg^{2+} free Phosphate Buffered Saline solution (PBS) (Sigma-Aldrich, Oakville, ON) to obtain a cell suspension containing approximately 1 x 10^6 cells per mL. A 15 μL aliquot of the cell suspension was mixed with an equivalent volume of 0.4% trypan blue (Sigma-Aldrich, Oakville, ON) and

loaded in a Bright-Line hemocytometer (Sigma-Aldrich, Oakville, ON) for cell counting via the aid of a fluorescence microscope (Olympus, Japan). Viable cells were determined by their ability to exclude trypan blue. When viewed under the microscope, viable cells had a clear cytoplasm whereas nonviable cells possessed a blue cytoplasm.

5.3.5 Statistical analysis

Following a completely randomized design, all transport studies were the results of multiple separate determinations using everted sac preparations isolated from laying hens on different occasions. Intestinal sections from the laying hens were cut into small parts (~4 cm) (depending on the number of treatment for each experiment) and were assigned to each treatment in a random manner (Experiment 1 = 5 parts, N = 8 hens, n = 8 replication for each measurement; Experiment 2 = 7 parts, N = 10 hens, n = 10 replication for each measurement; Experiment 3 = 7 parts, N = 7 hens, n = 7 replication for each measurement; Experiment 4 = 5 parts; N = 6 hens for each intestinal section, n = 6 replication for each measurement; Experiment 5 = 6 parts, N = 8 hens for each inhibitor, n = 8 replication for each measurement; Experiment 6 = 6 parts, N = 8 hens; n = 8 replication for each measurement). Using the SAS software (SAS Institute, Cary, NC), data from experiments 1, 2, 3, 5, and 6 were analyzed using regression analysis, whereas data from experiment 4 and the cell viability studies were subjected to ANOVA using the PROC GLM procedure. Differences between means for Experiment 4 and the cell viability studies were determined using Tukey's Honestly Significance Difference. Data from Experiment 1, 2, and 6 were log-10 transformed before analysis. Data were expressed as means ± SEM in nmoles per 100 gram of wet tissue weight per min. Differences with an α level of $P < 0.05$ were considered to be statistically significant.

The apparent kinetic constants for FA transport (K_m, V_{max}) were determined by fitting initial transport rates to the Michaelis-Menten equation [$v = (V_{max}[S]) / (K_m + [S])$] using non-linear regression analysis (Prism 4.0, Graphpad Software, Inc., San Diego, CA).

5.4 RESULTS

The mucosal to serosal uptake of 0.1 μM FA as a function of time was examined at pH 6.0 in the jejunal everted sacs of the laying hens to determine the linear phase of uptake (initial uptake rate) (**Figure 5.1**). Uptake of 0.1 μM FA increased linearly ($P < 0.001$) from 1 to 5 min and proceeded slowly after 5 min of incubation. While mucosal to serosal uptake of FA appeared to proceed in a more linear fashion between 1-2 min than between 2-5 min, the 5 min incubation time was chosen as the optimum time of incubation to balance the need to have sufficient FA transport activity present in the jejunum to allow the measurement of inhibition by 5-methylTHF, 10-formylTHF, and heme on FA transport rates. Although mucosal to serosal uptake of FA was linear in the first 5 min of incubation, the overall change in tendencies for uptake of 0.1 μM FA with time was best fitted to a quadratic model [Y (mucosal to serosal uptake) = 0.6394 + 1.5183X (time) – $0.6702X^2$ ($P < 0.001$, $R^2 = 0.8452$)], showing a decreasing rate of mucosal to serosal uptake as incubation time is increased.

With respect to the influence of FA concentration, mucosal to serosal rates of FA uptake are given in **Figure 5.2**. The transport of FA in the jejunal mucosa increased ($P < 0.05$) in a linear proportion in relation to FA concentrations from 0.0001 to 0.1 μM. Above 0.1 μM, the slope of the regression line was not significantly different from zero

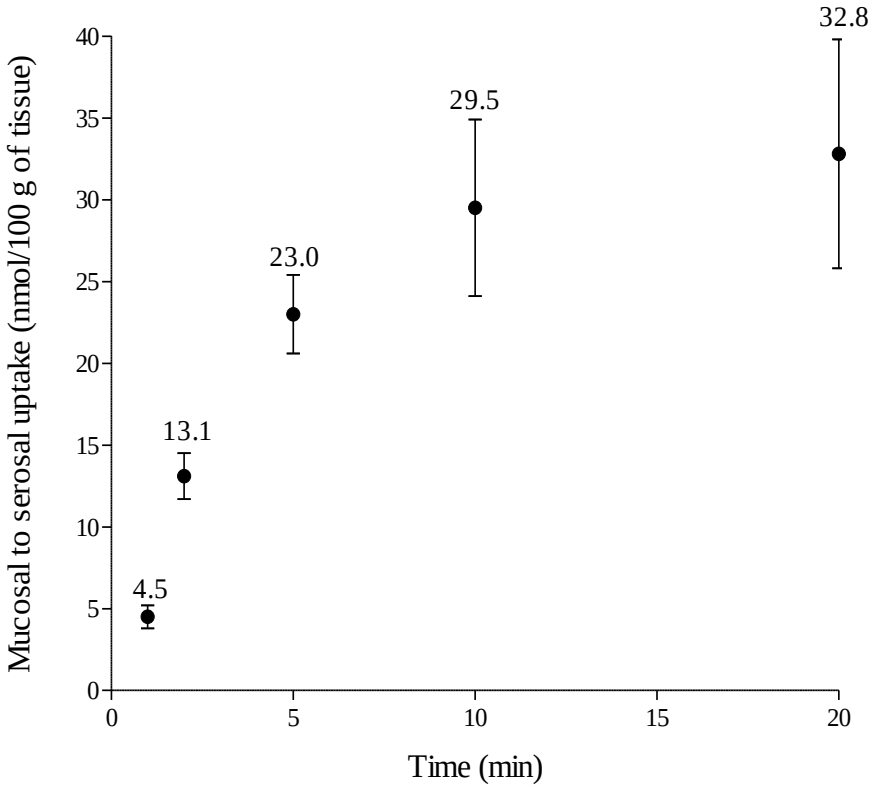

Figure 5.1. Time courses of the folic acid (FA) uptake in the everted sacs of the laying hens' jejunum (Experiment 1). Data represent means ± SEM (N = 8 hens, n = 8 replications for each measurement). The uptakes of 0.1 μM FA was evaluated at 41ºC and at pH 6.0 of the mucosal medium. Regression analysis of the data revealed a quadratic relationship between the mucosal to serosal uptake of 0.1 μM FA and time as described by the equation: [Y (mucosal to serosal uptake) = 0.6394 + 1.5183X (time) – $0.6702X^2$ ($P < 0.001$, $R^2 = 0.8452$).

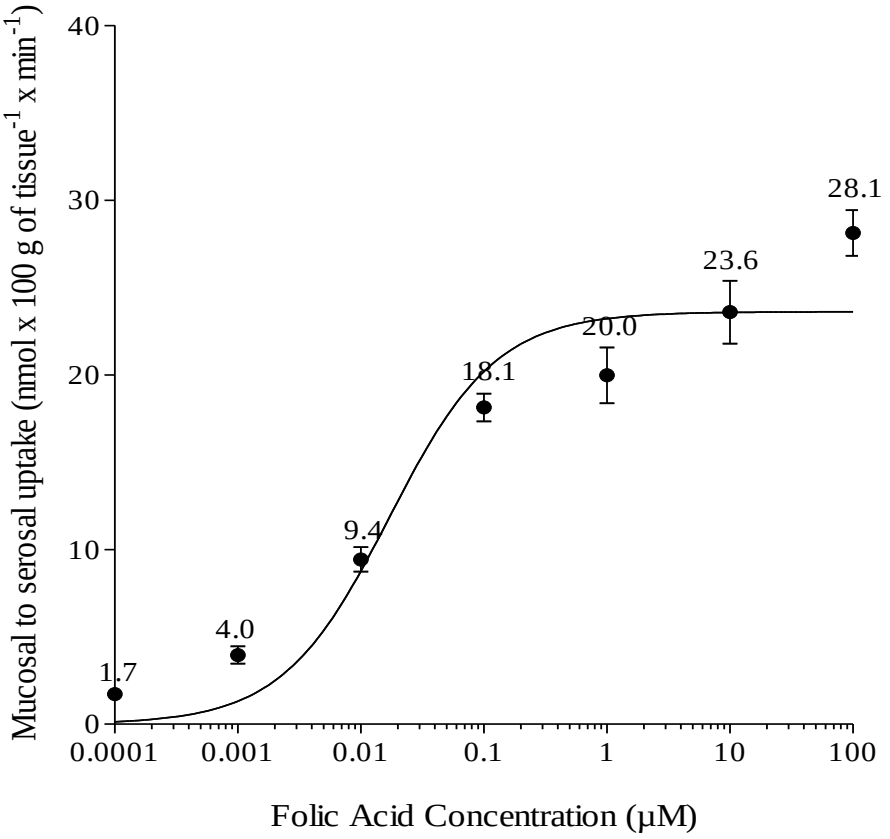

Figure 5.2. Concentration dependence of folic acid (FA) uptake in the everted sacs of the laying hens' jejunum (Experiment 2). Data represent means ± SEM (N = 10 hens, n = 10 replications for each measurement). The uptakes of FA were evaluated at 41ºC and at pH 6.0 of the mucosal medium for 5 min. The values of K_m (µM) and V_{max} (nmol x 100 g of tissue^{-1} x min^{-1}) ± SEM are 0.03 ± 0.004 and 22.7 ± 0.9, respectively, for FA after fitting with the Michaelis-Menten equation.

(P = 0.137). On the basis of these results, the trace concentration of 0.1 μM FA was chosen as the test substrate concentration for all the performed uptake experiments. The change in tendencies for mucosal to serosal uptake over the full range of FA concentrations was best fit to a quadratic model [Y (mucosal to serosal uptake) = 1.3153 + 0.1252X (concentration) – $0.0378X^2$ ($P < 0.001$, $R^2 = 0.9258$)], demonstrating a non linear response when FA concentrations in the mucosal medium was increased. With the evidence of a significant quadratic relationship, the data were also fit to the Michaelis-Menten equation, in order to calculate estimates of transport kinetics. The calculated K_m constant was 0.03 ± 0.004 μM, and the maximum velocity (V_{max}) was 22.7 ± 0.9, nmol x 100 g of $tissue^{-1}$ x min^{-1} ($R^2 = 0.9824$).

With respect to the effect of pH, the change in tendencies for mucosal to serosal uptake of FA at varying pH conditions was best fit to a quadratic model [Y (mucosal to serosal uptake) = -74.115 + 31.994X (pH) – $2.7483X^2$ ($P < 0.002$, $R^2 = 0.8198$)] (**Figure 5.3**). Therefore, by substituting the range of pH values to the best fitted model, we deduced that the mucosal to serosal uptake of 0.1 μM FA was maximum at pH 6.0. For this reason, succeeding transport assays were then carried out at this pH.

The distribution of FA transport along the intestine of the laying hen was increased in the upper half of the small intestine and decreased in the remaining lower half. Mucosal to serosal uptake of FA was highest in the jejunum, followed by the duodenum, and decreased *($P < 0.001$)* towards the ileum and cecum (**Figure 5.4**). Mucosal to serosal uptake of FA was also increased in the ileum *($P < 0.001$)* relative to cecum.

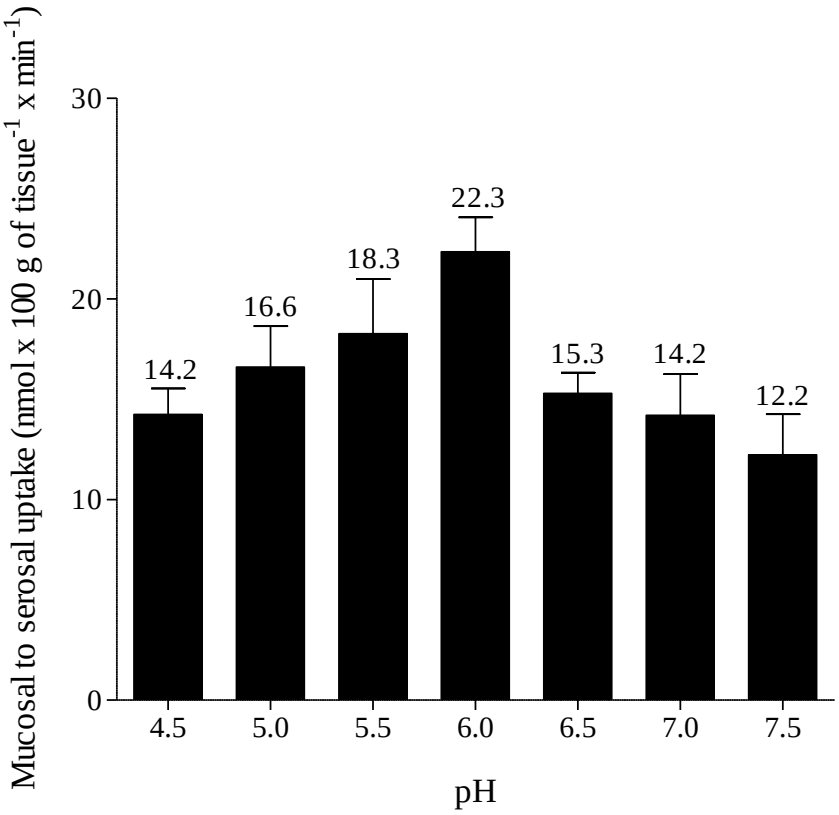

Figure 5.3. pH dependence of folic acid (FA) uptake in the everted sacs of the laying hens' jejunum (Experiment 3). Data represent means ± SEM (N = 7 hens, n = 7 replications for each measurement). The uptakes of 0.1 μM FA was evaluated at 41ºC for 5 min. The change in mucosal to serosal uptake of FA at varying pH conditions was described by the quadratic equation: [Y (mucosal to serosal uptake) = -74.115 + 31.994X (pH) – 2.7483X^2 ($P < 0.002$, $R^2 = 0.8198$).

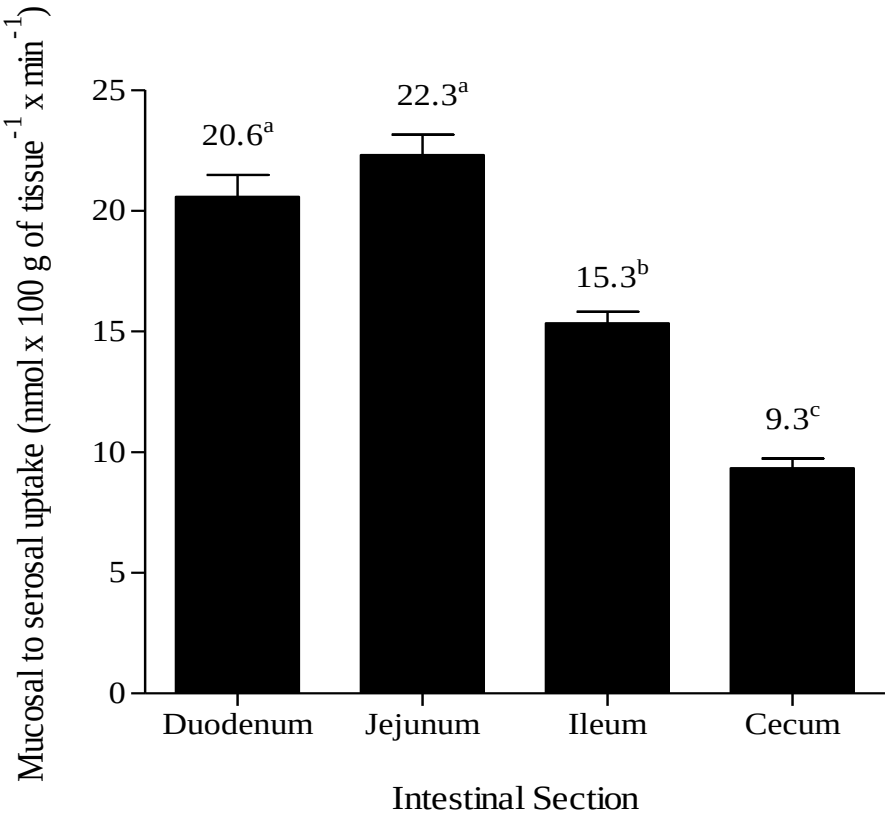

Figure 5.4. Uptakes of folic acid (FA) by everted sacs from various regions of the laying hens' small and large intestine (Experiment 4). Data represents means ± SEM (N = 6 hens, n = 6 replications for each measurement). The uptakes of 0.1 μM FA was evaluated at 41ºC and pH 6.0 for 5 min. [a-c]Data that do not share superscripts are significantly different ($P < 0.05$).

The addition of increasing concentrations of FCCP in the mucosal medium decreased the mucosal to serosal uptake of 0.1 μM FA in the jejunal everted sac of the laying hen. The extent of inhibition on mucosal to serosal uptake of FA was greatly manifested at higher FCCP concentrations (30, 40, and 50 μM FCCP) and best fit to a quadratic model [Y (mucosal to serosal uptake) = -2.1454 + 24.659X (FCCP concentration) $- 12.357X^2$ ($P < 0.05$, $R^2 = 0.6602$)] (**Figure 5.5**). Similarly, the mucosal to serosal transport of 0.1 μM FA in the jejunal everted sac of the laying hen was linearly decreased by the presence of 5-methylTHF ($P < 0.009$) and 10-formylTHF ($P < 0.05$); however, the mucosal to serosal transport of 0.1 μM FA was not significantly affected by heme ($P < 0.189$) (**Figure 5.6**). When inhibitors were added to the mucosal medium at 100 μM concentration, the percentage inhibition in the mucosal to serosal uptake of 0.1 μM FA was greatest with 5-methylTHF with a maximal decreased transport rate of 28% relative to control rates. The corresponding maximal decreased rates with 10-formylTHF and heme were 22.2% and 16.5%, respectively.

The trypan blue exclusion test was conducted to determine the viability of the intestinal everted sac preparations used in the uptake experiments. Isolated everted sacs taken from the section of the jejunum did not show differences in the percentage of viable cells when incubated for 0, 5, 10, and 20 min time periods. Percentage viability of the cells after 0, 5, 10 and 20 min incubation were 70.4, 68.7, 67.5, and 67.7%, respectively (**Figure 5.7**). Percentage viability of the cells in the jejunum (69.8%) and ileum (71.0%) section of the intestine were similar but increased *(*$P < 0.05$*)* when compared to the duodenum (62.1%) and cecum (63.2%) (**Figure 5.8**). Everted jejunal sacs incubated at 5

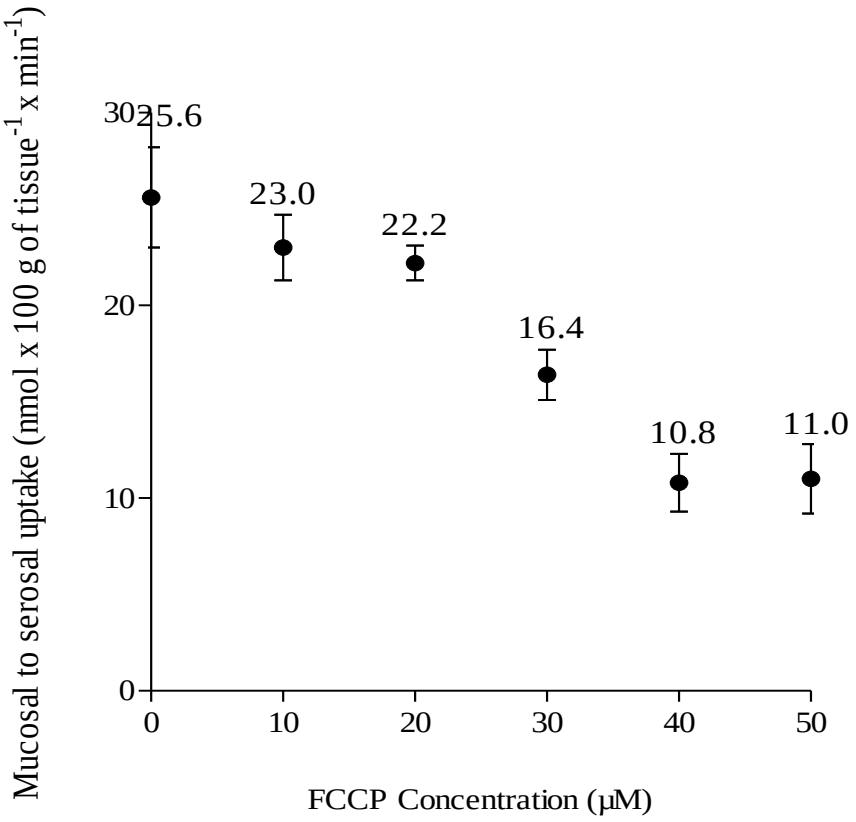

Figure 5.5. Effect of carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) on the uptake of folic acid (FA) in the everted sacs of the laying hens' jejunum (Experiment 5). Data represents means ± SEM (N = 8 hens, n = 8 replications for each measurement). The uptakes of 0.1 µM FA was evaluated in the presence and absence of FCCP at 41ºC and pH 6.0 for 5 min. Regression analysis of the data revealed a quadratic relationship between the mucosal to serosal uptake of 0.1 µM FA and FCCP concentration as described by the equation: [Y (mucosal to serosal uptake) = -2.1454 + 24.659X (FCCP concentration) – $12.357X^2$ ($P < 0.05$, $R^2 = 0.6602$)

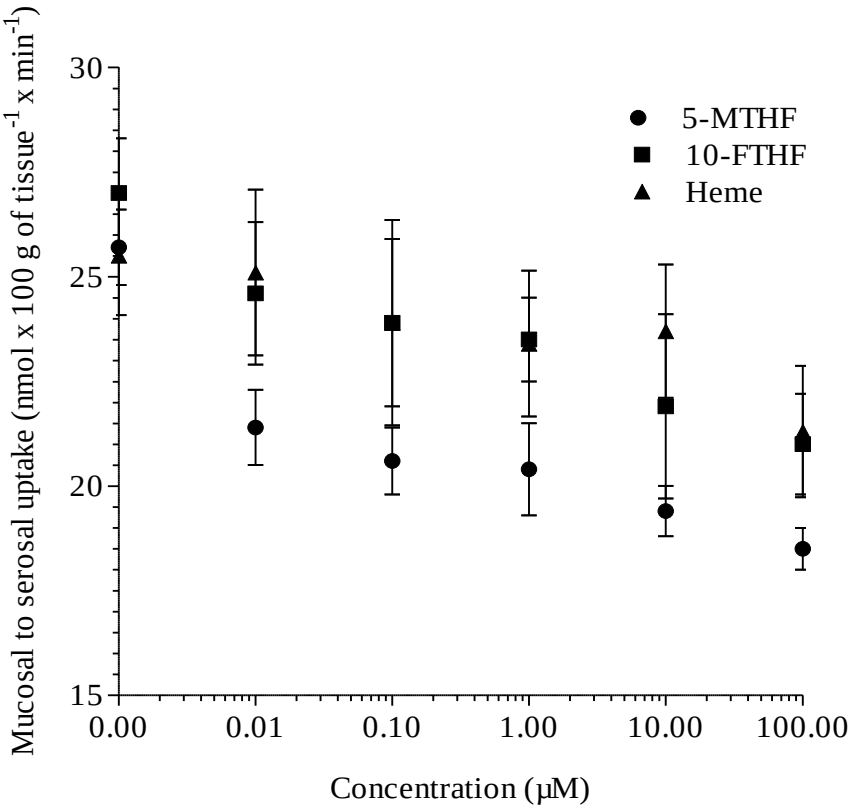

Figure 5.6. Effect of 5-methyltetrahydrofolate (5-methylTHF), 10-formyltetrahydrofolate (10-formylTHF), and heme on the uptake of FA in the everted sacs of the laying hens' jejunum (Experiment 6). Data represents means ± SEM (*n* = 8 replications for each measurement). The uptakes of 0.1 µM FA was evaluated in the presence and absence of 5-methylTHF, 10-formylTHF, and heme at 41ºC and pH 6.0 for 5 min. At 100 µM concentration of 5-methylTHF, 10-formylTHF, and heme in the mucosal medium, percentage inhibition in the mucosal to serosal uptake of 0.1 µM FA was 28%, 22.2%, and16.5%, respectively.

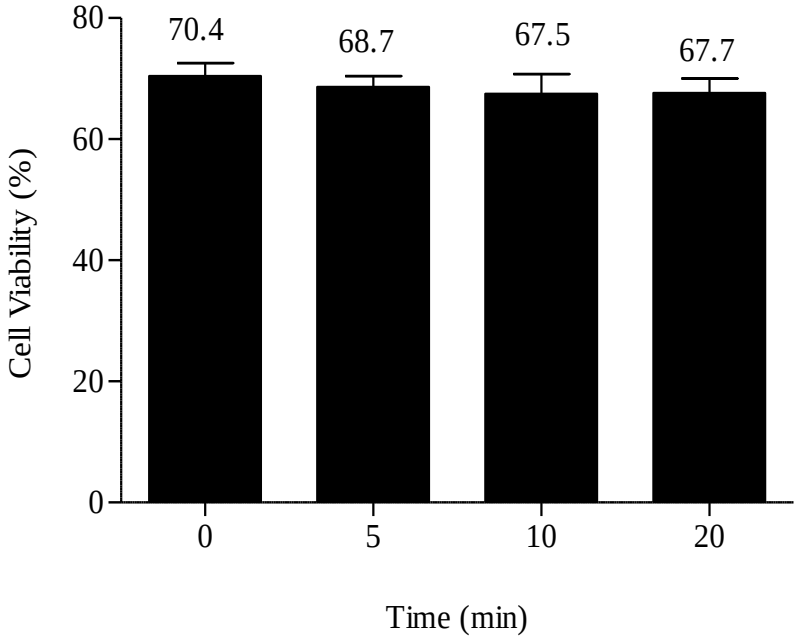

Figure 5.7. Percentage cell viability of the jejunal everted sac of the laying hen at different incubation time. Data represents means ± SE (*n* = 3). Cell viability was measured using the trypan blue assay.

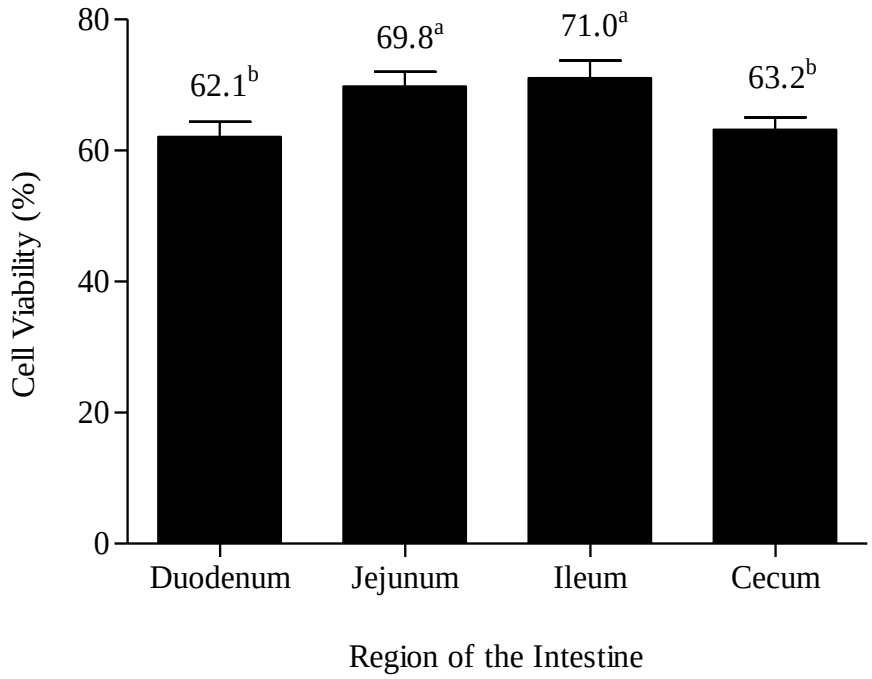

Figure 5.8. Percentage cell viability of the everted sac from different regions of the intestine of the laying hen. Data represents means ± SE (n = 3). Cell viability was measured using the trypan blue assay. [a-b]Data that do not share superscripts are significantly different ($P < 0.05$).

min in increasing concentrations of FCCP did not differ in the percentage of viable cells. Percentage viability of the cells at 0, 10, 20, 30, 40, and 50 µM FCCP were 70.1, 68.5, 70.3, 71.0, 68.5, and 67.4%, respectively (**Figure 5.9**).

5.5 DISCUSSION

The characteristics of FA transport in the intestine of the laying hen were studied and the influence of naturally occurring folate derivatives and heme on FA absorption was further investigated. The absorption of FA in the intestine is believed to be one of the major factors that affect the transfer of dietary FA into the egg. It has long been determined that folate is absorbed in the intestine by a specific carrier-mediated transport system (Steinberg, 1984; Sirotnak and Tolner, 1999); however, the identity of the putative folate transporter had not been established. We have recently determined the presence and expression patterns of RFC and PCFT in the digestive tract of the laying hens (Jing et al., 2009; 2010), however, functional studies had not been previously conducted. In the present study, everted intestinal sacs were chosen as an *in vitro* model to characterize intestinal absorption of FA in laying hens because they offered the opportunity of investigating the initial stages of FA absorption under closely controlled conditions. Ball (2006) has noted that folate absorption studies involving whole animal models are often difficult to interpret due to problems associated with the entero-hepatic circulation of folate via bile fluid and the complicating effect of changes in digesta transit time. The *in vitro* everted sac model system has been shown to be rapid and useful in predicting the trend of the absorptive response in intact animals (Hill et al., 1987).

As a function of FA concentrations, the rate of increase in mucosal to serosal uptake of FA in the jejunal section of the small intestine of the laying hen diminished at

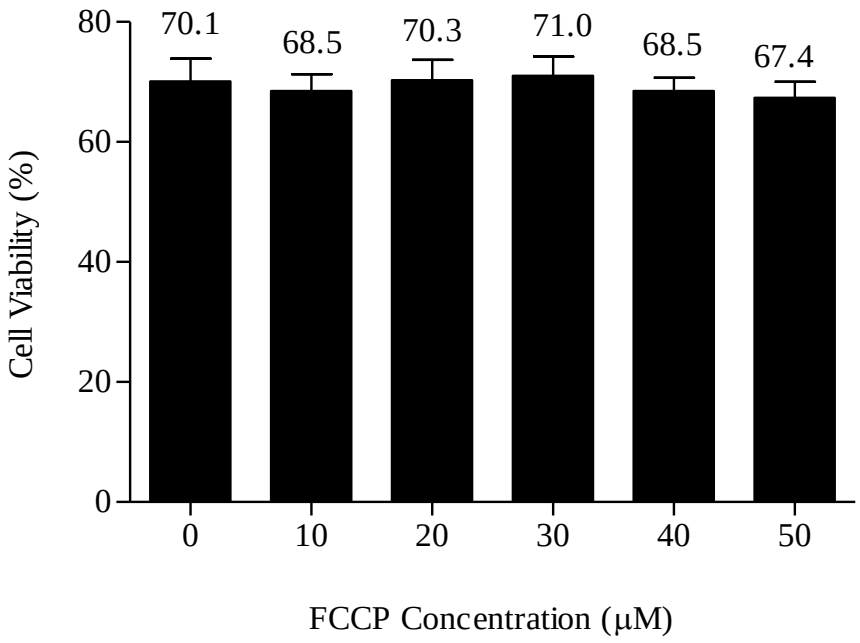

Figure 5.9. Percentage cell viability of the jejunal everted sac of the laying hen at different carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) concentrations. Data represents means ± SE (*n* = 3). Cell viability was measured using the trypan blue assay.

higher FA concentrations (> 0.1 µM). This pattern is reminiscent of the response demonstrated by laying hens for egg folate concentration when dietary FA supplementation increased (House et al., 2002; Hebert et al., 2005). It was previously reported by Hebert et al. (2005) that increasing supplementation of FA at 2, 4, 8, 16, 32, 64 or 128 mg per kg of laying hens diet did not lead to a consistent increase in egg folate concentration. They noted that while the amount of folate in egg was dramatically enriched at lower supplementation rates (2-4 mg FA per kg of the diet); higher FA supplementation did not result to proportional folate enrichment, instead, it demonstrated features of saturation. Therefore, on the basis of these results, we surmise that the observed saturation in egg folate concentration after increased dietary supplementation of FA may be partly attributed to limitations in FA absorption in the intestine. At increased FA concentration in the mucosal medium, the maximum capacity of the intestinal folate transporter for FA uptake might have been achieved. This observation is in line with our earlier findings which demonstrated that neither the metabolism of dietary derived FA into its biologically active form of folate, the 5-methylTHF (Tactacan et al., 2010), nor the capacity for transfer of circulating blood 5-methylTHF into the egg (House et al., 2002; Hebert et al., 2005) are restricting the capacity of the laying hens for deposition of folate into the egg.

Folate transport activity with optimal function at acidic pH was first identified for intestinal folate absorption (Mason and Rosenberg, 1994). Optimal transport at acidic condition is a distinct feature of intestinal folate transporter which separates it from other cellular folate transporters (Said, 2004). In the present study, the mucosal to serosal uptake of FA as a function of pH appeared to involve a pH-dependent transport process,

demonstrating maximum transport at the acidic condition of pH 6.0. The presence of FCCP in the mucosal medium, a dissipater of H^+ gradients, confirmed this observation. With increasing concentration of FCCP in the intestinal mucosa, the mucosal to serosal uptake of FA was extensively reduced. Therefore, the transport of FA across the intestinal wall of the laying hen may involve a H^+-coupled transport process. These results are consistent with those observed in other species (Qiu et al., 2007; Inoue et al., 2008), and may provide evidence to suggest that proton-coupled folate transporter, which utilized H^+ gradient to transport folate, plays a role in the intestinal absorption of FA in the laying hen.

Proton-coupled folate transporter and RFC are expressed along the entire intestinal tract of the laying hen (Jing et al., 2009; 2010) and rat (Inoue et al., 2008). In the present study, transport of FA was also observed in the duodenal, jejunal, ileal, and cecal section of the chicken intestine. The distribution profile for FA transport activity was highest in the jejunum and duodenum and decreased as it approaches the ileum and cecum. Previous studies have also reported the existence of higher transport activity for FA in the upper half and lower transport activity for FA in the lower portion of the small intestine (Qiu et al., 2006; Inoue et al., 2008). Folate transport with optimum activity at the proximal portion of the small intestine is consistent with the acidic pH in the micro-environment of the absorptive surface of the upper small intestine (Said et al., 1987; McEwan et al., 1990; Ikuma et al., 1996) while the lower transport activity in the ileum and cecum is consistent with the more alkaline condition in this region of the intestine (Engberg et al., 2002). The presence of transport activity of FA in the ceca, coupled with our previous demonstration of the presence of mRNA for both RFC and PCFT in the ceca

(Jing et al., 2009; 2010) provide evidence that folate supplied by commensal microorganisms in the ceca may contribute to the folate status of the laying hen. The role of endogenous folate supply in contributing to the folate status of the host has been proposed by others (Rong et al., 1991; Asrar and O'Connor, 2005). Gut microflora can synthesize considerable amounts of folate, and a significant portion of this folate exists in the lumen in the absorbable monoglutamate form (5-methylTHF) (Kim et al., 2004). The current demonstration of a measurable uptake of folate from cecal everted sacs has the potential to make significant contributions to our understanding of the extent to which nutrients derived from microbes contribute to daily requirements of the laying hen.

Beyond its low pH optimum, another distinguishing feature of intestinal folate transport is its demonstrated affinity not only for FA but also for the natural folate derivatives (Zhao et al., 2007). Because transport is non-specific, existence of different folate compounds in the lumen may result in potential interactions during cellular transport and absorption in the intestine. In the present study, the absorption of FA was impeded by the presence of 5-methylTHF and 10-formylTHF in the mucosal medium. 5-methyltetrahydrofolate was a more potent inhibitor of FA than 10-formylTHF. Another compound heme, a dietary source of iron, was investigated because PCFT was originally identified as a heme carrier protein (Shayeghi et al., 2005). However, the results of the uptake study provided evidence that folate transporters exhibited a very low affinity for heme, as compared to the two derivatives of folate. This result is physiologically relevant considering that these micronutrients may negatively influence the absorption of dietary FA and its eventual transfer to tissues, including the egg. Both 5-methylTHF and 10-formlyTHF are the two most predominant folate derivatives found in plant-based feed

ingredients (Bagley and Shane, 2005) while heme is found in significant amount in diets containing animal-based ingredients, in particular meat and bone meal.

In summary, the mucosal to serosal uptake of FA in the jejunal everted sac of the laying hen has been characterized. The rate of increase in mucosal to serosal uptake of FA in the jejunal section of the small intestine of the laying hen diminished at higher FA concentrations. The transport of FA demonstrated maximum activity at acidic pH but was markedly reduced when H^+ gradients were eliminated. This may indicate an H^+-coupled transport process, a feature consistent with the functional characteristic of the PCFT. Folic acid was absorbed in all regions of the intestine showing greatest transport activity in the upper half and lowest transport activity in the lower half of the small intestine. Although of lesser capacity, the absorption of FA in the ceca raises the possibility that bacterially-derived folate may serve as an alternative source of exogenous folate for the chicken. Natural folate derivatives like 5-methylTHF and 10-formlyTHF, as well as a molecular compound like heme, impeded FA absorption thus potentially reducing its metabolic availability to tissues.

CHAPTER 6

MANUSCRIPT 3

The regulation of intestinal folic acid absorption in the laying hen supplemented with increased levels of dietary folic acid

6.1 ABSTRACT

Different aspects of dietary folic acid (FA) absorption in the intestine of the laying hen have been previously characterized. Much less is known however about the regulation of this process. To this end, a study was conducted to evaluate the effect of increased dietary folic acid (FA) supplementation on the regulation of intestinal FA absorption and the gene expression of the intestinal folate transporters, the proton coupled folate transporters (PCFT) and the reduced folate carrier (RFC) in the laying hen. The production performance and indices of folate status were also investigated. Twenty-four Shaver White hens at 34 wk of age were randomly assigned to receive 1 of 3 dietary treatments: 1) basal diet with no supplemental folate (n = 8), 2) basal diet + 10 mg/kg of crystalline FA (n = 8), and 3) basal diet + 100 mg/kg crystalline FA (n = 8). A completely randomized design with 3 dietary treatments was used. Data were subjected to PROC GLM, *t*-test, and correlation procedures of SAS. The effect of FA supplementation (10 or 100 mg FA/kg of diet) was not reflected in the production performance, but was manifested in the indices of folate status of the laying hen. Relative to the control-fed birds, egg and plasma folate concentrations increased (P < 0.001), while plasma homocysteine concentration decreased (P < 0.011) in birds fed with 10 or 100 mg FA/kg diet. There were no significant differences in the indices of folate status in birds supplemented with dietary FA. With respect to FA absorption, the mucosal to serosal uptake of FA in the duodenum was down-regulated (P < 0.002), but the mRNA levels of the duodenal PCFT and RFC genes were not affected when birds were fed with 10 or 100 mg FA/kg diet. In the jejunum, the mucosal to serosal uptake of FA as well as the mRNA levels of the PCFT and RFC genes were not influenced by increased FA supplementation.

Overall, these data demonstrate that supplementation of increased levels of dietary FA resulted in down-regulation of FA absorption in the duodenum, but not in the jejunum of the laying hen. This down-regulation was not associated with decreased mRNA expressions of the duodenal PCFT and RFC genes. Therefore, a post-transcriptional or translational regulation of the intestinal folate transporters may be involved in the down-regulation of duodenal FA absorption during increased supplementation of dietary FA.

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Keywords: folic acid, reduced folate carrier, proton-coupled folate transporter, gene expression, laying hens

6.2 INTRODUCTION

Certain intestinal nutrient transporters are known to be adaptively regulated by their own substrates (Karassov and Diamond, 1987; Ferraris and Diamond, 1989; Said and Khan, 1993). In response to substrate concentration, the number of nutrient transporters in the intestinal membrane may increase or decrease as appropriate. For example, dietary deficiency of biotin and riboflavin has been shown to lead to up-regulation in intestinal uptake of these vitamins (Ferraris and Diamond, 1989; Said and Khan, 1993), whereas a decrease in D-glucose and amino acid intake leads to down-regulation in their intestinal uptake (Karassov and Diamond, 1987).

With respect to folate transport, its movement across the cell membrane of the intestinal epithelium is also regulated by the level of folate concentration in the diet. Dev et al. (2010) demonstrated that intestinal uptake of folic acid (FA) was down-regulated in the rat brush-border membrane vesicles when maintained under the condition of folate over-supplementation (20 mg FA/kg diet). This was accompanied by a significant decrease in the protein, but not in the mRNA levels of the rat reduced folate carrier (RFC) and the proton-coupled folate transporter (PCFT), two major transporters of folate in the intestine. Similarly, Jing et al. (2009; 2010) did not find significant differences in the mRNA levels of the PCFT and RFC genes in the small intestine of the laying hens supplemented with an increased level of dietary FA (0 vs. 10 mg FA/kg of the diet). Meanwhile, dietary folate deficiency leads to a significant upregulation in intestinal FA transport (Said et al., 2000). When rats were fed a folate deficient diet, an upregulation in transepithelial FA transport coupled by a marked increase in the mRNA and protein

levels of RFC were observed (Said et al., 2000). Additionally, Qiu et al. (2007) reported that PCFT mRNA levels increased in the proximal small intestine of mice when fed a folate-deficient diet versus a folate-replete diet.

In laying hens, an *in vitro* everted sac study conducted to characterize the transport of FA in the intestine demonstrated that the rate of FA transport in the jejunal everted sac was influenced by the level of mucosal FA concentration (Tactacan et al., 2011). The movement of FA across the intestinal mucosa occurred at a faster rate at lower FA concentrations but started to diminish at higher FA concentrations. In folate-enriched eggs, the efficiency of folate incorporation in the egg also diminished when FA levels in the diet were increased (House et al., 2002; Hebert et al., 2005). Therefore, taken together, it appears that the rate of dietary FA absorption in the small intestine of the laying hen determines its bioavailability for deposition in the egg. However, relative to our current understanding of the mechanisms involved in the absorption of FA in the laying hen intestine, very little is known about its regulation. Given the interest in determining the factors regulating the transfer of dietary FA into the egg, this study was conducted to determine the effect of increased dietary FA supplementation on the molecular and functional characteristics of dietary FA absorption in the small intestine of the laying hen.

6.3 MATERIALS AND METHODS

6.3.1 General

Shaver White laying hens (Manitoba Perfect Pullets, Rosenort, Manitoba) were kept in confinement housing under semi-controlled environmental conditions and were exposed to a 16-h photoperiod. Twenty-four birds were housed individually; the cage

dimensions were 25.4 cm x 40.6 cm, providing 1,032 cm^2 per bird. Feed and water were available to permit *ad libitum* consumption. 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).

6.3.2 Diets

The wheat-based basal diet was formulated to meet the recommendations for laying hens consuming 100 g of feed per day (NRC, 1994; **Table 6.1**). The diet was analyzed in duplicate to determine the CP, total P, and Ca concentrations. Nitrogen for CP analysis was measured using a nitrogen analyzer (NS-2000, Leco Corporation, St. Joseph, MI), whereas samples for calcium and total phosphorus analyses were prepared using the AOAC (1990) procedures (method 990.08) and were analyzed using an inductively coupled plasma mass spectrometer (Varian Inc., Palo Alto, CA). The NRC (1994) reports 0.25 mg FA/kg of diet as the requirement for laying hens. The basal diet included no crystalline FA or commercially produced 5-methyltetrahydrofolate (5-methylTHF), a practice consistent with industry standards (BASF, 2000). Diets were offered daily, and the test diets were stored in the dark at 4^oC during the course of the trial.

6.3.3 Experimental approach

For 2 wk before the commencement of the study, 48 healthy Shaver White hens (32 wk of age) were monitored for egg production, and the 24 highest-producing hens (percentage hen-day egg production of 94.6 ± 3.72) were selected for the experiment. Percentage hen-day egg production and egg weight of the selected hens were not

Table 6.1. Composition of the basal wheat-based laying hen diet.

Item	Amount
Ingredients	
Wheat (16.1% CP)	59.00
Soybean meal (45.1% CP)	20.80
Limestone (38% Ca)	10.00
Vegetable oil (8800 kcal/kg ME)	6.80
Monocalcium phosphate	1.70
Vitamin premix	1.00
Mineral premix	0.50
DL-Methionine	0.18
Antioxidant	0.02
Nutrient composition	
CP, % (calculated)	19.00
CP, % (analyzed)	18.90
ME, kcal/kg	2,950.00
Ca, % (calculated)	4.20
Ca, % (analyzed)	4.44
Available phosphorus, % (calculated)	0.46
Total phosphorus, % (analyzed)	0.72
Lysine, % (calculated)	0.88
Methionine, % (calculated)	0.47
Methionine + cysteine, % (calculated)	0.85
Folate, mg/kg (analysed)	5.01

[1]Provided per kilogram of diet: 11000 IU of vitamin A, 3000 IU of vitamin D_3, 20 IU of vitamin E, 3 mg of vitamin K_3 (as menadione), 0.02 mg of vitamin B_{12}, 6.5 mg of riboflavin, 10 mg of calcium pantothenate, 40.1 mg of niacin, 0.2 mg of biotin, 10 mg of niacin, 2.2 mg of thiamine, 4.5 mg of pyridoxine, 1000 mg of choline, and 125 mg of ethoxyquin (antioxidant).

[2]Provided per kilogram of diet: 66 mg of Mn (as manganese oxide); 70 mg of Zn (as zinc oxide); 80 mg of Fe (as ferrous sulfate); 10 mg of Cu (as copper sulfate); 0.3 mg of Se (as sodium selenite); 0.4 mg I (as calcium iodate), and 0.67 mg of iodized salt.

significantly different before the start of the feeding period. At 34 wk of age, the selected hens were placed individually into battery cages and were randomly assigned to receive 1 of 3 dietary treatments: 1) basal diet with no supplemental folate (n = 8), 2) basal diet + 10 mg/kg crystalline FA (Shircks Laboratories, Jona, Switzerland; n = 8), and 3) basal diet + 100 mg/kg crystalline FA (n = 8). The diets were fed for a 7-d adjustment period followed by a 21-d collection period. All birds were weighed individually at the start and the end of the 28-d experiment and feed consumption for each cage unit was measured for average daily feed intake and feed efficiency calculations. Feed efficiency was calculated as grams of feed per gram of egg mass produced. Egg production was recorded daily and calculated as percentage hen-day egg production. All eggs laid from d 1 to d 21 of the collection period were weighed to give an average egg weight and were processed for egg folate determination.

Blood samples were collected daily from d 14 to d 21 of the collection period. A 2-mL blood was taken from each bird via wing venipuncture using a 3-mL syringe with a 23-gauge needle. The collected blood was transferred to a 2-mL sterile syringe containing 50 µL of porcine heparin saline solution (68.6 USP units) and was placed on ice to cool. Plasma was separated from the heparinized blood by centrifugation at 12,000 x g for 5 min. After the blood collection, one bird from each treatment was killed by cervical dislocation so that three birds were killed per d for 8 days. Duodenal and jejunal segments from each slaughtered birds were isolated immediately for use in the FA uptake study. Small portions of duodenal, jejunal, and cecal samples were rinsed with ice-cold PBS, and frozen as aliquots in liquid nitrogen. Plasma, duodenal, jejunal, and cecal tissue samples were stored at -80^{o}C until analysis.

6.3.4 Analysis of dietary folate content

The analysis of the basal and folate-supplemented diets was performed by Medallion Labs (Medallion Labs, Plymouth Avenue, MN). In brief, feed samples were homogenized with a 50 m*M* CHES-HEPES buffer with 2% ascorbic acid and 0.2 *M* 2-mercaptoethanol (pH 7.8) and stored at -80°C until analysis. A homogenized sample was weighed and an internal standard of isotopic FA analog (^{13}C-FA) and phosphate buffer were added. Amylase and protease enzymes were used to break down the matrix for extraction of the folates. Rat plasma conjugase was used to deconjugate the polyglutamate forms of folates to monoglutamates. The extract was cleaned using solid phase extraction (SPE) and the cleaned extract was analyzed by Ultra-high Performance Liquid Chromatography-Tandem Mass Spectrometry (UPLC-MS/MS). Individual vitamers of folates were quantified and the total folates determined were expressed as FA.

6.3.5 Extraction and analysis of egg yolk folate content

The extraction and analysis of the egg yolk folate content was performed as described previously (House et al., 2002). In brief, eggs were weighed, placed in boiling water for 10 min, cooled, and the yolks were separated, weighed, and retained for analysis by storing at -80°C. Previous research has documented that more than 95% of the folate in egg is located in the yolk (Sherwood et al., 1993), and we have confirmed this from our previous study (House et al., 2002). Egg folate in the form of 5-methylTHF, the major form of folate in eggs (Seyoum and Selhub, 1998), was extracted into an ascorbate buffer (pH 7.8). The extracts were analyzed for 5-methylTHF via reverse-phase HPLC with fluorescence detection, using the method of Vahteristo et al. (1997). An

external standard curve with purified 5-methylTHF was used to quantify egg folate concentrations. The inter- and intra-assay CV for determinations was less than 2%, and recovery of 5-methylTHF added to egg yolk was 99%.

6.3.6 Analysis of plasma folate and plasma homocysteine

Plasma folate concentrations were determined through the use of a competitive binding assay, SimulTRAC-S Radioassay Vitamin B_{12}[^{57}Co]/Folate [^{125}I] purchased from MP Biomedical (catalog no. 06B254932), according to the manufacturer's recommended protocol. Plasma homocysteine was determined by reverse-phase HPLC with fluorescence detection, using the method of Araki and Sako (1987), as modified by Gilfix et al. (1997).

6.3.7 Folic acid uptake in everted intestinal sacs

Folic acid uptake experiments were conducted using everted sacs from the small intestine of laying hens following the same procedure as described previously (Tactacan et al., 2011). In brief, chickens were killed by cervical dislocation. From their abdominal cavity, duodenal and jejunal segments were immediately isolated and rinsed free of digestive contents using oxygenated physiological saline. Intestinal segments of approximately 4 cm in length from each region were cut and everted by a glass rod. The test solution was prepared in Krebs-Ringer-bicarbonate buffer (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$) added with 20 mM HEPES (pH 6.0), 10 mM glucose, 15 mM sodium ascorbate, and oxygenated with 95% O_2 – 5% CO_2 gas. To the test solution trace amounts of [^{3}H] FA (37 MBq per mL) (Moravek Biochemicals Inc., Brea, CA), [1,2-^{14}C]polyethylene glycol (PEG) 4000 (1.850 MBq per mL) (American Radiolabeled Chemicals, St. Louis, MO) as a non-absorbable marker,

and also unlabeled FA (Shircks Laboratories, Jona, Switzerland) to adjust FA concentration to 0.1 μM, the optimum substrate concentration for FA transport obtained from our previous study (Tactacan et al., 2011). The everted sacs were incubated for 5 min which is within the initial linear rate of uptake (Tactacan et al., 2011) in 20 mL of a test solution at a temperature of 41^{o}C and a shaking rate of 100 strokes per min. Uptake was stopped by rinsing the everted sac briefly in ice-cold saline. Sac content (serosal fluid) was drained into individual scintillation vials and counted for radioactivity. Uptake in the mucosal tissue of the sac was evaluated by determining the radioactivity after solubilisation of the tissue sample using 2 mL of Soluene-350 (Perkin Elmer Inc., Waltham, MA) as a tissue solubilizer. Mucosal to serosal uptake of FA which constitutes the overall transport processes (apical transport, intracellular processing, basolateral transport) of FA across the intestinal wall was calculated by adding uptake in the mucosal tissue and the serosal transfer (Smyth, 1974) and expressed per 100 g of wet tissue weight of intestinal segment.

6.3.8 RNA isolation

Isolation of RNA from intestinal tissue samples was performed with the RNeasy Mini Kit (Qiagen Canada, Mississauga, ON, Canada) as described previously (Jing et al, 2009; 2010). In brief, a total of 20-30 mg of frozen intestinal tissue samples were thawed at room temperature and homogenized for 1 min in 600 μL of Buffer RLT (Qiagen Canada, Mississauga, ON, Canada) (lysis reagent). The homogenates were transferred to 1.5 mL tubes to which 200 μL of chloroform were added. After the addition of chloroform, the homogenate was separated into aqueous and organic phases by centrifugation. The RNA-containing the aqueous phase was extracted and ethanol was

added to provide appropriate binding conditions. The samples were applied to a silica membrane and after several washes, RNA was eluted in RNase-free water. Total RNA concentrations were measured at 260 nm on a DU800 Spectrophotometer (Beckman Coulter Canada Inc., Mississuage, ON, Canada), and the absorbance ratio at wavelength 260/280 nm was within 1.7-2.1. The RNA preparations were then treated with DNA digestion through the use of a TURBO DNA-free^{TM} Kit (Applied Biosystems Inc., Foster City, CA, USA), in order to get rid of DNA contamination in the RNA samples.

6.3.9 Reverse transcription and real-time PCR analysis

Using the SuperScript Vilo cDNA synthesis kit (Invitrogen Canada Inc., Burlington, ON, Canada), 1 μg of total RNA from the intestinal tissue samples were reversely transcribed into cDNA. The RT conditions for each cDNA synthesis were 25^{o}C for 10 min, 42^{o}C for 90 min, and 85^{o}C for 5 min. The resulting cDNAs were used as the template for PCR expression analysis.

As described previously (Jing et al., 2009; 2010), the primer sequences used in the real-time were as follows: PCFT (amplicon length: 153 bp) sense primer, 5'-GGCTGTGCTCACTTGTGGCTA-3'; PCFT antisense primer, 5'-GAAGGTTGGAGTCCTGGATTTCTAT-3'; RFC (amplicon length: 103 bp) sense primer, 5′-CGGGGCTGCTGCTATTCAT-3′; RFC antisense primer, 5′ ATAGCGATGGGAACCAGAAACT-3′; β-actin (amplicon length: 205 bp) sense primer, 5'-CAACACAGTGCTGTCTGGTGGTA-3'; β-actin antisense primer, 5'-ATCGTACTCCTGCTTGCTGATCC-3'. The specificity of the primers was tested by performing a BLASTN search against the genomic National Center for Biotechnology Information database.

The gene expression was quantified by SYBR green real time PCR as described previously (Jing et al., 2009; 2010). The qRT-PCR was performed using Fast SYBR Green Master Mix (Applied Biosystems Inc., Foster, CA, USA), and SYBR green was used as the detection dye. The cycling conditions were as follows: 20 sec at 95^{o}C, 40 cycles of denaturation at 95^{o}C for 3 sec, and combined annealing and extension at respective annealing temperature (60^{o}C for both PCFT and RFC; 62^{o}C for β-actin) for 30 sec, then followed by one three-segment cycle of product melting (95^{o}C/15 sec, 60^{o}C/1 min, 95^{o}C/15 sec). Dissociation curves confirmed the specific amplification of the PCFT, RFC, and β-actin cDNA and the absence of nonspecific products. Real-time PCR was performed in triplicate in 20-μL reactions on a STEP ONE Real-Time PCR System (Applied Biosystem Inc., Foster, CA, USA). β-actin was used as a reference control to normalize quantification of the PCFT and RFC mRNA. A sample without the cDNA template was used to verify that the master mix was free from contaminants.

The mRNA expression of target genes (PCFT, RFC and β-actin) was calculated with the comparative threshold cycle (*C*t) method. The difference between *C*t values was calculated for each mRNA by taking the mean *C*t of triplicate reactions and subtracting the mean *C*t to the reference mRNA (β-actin mRNA) (Δ $Ct=Ct_{\text{target gene}}-Ct_{\beta\text{-actin}}$). All treated samples were then normalized to the ΔCt value of a calibrator sample to obtain a $\Delta\Delta Ct$ value ($\Delta Ct_{Test}-\Delta Ct_{Calibrator}$). In the present study, the basal diet was chosen as the calibrator sample in order to evaluate the differential mRNA expression of target genes during FA supplementation. Fold differences in the mRNA expression of the treated samples relative to the calibrator sample is calculated by $2^{-\Delta\Delta Ct}$, which is usually

expressed as a range due to the result of incorporating the standard deviation of the ΔΔ*C*t value (ΔΔ*C*t ± SD). Therefore, using the $2^{-\Delta\Delta Ct}$ method, data are presented as the fold-change in gene expression of the treated samples normalized to the endogenous reference gene (β-actin) and relative to the calibrator sample.

6.3.10 Statistical analysis

A completely randomized design with 3 dietary treatments was used. Production performance, indices of folate status, and FA uptake data were subjected to ANOVA using the PROC GLM procedure of SAS software. Data are presented as least squares means ± SEM, with differences between means determined using Tukey's Honestly Significance Difference. mRNA gene expression data were subjected to *t*-test procedure (SAS Institute, Cary, NC). Correlations between the mucosal to serosal uptake of FA and the mRNA gene expression of the intestinal folate transporters at increased levels of dietary FA supplementation were evaluated by Pearson correlation procedures. Differences with an α level of $P < 0.05$ were considered to be statistically significant.

6.4 RESULTS

The results from the feed folate analysis confirm the actual folate content of the 2 folate supplemented diets (10 mg FA/kg = 11.8 mg FA/kg; 100 mg FA/kg = 91.2 mg FA/kg). However, the analyzed FA content of the basal diet (5.01 mg FA/kg) was greater than what we have expected (1.5 mg FA/kg) from our previous studies. Nevertheless, the difference in the FA levels between the basal and the FA supplemented diets were still substantial enough to elicit significant response in the egg folate concentration and indices of folate status in the laying hens (as discussed in the following section).

The effect of supplementing 10 or 100 mg FA/kg of basal laying hen diet was not reflected in the production performance of the laying hen, but was manifested in the egg folate concentration and their indices of folate status (**Table 6.2**). Percentage hen-day egg production, feed consumption, egg weight, feed efficiency, initial, and final bodyweights were similar in the laying hen fed the 3 dietary treatments. However, the egg and plasma folate concentrations of birds fed 10 or 100 mg FA supplemented diets increased ($P < 0.001$) compared to birds fed the control diet. In contrast, plasma homocysteine concentration of birds fed the FA supplemented diets decreased ($P < 0.011$) relative to birds fed the control diet. There were no significant differences in the indices of folate status in birds supplemented with 10 or 100 mg FA/kg of diet.

With respect to measures of FA absorption, the mucosal to serosal uptake of FA was decreased ($P < 0.002$) in the duodenum of the laying hen when 10 or 100 mg FA were supplemented in the basal diet at a per kg basis (**Figure 6.1**). This decrease was not associated with down-regulation in the duodenal mRNA levels of the PCFT (**Table 6.3**) and RFC (**Table 6.4**) genes, as both genes were expressed similarly in all of the 3 dietary treatments. However, the mucosal to serosal uptake of FA was moderately correlated ($r^2 = 0.554 – 0.731$) with the mRNA expression levels of the duodenal PCFT and RFC genes at 0, 10, or 100 mg/kg dietary supplementation of FA (**Table 6.5**). In the jejunum, the mucosal to serosal uptake of FA (**Figure 6.1**) as well as the mRNA levels of the PCFT (**Table 6.6**) and RFC (**Table 6.7**) genes were not influenced by increased FA supplementation. However, the jejunal uptake of FA ($P < 0.205$) and the mRNA level of the jejunal PCFT gene ($P < 0.160$) showed consistent tendencies for down-regulation as dietary FA was increased. The mucosal to serosal uptake of FA yielded moderate

Table 6.2. Production performance of 38 week old laying hens supplemented with 0, 10, or 100 mg FA/kg diet.

	Diet[2]				
Response[1]	0 mg FA	10 mg FA	100 mg FA	SEM[3]	P-value[4]
Egg production, %	97.3	100.0	98.2	1.69	0.531
Feed consumption, g	95.8	103.1	97.6	3.68	0.365
Egg weight, g	61.3	62.1	60.9	1.33	0.816
Feed Efficiency, g of feed/g of egg	1.61	1.66	1.63	0.06	0.833
Initial BW, kg	1.67	1.70	1.68	0.20	0.602
Final BW, kg	1.56	1.63	1.62	0.03	0.183
Egg folate, μg/egg	34.6[b]	65.5[a]	62.2[a]	1.33	<0.001
Plasma folate, ng/mL	79.2[b]	124.5[a]	146.7[a]	10.93	<0.001
Plasma homocysteine, μmol/L	11.85[a]	9.19[b]	8.86[b]	0.69	0.011

[a,b]Data within a row that do not share superscripts are significantly different ($P < 0.0.5$)
[1]Data are presented as least square means.
[2]Total measured folate activity (expressed as mg FA equivalents/kg of diet): 0 mg FA = 5.01; 10 mg FA = 11.80; 100 mg FA = 91.2.
[3]n = 8/treatment.
[4]P-value for main effect of diet.

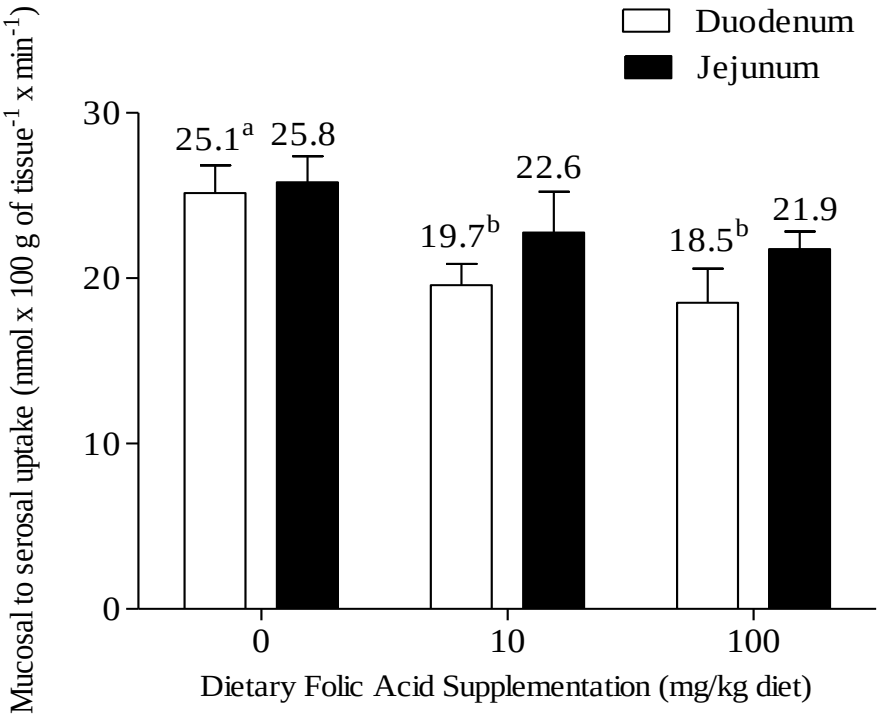

Figure 6.1. Folic acid (FA) uptake in the everted duodenum and jejunum of the laying hens fed increasing levels of dietary FA. Data represents means ± SEM (n = 8). The uptakes of 0.1 μM FA was evaluated at 41ºC and pH 6.0 for 5 min. [a-b]Data that do not share superscripts are significantly different ($P < 0.05$).

Table 6.3. Fold change expression of the duodenal proton-coupled folate transporter (PCFT) in laying hens supplemented with 0, 10, and 100 mg FA/kg of diet.

Diet[1]	PCFT Average C_T	β-actin Average C_T	ΔC_T[2] PCFT - β-actin	$\Delta\Delta C_T$[3] ΔC_T treated - ΔC_T calibrator	Fold difference relative to 0 mg FA[4]
0 mg FA	26.66 ± 0.20	20.99 ± 0.54	5.67 ± 0.58	0.00 ± 0.46	1 (0.73-1.38)
10 mg FA	26.62 ± 0.13	20.91 ± 0.43	5.71 ± 0.45	0.04 ± 0.44	1.01 (0.72-1.32)
100 mg FA	26.11 ± 0.11	20.57 ± 0.28	5.54 ± 0.30	-0.13 ± 0.35	1.12 (0.86-1.39)

[1]Total measured folate activity (expressed as mg FA equivalents/kg of diet): 0 mg FA = 5.01; 10 mg FA = 11.80; 100 mg FA = 91.2.
[2]ΔC_T is determined by subtracting the average β-actin C_T from the average PCFT C_T.
[3]The calculation of $\Delta\Delta C_T$ involves the subtraction of the $\Delta C_{T\,calibrator}$ sample (0 mg FA = calibrator sample) to the $\Delta C_{T\,treated}$ samples (10 mg FA and 100 mg FA = treated samples).
[4]The range given for the fold difference in the mRNA expression of the PCFT gene in the treated samples relative to the calibrator sample was determined by $2^{-\Delta\Delta Ct}$ with $\Delta\Delta C_T$ ± SD (n = 8).

Table 6.4. Fold change expression of the duodenal reduced folate carrier (RFC) in laying hens supplemented with 0, 10, and 100 mg FA/kg of diet.

Diet[1]	RFC Average C_T	β-actin Average C_T	ΔC_T[2] RFC - β-actin	$\Delta\Delta C_T$[3] ΔC_T treated - ΔC_T calibrator	Fold difference relative to 0 mg FA[4]
0 mg FA	24.24 ± 0.42	20.99 ± 0.54	3.25 ± 0.68	0.00 ± 0.77	1 (0.59-1.71)
10 mg FA	24.03 ± 0.09	20.91 ± 0.43	3.12 ± 0.44	-0.13 ± 0.49	1.14 (0.78-1.54)
100 mg FA	23.89 ± 0.09	20.57 ± 0.28	3.32 ± 0.29	0.07 ± 0.20	0.96 (0.83-1.09)

[1]Total measured folate activity (expressed as mg FA equivalents/kg of diet): 0 mg FA = 5.01; 10 mg FA = 11.80; 100 mg FA = 91.2.
[2]ΔC_T is determined by subtracting the average β-actin C_T from the average RFC C_T.
[3]The calculation of $\Delta\Delta C_T$ involves the subtraction of the $\Delta C_{T\,calibrator}$ sample (0 mg FA = calibrator sample) to the $\Delta C_{T\,treated}$ samples (10 mg FA and 100 mg FA = treated samples).
[4]The range given for the fold difference in the mRNA expression of the RFC gene in the treated samples relative to the calibrator sample was determined by $2^{-\Delta\Delta Ct}$ with $\Delta\Delta C_T$ ± SD (n = 8).

Table 6.5. The correlation coefficient (r^2) between the mucosal to serosal uptake of FA and the mRNA expression levels of the duodenal and jejunal proton-coupled folate transporter (PCFT) and reduced folate carrier (RFC) at 0, 10, and 100 mg FA/kg supplementation.

Intestinal Folate Transporter mRNA Expression	Mucosal to Serosal Uptake		
	0 mg FA	10 mg FA	100 mg FA
Duodenal PCFT mRNA expression	0.725**	0.731**	0.728**
Duodenal RFC mRNA expression	0.554*	0.580*	0.572*
Jejunal PCFT mRNA expression	0.825***	0.781***	0.804***
Jejunal RFC mRNA expression	0.619**	0.597*	0.753***

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Table 6.6. Fold change expression of jejunal proton-coupled folate transporter (PCFT) in laying hens supplemented with 0, 10, and 100 mg FA/kg of diet.

Diet[1]	PCFT Average C_T	β-actin Average C_T	ΔC_T[2] PCFT - β-actin	$\Delta\Delta C_T$[3] ΔC_T treated - ΔC_T calibrator	Fold difference relative to 0 mg FA[4]
0 mg FA	28.84 ± 0.37	18.99 ± 0.14	9.85 ± 0.40	0.00 ± 0.34	1 (0.79-1.27)
10 mg FA	29.31 ± 0.40	19.23 ± 0.15	10.08 ± 0.43	0.23 ± 0.49	0.89 (0.61-1.20)
100 mg FA	29.45 ± 0.67	19.05 ± 0.18	10.41 ± 0.69	0.56 ± 0.62	0.73 (0.44-1.04)

[1]Total measured folate activity (expressed as mg FA equivalents/kg of diet): 0 mg FA = 5.01; 10 mg FA = 11.80; 100 mg FA = 91.2.
[2]ΔC_T is determined by subtracting the average β-actin C_T from the average PCFT C_T.
[3]The calculation of $\Delta\Delta C_T$ involves the subtraction of the $\Delta C_{T\,calibrator}$ sample (0 mg FA = calibrator sample) to the $\Delta C_{T\,treated}$ samples (10 mg FA and 100 mg FA = treated samples).
[4]The range given for the fold difference in the mRNA expression of the PCFT gene in the treated samples relative to the calibrator sample was determined by $2^{-\Delta\Delta Ct}$ with $\Delta\Delta C_T$ ± SD (n = 8).

Table 6.7. Fold change expression of jejunal reduced folate carrier (RFC) in laying hens supplemented with 0, 10, and 100 mg FA/kg of diet.

Diet[1]	RFC Average C_T	β-actin Average C_T	ΔC_T[2] RFC - β-actin	$\Delta\Delta C_T$[3] ΔC_T treated - ΔC_T calibrator	Fold difference relative to 0 mg FA[4]
0 mg FA	25.19 ± 0.20	18.99 ± 0.14	6.20 ± 0.24	0.00 ± 0.22	1 (0.86-1.16)
10 mg FA	25.51 ± 0.06	19.23 ± 0.15	6.28 ± 0.16	0.08 ± 0.11	0.95 (0.88-1.02)
100 mg FA	25.29 ± 0.44	19.05 ± 0.18	6.24 ± 0.48	0.04 ± 0.35	0.99 (0.76-1.24)

[1]Total measured folate activity (expressed as mg FA equivalents/kg of diet): 0 mg FA = 5.01; 10 mg FA = 11.80; 100 mg FA = 91.2.
[2]ΔC_T is determined by subtracting the average β-actin C_T from the average RFC C_T.
[3]The calculation of $\Delta\Delta C_T$ involves the subtraction of the $\Delta C_{T\,calibrator}$ sample (0 mg FA = calibrator sample) to the $\Delta C_{T\,treated}$ samples (10 mg FA and 100 mg FA = treated samples).
[4]The range given for the fold difference in the mRNA expression of the RFC gene in the treated samples relative to the calibrator sample was determined by $2^{-\Delta\Delta Ct}$ with $\Delta\Delta C_T$ ± SD (*n* = 8).

correlations (r^2 = 0.597 – 0.825) with the mRNA expression levels of the jejunal PCFT and RFC genes at 0, 10, and 100 mg/kg dietary supplementation of FA (**Table 6.5**). In the cecum, the mRNA levels of the PCFT (P < 0.229) (**Table 6.8**) and RFC (P < 0.124) (**Table 6.9**) genes also showed the same tendencies for down-regulation, however, the decrease in the mRNA levels of these transporters were not significant.

6.5 DISCUSSION

Our objective in this study was to examine the effects of supplementing increased dietary levels of FA on the molecular and functional characteristics of FA absorption in the intestine of the laying hen. This is in connection with our *in vitro* intestinal everted sac study (Tactacan et al., 2011) where we found that the rate of FA transport in the small intestine of the laying hen diminished when the mucosal concentration of FA was increased. We then surmised that the extent to which increased levels of dietary supplemented FA is absorbed in the intestine will be crucial in determining the capacity of the laying hen to transfer folate into the egg. Similar to other water-soluble vitamins (Karasov and Diamond, 1987; Ferraris and Diamond, 1989; Said and Khan, 1993), the capacity for folate absorption in the intestine has been shown to be regulated by its level in the diet (Ashokkumar et al., 2007; Qiu et al., 2007). Because the production of folate-enriched eggs involved the supplementation of dietary FA at supraphysiological levels, the present study was designed to determine the mechanism by which intestinal FA transport is physiologically regulated by increased levels of FA supplementation in the laying hen diet.

In the present study, the measures of laying hen performance were not influenced

Table 6.8. Fold change expression of cecal proton-coupled folate transporter (PCFT) in laying hens supplemented with 0, 10, and 100 mg FA/kg of diet.

Diet[1]	PCFT Average C_T	β-actin Average C_T	ΔC_T[2] PCFT - β-actin	$\Delta\Delta C_T$[3] ΔC_T treated - ΔC_T calibrator	Fold difference relative to 0 mg FA[4]
0 mg FA	30.05 ± 0.42	17.39 ± 0.47	12.66 ± 0.63	0.00 ± 0.73	1 (0.60-1.66)
10 mg FA	30.65 ± 0.21	17.84 ± 0.11	12.81 ± 0.24	0.15 ± 0.25	0.91 (0.76-1.07)
100 mg FA	30.88 ± 0.25	17.76 ± 0.46	13.12 ± 0.52	0.46 ± 0.44	0.75 (0.54-0.99)

[1]Total measured folate activity (expressed as mg FA equivalents/kg of diet): 0 mg FA = 5.01; 10 mg FA = 11.80; 100 mg FA = 91.2.
[2]ΔC_T is determined by subtracting the average β-actin C_T from the average PCFT C_T.
[3]The calculation of $\Delta\Delta C_T$ involves the subtraction of the $\Delta C_{T_{calibrator}}$ sample (0 mg FA = calibrator sample) to the $\Delta C_{T_{treated}}$ samples (10 mg FA and 100 mg FA = treated samples).
[4]The range given for the fold difference in the mRNA expression of the PCFT gene in the treated samples relative to the calibrator sample was determined by $2^{-\Delta\Delta Ct}$ with $\Delta\Delta C_T$ ± SD (n = 8).

Table 6.9. Fold change expression of cecal reduced folate carrier (RFC) in laying hens supplemented with 0, 10, and 100 mg FA/kg of diet.

Diet[1]	RFC Average C_T	β-actin Average C_T	ΔC_T[2] RFC-β-actin	$\Delta\Delta C_T$[3] ΔC_T treated - ΔC_T calibrator	Fold difference relative to 0 mg FA[4]
0 mg FA	25.17 ± 0.19	17.39 ± 0.47	7.78 ± 0.51	0.00 ± 0.45	1 (0.73-1.37)
10 mg FA	25.88 ± 0.11	17.84 ± 0.11	8.03 ± 0.16	0.25 ± 0.22	0.85 (0.72-0.98)
100 mg FA	26.03 ± 0.18	17.76 ± 0.46	8.28 ± 0.49	0.50 ± 0.36	0.73 (0.55-0.91)

[1]Total measured folate activity (expressed as mg FA equivalents/kg of diet): 0 mg FA = 5.01; 10 mg FA = 11.80; 100 mg FA = 91.2.
[2]ΔC_T is determined by subtracting the average β-actin C_T from the average RFC C_T.
[3]The calculation of $\Delta\Delta C_T$ involves the subtraction of the $\Delta C_{T_{calibrator}}$ sample (0 mg FA = calibrator sample) to the $\Delta C_{T_{treated}}$ samples (10 mg FA and 100 mg FA = treated samples).
[4]The range given for the fold difference in the mRNA expression of the RFC gene in the treated samples relative to the calibrator sample was determined by $2^{-\Delta\Delta Ct}$ with $\Delta\Delta C_T$ ± SD (n = 8).

by increased levels of dietary FA supplementation. This result is consistent with reports from previous studies that dietary supplementation of FA at increased levels did not affect the production performance of the laying hens (House et al., 2002; Hebert et al., 2005; Roth-Maier and Böhmer, 2007; Bunchasak and Kachana, 2009; Dickson et al., 2010). House et al. (2002) and Hebert et al. (2005) noted that it is the lack of demonstrable impacts on the production performance of the laying hen that has generally limited the inclusion of FA in layer diets. Surveys conducted on commercial feed mills regarding the supplementation of FA in the laying hen diet supported this observation (BASF, 2000).

With respect to egg folate concentration and indices of folate status in the laying hen, our results were again consistent with findings from previous studies (Sherwood et al., 1993; House et al., 2002; Hebert et al., 2005; Roth-Maier and Böhmer, 2007; Bunchasak and Kachana, 2009; Hoey et al., 2008; Dickson et al., 2010; Tactacan et al., 2010). As expected, the supplementation of FA in the laying hen diet increased the egg folate concentration. The concentration of folate in egg in birds supplemented with FA (10 or 100 mg FA/kg diet) increased by approximately twice as much relative to birds fed the control diet. However, this increase was similar between 10 or 100 mg FA/kg supplemented diets, demonstrating the typical saturable characteristic of egg folate concentration when the dietary supplementation of FA was increased (Sherwood et al., 1993; House et al., 2002; Hebert et al., 2005; Roth-Maier and Böhmer, 2007; Bunchasak and Kachana, 2009; Hoey et al., 2008; Dickson et al., 2010). Hebert et al. (2005) for instance supplemented as much as 128 mg FA/kg of the laying hen diet but did not find any significant increased in egg folate concentration beyond the level achieved when FA

was supplemented at 4 mg/kg of the diet. Accordingly, the plasma folate concentration paralleled the observed response in egg folate concentration over the three dietary treatments studied. The proportionate increase between plasma folate and egg folate concentrations indicated that circulating blood folates are efficiently transferred into the egg (Sherwood et al., 1993; House et al., 2002; Hebert et al., 2005). This is because blood folate serves as the precursor pool for the deposition of folate in the egg (Sherwood et al., 1993). In contrast, the plasma homocysteine concentration decreased significantly with dietary FA supplementation. The biologically active form of folate, 5-methylTHF, participates in a reaction that involves the remethylation of homocysteine to methionine (Scott and Weir, 1994). This folate-dependent reaction consequently decreased the homocysteine concentration in the blood.

In examining the effect of increased FA supplementation levels on the intestinal folate absorption process of the laying hen, we employed the well established technique of the *in vitro* intestinal everted sac model. This model was chosen because a previous study from our laboratory (Tactacan et al., 2011) demonstrated its suitability as an excellent *in vitro* model system in studying the different aspects of the intestinal folate uptake process in the laying hen. In the present study, the increased supplementation (10 or 100 mg FA/kg of diet for 21 d) of FA in the laying hen diet led to a significant down-regulation in the rate of FA absorption in the duodenum. However, this was not associated with a similar down-regulation in the duodenal mRNA expression levels of folate transporters PCFT and RFC, as both were equivalently expressed regardless of the supplemented FA levels in the diet. Relative to birds fed the control diet (0 mg FA/kg diet), Jing et al. (2009; 2010) observed no significant differences in the mRNA levels of

the duodenal PCFT and RFC genes in laying hens supplemented with 10 mg FA/kg of the diet. Accordingly, Dev et al. (2010) also did not find a significant change in the intestinal mRNA levels of the PCFT and RFC genes in FA-oversupplemented (20 mg/kg of diet) rats, in spite of the observed down-regulation in the intestinal uptake of FA during acute (10 d) FA-oversupplementation. Rather, they found a parallel decrease in the protein levels of both the PCFT and RFC transporters proportionate to the down-regulation in the intestinal uptake of folate during acute FA-oversupplementation. In the present study, strong positive relationships between the mRNA levels of the duodenal folate transporters (PCFT and RFC) and the rate of duodenal FA absorption were detected when both were subjected to correlation analysis. However, because the mRNA expression of the duodenal PCFT and RFC genes did not parallel the observed down-regulation in the duodenal absorption of FA when dietary FA was increased, it can be deduced that the down-regulation in the duodenal absorption of FA was not regulated by the folate transporters at the level of transcription. Although the protein levels of the duodenal PCFT and RFC were not determined, the current results indicated the potential involvement of another regulatory mechanism, possibly a post-transcriptional or translational regulation.

In the jejunum, the absorption of FA and the mRNA expression levels of the PCFT and RFC genes were not influenced by increased dietary FA supplementation. However, the jejunal absorption of FA as well as the mRNA expression of the jejunal PCFT gene showed similar tendencies for down-regulation as FA level in the diet was increased. Jing et al. (2009; 2010) observed that the jejunal mRNA expression of the PCFT and RFC genes were not influenced by the addition of 10 mg FA/kg of the basal

laying hen diet. In the present study, moderate correlation coefficients between the mRNA expression of the jejunal PCFT gene and the rate of jejunal FA absorption were detected when both variables were subjected to correlation analysis. Coupled with the similar tendencies for down-regulation in the rate of FA absorption and the mRNA expression of the PCFT gene in the jejunum, this result may hold some degree of biological significance. In our *in vitro* study, the uptake of FA across the intestinal wall of the laying hen demonstrated features of transport resembling a PCFT transport characteristic (Tactacan et al., 2011). As to whether the tendency for down-regulation in the uptake of FA and the expression of the PCFT gene in the jejunum indicated a more significant role for PCFT than RFC in the absorption of dietary FA in the laying hen, remains to be determined in future studies.

The cecal mRNA expression levels of the PCFT and RFC genes were not affected by increasing dietary FA supplementation. However, there was a consistent trend for mRNA expression of the cecal PCFT and RFC genes for down-regulation as dietary FA supplementation was increased. We demonstrated previously that a considerable amount of folate may be absorbed in the cecal everted sacs of the laying hen (Tactacan et al., 2011). Other studies using different animal models have shown that a portion of folate absorbed in the cecum is incorporated into the various tissues of the body (Rong et al., 1991; Asrar and O'Connor, 2005). Assuming that the decrease in the mRNA levels of PCFT and RFC in the cecum translates into a corresponding decrease in the functional activity of the folate transport process in this region of the gut, it follows that the portion of the absorbed bacterially-synthesized folate in the cecum of the laying hen may be compromised.

In summary, our study demonstrated that supplementation of increased levels of dietary FA resulted in a down-regulation of FA absorption in the duodenum, but not in the jejunum of the laying hen. This down-regulation was not associated with a decreased mRNA expression of the duodenal PCFT and RFC genes. Therefore, a post-transcriptional or translational regulation of the intestinal folate transporters may be involved in the down-regulation of duodenal FA absorption during increased supplementation of dietary FA. This down-regulation may contribute to the saturation of egg folate concentration when levels of FA in the laying hen diet are increased.

CHAPTER SEVEN

GENERAL DISCUSSION

The objective of this study was to investigate the factors involved in the regulation of dietary folic acid (FA) deposition in the egg and characterize the mechanism by which they may influence the level of egg folate concentration. This study was initiated by findings from our group and from several others (Sherwood et al., 1993; House et al., 2002; Hebert et al., 2005; Roth-Maier and Böhmer, 2007; Bunchasak and Kachana, 2009; Hoey et al., 2009; Dickson et al., 2010) which showed that folate deposition in the chicken egg achieved a characteristic saturation when supplementation of FA in the laying hen diet was increased. Because folate-enriched eggs represent a viable option to increase the population intake of natural folate, a better understanding of the factors regulating the deposition of dietary FA into the egg may provide valuable insights on how the level of folate in eggs may be further increase. At the current level of folate enrichment, an average size egg (60 g) can provide approximately 33-40%, 12.5-15%, and 8-10% of the recommended daily allowance (RDA) for toddlers, adults, and pregnant women, respectively. However, because concern about cholesterol intake remains to influence the consumption of eggs in the general public, increasing the level of folate in eggs will increase its contribution on folate RDA, notwithstanding their limited consumption. At the present time, in spite of the recognized saturable characteristic of FA deposition in the egg, studies designed to identify and understand the factors that are influencing the saturation of folate in eggs are lacking. Therefore, the current study addresses this gap by providing information on the biochemical and

molecular mechanisms by which regulatory factors influence the deposition of dietary FA in eggs. This study may also potentially serve as a model for the establishment of novel strategies geared towards the enrichment of eggs with other essential nutrients.

7.1 Production performance of the laying hen in response to dietary FA and 5-methyltetrahydrofolate (5-methylTHF) supplementation

Consistent with results from previous studies, our current results demonstrated that the supplementation of FA in the laying hen diet did not influence the production performance of the laying hen (House et al., 2002; Hebert et al., 2005; Roth-Maier and Böhmer, 2007; Bunchasak and Kachana, 2009; Dickson et al., 2010; Hebert et al., 2011, in press). Measures of production consisting of egg production, feed consumption, egg weight, and feed efficiency were similar in laying hens supplemented with 0 or 10 (Chapter 4), and 0, 10, or 100 mg FA/kg of the diet (Chapter 6). House et al. (2002) and Hebert et al. (2005) noted that it is the lack of demonstrable impacts on production performance that has generally limited the inclusion of FA in laying hen diets. Interestingly, the supplementation of 11.3 mg 5-methylTHF/kg of the diet (equimolar concentration as 10 mg FA/kg diet) resulted in the production of heavier eggs than the control fed birds, and the improvement of feed efficiency as compared to birds fed with equimolar FA and birds fed the control diet. For reasons not totally clear, these results however, may indicate a more important role for 5-methylTHF in highly producing laying hens. Theoretically, a high requirement for amino acid and nucleotide biosynthesis is obligatory for laying hens during the period of peak egg production. Because the supplemented amount of folate is much higher than the folate requirement of the laying hen (NRC, 1994), perhaps blood and tissue indices might have plateaued; however, the

presence of different forms of folate may be affecting the efficiency of usage. However, this remains to be determined in a much larger and longer-term production study.

7.2 Egg folate concentration and indices of folate status in response to dietary FA supplementation

Our current study supports previous findings that it is possible to produce eggs with enriched total folate content by feeding laying hens a diet supplemented with FA (Sherwood et al., 1993; House et al., 2002; Hebert et al., 2005; Roth-Maier and Böhmer, 2007; Bunchasak and Kachana, 2009; Hoey et al., 2009; Dickson et al; 2010; Hebert et al., 2011, in press). Egg folate concentrations of birds fed the FA supplemented diets in Chapter 4 (10 mg FA/kg diet) and Chapter 6 (10 or 100 mg FA/kg diet) were almost doubled compared to eggs from birds fed the control diet. Consistent with the previous studies, the supplementation of FA at 100 mg/kg diet did not lead to any further significant increase in egg folate concentration when compared to laying hens fed with 10 mg FA/kg diet. Dose response studies by Sherwood et al. (1993), House et al. (2002), and Hebert et al. (2005) demonstrated that saturation in egg folate levels occurred at FA concentrations of around 2-4 mg/kg in the laying hen diet. A more recent study by Dickson et al. (2010) reported a 4 mg FA/kg of diet as the optimal dietary FA level required for maximal egg folate deposition.

With respect to indices of folate status, our results were again in agreement with results from earlier studies (Sherwood et al., 1993; House et al., 2002; Hebert et al., 2005; Roth-Maier and Böhmer, 2007; Bunchasak and Kachana, 2009; Hoey et al., 2008; Dickson et al., 2010; Hebert et al., 2011, in press). The blood folate concentration of the laying hen increased in parallel proportion with egg folate concentration when dietary FA

supplementation was increased (Chapter 4 and 6). It is the proportionate response between blood folate and egg folate concentrations that led previous workers to believe that the saturation in egg folate concentration is not due to limitations in the transport processes from blood to the egg. Because the blood serves as the precursor pool for folate deposition in the egg, the proportionate relationship between the two folate compartments indicated an effective transport mechanism of circulating blood folate into the egg. In contrast, blood homocysteine concentration decreased with increasing FA supplementation (Chapter 4 and 6). The biologically active form of folate, 5-methylTHF; reduced blood homocysteine concentration by participating in the remethylation of homocysteine to yield methionine (Bagley and Shane, 2005).

7.3 Egg folate concentration and indices of folate status in response to dietary supplementation of 5-methylTHF

An equimolar concentration of FA and 5-methylTHF were fed to the laying hen (Chapter 4) to determine their ability to metabolically convert FA into its biologically active form in the egg, the 5-methylTHF. Because 5-methylTHF is the biologically active form of folate, it does not need to undergo the metabolic processes of reduction and methylation reactions required for FA, prior to its deposition in the egg. Thus, if the capacity of the laying hen to convert FA to 5-methylTHF is limited, the response to FA supplementation in terms of egg folate concentration and indices of folate status will be restricted.

The egg folate concentration and indices of folate status (serum folate, plasma homocysteine, and liver folate concentrations) in the laying hen responded similarly with dietary FA or 5-methylTHF supplementation (Chapter 4). This indicated that FA is not

only as effective as 5-methylTHF in influencing the concentration of folate in egg but also in influencing the indices of folate status in the laying hen. To our knowledge, the extent to which the laying hen can metabolize the increased dietary level of FA into its biologically active form in the egg has not been previously elucidated. The result of the present study provided evidence that laying hens can readily convert elevated level of dietary FA into 5-methylTHF.

7.4 Activities of folate-dependent enzymes in response to FA and 5-methylTHF supplementation

The overall activities of the folate-dependent enzymes with the exception of hepatic dihydrofolate reductase activity (DHFR) were similar between FA and 5-methylTHF supplemented birds (Chapter 4). Dihydrofolate reductase facilitates the reduction of FA into dihydrofolate (DHF) and tetrahydrofolate (THF) through transfer of hydride molecules from NADPH (Benkovic and Hammes-Schiffer, 2003). In humans, the low activity of DHFR has been attributed as the primary reason for the presence of unmetabolized FA in the systemic circulation during periods of high FA intake (Bailey et al., 2003; Wright et al., 2007). Thus, DHFR is an important regulatory enzyme in the metabolism of FA into its biologically active forms. As hepatic DHFR activity increased in the laying hen when the level of supplemented FA in the diet was increased, the result of the present study demonstrated that laying hens possess the inherent ability to modulate the activity of an important enzyme required to metabolically convert elevated supply of FA into its biologically active forms in the egg. This may explain the low levels of FA detected in eggs (Vahteristo et al., 1997; Seyoum and Selhub, 1998; McKillop et al., 2003; Hoey et al., 2009).

Relative to the control-fed birds, the hepatic serine hydroxymethyltransferase (SHMT) and methionine synthase activities (MS) were increased and decreased respectively, in FA and 5-methlyTHF-fed birds. Tetrahydrofolate in concert with serine and vitamin B_6 initiates the SHMT-catalyzed reaction resulting in the formation of 5, 10-methylenetetrahydrofolate (5, 10-MTHFR) and glycine. Tetrahydrofolate is either synthesized from the reduction of FA by DHFR or by the transfer of a methyl group from 5-methylTHF to homocysteine during methionine synthesis (Geller and Kotb, 1989). On the other hand, MS activity is regulated by the blood homocysteine concentration (Scott and Weir, 1994). Because plasma homocysteine was lower in folate-fed birds than the control fed-birds, hepatic MS activity in FA and 5-methylTHF supplemented birds was not increased. Therefore, these results demonstrated that the form of folate as well as the level of supplementation has implications for the activity of key enzymes responsible for folate metabolism in the laying hen.

7.5 The rate of intestinal FA absorption with increasing FA concentration

An *in vitro* everted sac intestinal model was used to functionally characterize the intestinal absorption of FA in the laying hen. This technique was chosen owing to the difficulty of interpreting folate absorption studies involving whole animal models due to problems associated with the enterohepatic circulation of folate via bile fluid and the complicating effect of changes in digesta transit time. The *in vitro* everted sac model has been shown to be rapid and useful in predicting the trend of the absorptive process in intact animals (Hill et al., 1987).

The rate of intestinal FA absorption in the jejunal everted sac of the laying hen occurred faster at lower FA concentration but diminished at higher concentration of FA

in the intestinal mucosal medium (Chapter 4). Regression analysis of the dataset on FA absorption over the full range of FA concentrations revealed a quadratic relationship following the equation [Y (mucosal-to-serosal uptake) = 1.3153 + 0.1252X (concentration) – $0.0378X^2$ ($P < 0.001$, $R^2 = 0.92580$], demonstrating the nonlinear response in absorption as concentration was increased from 0.0001 to 100 μM FA. When fitted to the Michaelis-Menten equation to calculate for estimates of transport kinetics, the calculated *K*m constant was 0.03 ± 0.004 μM and the maximum velocity (*V*max) was 22.7 ± 0.9 nmol x 100 g of $tissue^{-1}$ x min^{-1} ($R^2 = 0.9824$). The high correlation coefficient with the Michaelis-Menten kinetics implies a carrier-mediated transport system, consistent to the generally accepted mode of folate transport (Steinberg, 1984; Sirotnak and Tolner, 1999). Consequently, an evaluation of intestinal FA absorption during periods of increased dietary FA supplementation in the laying hens was conducted (Chapter 6). The production of folate-enriched eggs employs the manipulation of dietary folate concentration through supplementation of supraphysiological levels of FA in the laying hen diet. Results of the study revealed a down-regulation in the rate of duodenal FA absorption and a tendency for a down-regulation of FA absorption in the jejunum of the laying hen when dietary FA supplementation was increased. Dev et al. (2010) also demonstrated that intestinal uptake of FA was down-regulated in rat brush-border membrane vesicles when maintained under the condition of folate over-supplementation. Therefore, consistent with the literature, our two studies revealed that the rate of FA absorption in the small intestine of the laying hens diminished with increased dietary FA supplementation. This response is reminiscent of the pattern demonstrated by the laying hens for egg folate concentration when dietary FA concentration was increased

(Sherwood et al., 1993; House et al., 2002; Hebert et al., 2005; Roth-Maier and Böhmer, 2007; Bunchasak and Kachana, 2009; Hoey et al., 2009; Dickson et al; 2010; Hebert et al., 2011, in press; Chapter 4; Chapter 6). Thereby, it is possible that the observed saturation in egg folate concentration when dietary supplementation of FA was increased may be attributed to the diminished rate of FA absorption in the intestine.

7.6 Intestinal transporters of dietary FA in the laying hen

Overall, 3 major folate transporters may facilitate folate transport across the intestinal cell membrane. They are the folate receptor (FR), reduced folate transporter (RFC), and the proton-coupled folate transporter (PCFT). An earlier study reported that FR is negligibly expressed in the small intestine (Steinberg et al., 1994), therefore its contribution to folate absorption in the intestine has never been considered in subsequent studies. Members of our group have investigated the mRNA expression of RFC and PCFT in the digestive tract of the laying hen and found that both transporters are widely expressed in the entire region of the small and large intestines (Jing et al., 2009; 2010). These studies were the first to confirm that both folate transporters are present in the intestine of the laying hen. However, whether their expression implies functional activity for absorption of dietary folate remains to be demonstrated.

The rate of FA absorption in the jejunum of the laying hen was maximal at pH 6.0 and minimal at pH 7.5 (Chapter 5). This transport feature may indicate a H^+-gradient dependent transport process as transport rates appeared to favour FA transport at acidic pH conditions (pH 5.5-6.0). Indeed, H^+-gradient dependency was confirmed when carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP), an ionophore which dissipates H^+ gradients accross the cell membrane was added in the mucosal medium.

With increasing concentration of FCCP in the mucosal medium, the rate of FA transport was extensively reduced; thus providing evidence that transport of FA in the intestine of the laying hen involved a H^+-coupled transport mechanism. Proton-coupled folate transporter transports folate across the cell membrane using a H^+-coupled mechanism. The Michaelis-Menten kinetics of FA transport which indicated a K_m (μM) and a V_{max} (nmol x 100 g of tissue^{-1} x min^{-1}) value of 0.03 ± 0.004 and 22.7 ± 0.9, respectively, also resembles a PCFT transport more than an RFC transport. The proton- coupled folate transporter is known to have very high affinity to FA, while RFC prefers reduced folate derivatives like 5-methylTHF as substrate (Zhao et al., 2009). However, in the current study, it is rather difficult to assess the contribution of PCFT in FA absorption of the laying hen based solely on the observed transport kinetic similarities. One of the limitations in the use of the everted sac technique is that kinetic parameters of the measured transport can only be calculated for the overall transport across the intestinal wall. Transport in the everted sac includes the apical uptake of substrate, intracellular processing or metabolism of substrate, basolateral release of substrate, and the possible secretion of the substrate back to the luminal side. In addition, everted sacs contain neurons of the enteric nervous system and components of the immune system; both are known to influence transport and processing of substrates across the intestinal wall (Hill et al., 1987). Therefore, the measured transport of FA is a result of all the processes which occur in the intestinal wall and not solely the action of one folate transporter.

7.7 Distribution of FA absorption in the intestine of the laying hen

The absorption of FA was observed in the duodenal, jejunal, ileal, and cecal sections of the intestine of the laying hen. Jing et al. (2009, 2010) also reported the

expression of PCFT and RFC along the entire intestinal tract of the laying hen. The distribution profile of FA transport was highest in the duodenum and jejunum and decreased as it approached the ileum and cecum. Previous studies have also reported the existence of higher transport activity for FA in the upper half of the small intestine and lower transport activity for FA in the lower portion of the small intestine (Qiu et al., 2006; Inoue et al., 2008). Folate transport with optimal activity at the proximal portion of the small intestine is consistent with the acidic pH in the microenvironment of the absorptive surface of the upper small intestine (Said et al., 1987; McEwan et al., 1990; Ikuma et al., 1996), whereas the lower transport activity in the ileum and cecum is consistent with the alkaline condition in this region of the intestine (Engberg et al., 2002). The presence of transport activity of FA in the ceca, coupled with our previous demonstration of the presence of mRNA for both RFC and PCFT in the ceca (Jing et al., 2009; 2010) provide evidence that folate supplied by commensal microorganisms in the ceca may contribute to the folate status of the laying hen. The role of endogenous folate supply in contributing to the folate status of the host has been demosntrated by others (Rong et al., 1991; Asrar and O'Connor, 2005). Gut microflora can synthesize considerable amounts of folate, and a significant portion of this folate exists in the lumen in the absorbable monoglutamate form (5-methylTHF) (Kim et al., 2004). The current demonstration of a measurable uptake of folate from cecal everted sacs has the potential to make significant contributions to our understanding of the extent to which nutrients derived from microbes contribute to daily requirements of the laying hen.

7.8 Inhibition of intestinal FA absorption by natural folate derivatives and heme

Because the intestinal transport of folate is non-specific (Zhao et al., 2007), the existence of different folate compounds in the lumen may result in potential interactions during cellular transport and absorption of dietary FA in the intestine. Indeed, the absorption of FA was impeded by the presence of 5-methylTHF and 10-formylTHF in the mucosal medium. 5-methyltetrahydrofolate was a more potent inhibitor of FA than 10-formylTHF. Another compound heme, a dietary source of iron, was investigated because PCFT was originally identified as a heme carrier protein (Shayeghi et al., 2005). However, compared to 5-methylTHF and 10-formylTHF, the absorption of FA was less inhibited by heme. This result is physiologically relevant considering that these micronutrients may negatively influence the absorption of dietary FA and its eventual transfer to tissues, including the egg. Both 5-methylTHF and 10-formlyTHF are the two most predominant folate derivatives found in plant-based feed ingredients (Bagley and Shane, 2005) while heme is found in significant amount in diets containing animal-based ingredients, in particular meat and bone meal.

7.9 Regulatory mechanism of intestinal FA absorption in period of increased dietary FA supplementation

Increasing the levels of dietary FA supplementation led to a down-regulation in the duodenal, but not in the jejunal absorption of FA in the laying hen. This down-regulation was not associated with a decreased mRNA expression of the duodenal PCFT and RFC genes. However, strong positive relationships between the expression of the folate transporters and the rate of FA absorption in the duodenum were detected when both were subjected to correlation analysis. Since this relationship was not evident when the dietary FA levels were increased, it can be deduced that the down-regulation in the

duodenal absorption of FA was not regulated by the expression of the PCFT and RFC genes at the level of transcription. Although the protein levels of PCFT and RFC were not determined in the current study, the lack of evident association between the mRNA expression of these transporters and the duodenal absorption of FA when its levels in the diet were increased may indicate the possible involvement of another regulatory mechanism, possibly a post-transcriptional or translational regulation.

CHAPTER EIGHT

CONCLUSIONS AND FUTURE STUDIES

The following conclusions can be drawn from the present research:

1. Production performance (hen-day egg production, feed consumption, egg weight, and feed efficiency) of the laying hen was not affected by dietary FA supplementation.
2. Supplementation of dietary 5-methylTHF led to the production of heavier eggs and to the improvement of feed efficiency in the laying hens.
3. Supplementation of FA in the laying hen diet increased egg folate and blood folate concentrations, and decreased plasma homocysteine concentration.
4. Supplementation of FA and 5-methylTHF in the laying hen diet had similar influence on egg folate concentration, indices of folate status, and overall activities of folate-dependent enzymes.
5. Supplementation of FA in the laying hen diet increased serum folate and decreased plasma homocysteine concentrations in Shaver White compared to Shaver Brown hens.
6. The absorption of FA in the jejunal everted sac of the laying hen decreased with increasing FA concentration, was maximal at acidic pH condition, and was inhibited by natural folate derivatives and heme.
7. The absorption of FA was observed in the entire intestinal tract of the laying hen, being highest in the duodenum and jejunum and decreased in the ileum and cecum.

8. Increasing levels of dietary FA supplementation led to a down-regulation in the absorption of FA in the duodenum of the laying hen. This down-regulation was not associated with a decrease in mRNA expression of the duodenal PCFT and RFC genes.
9. The jejunal absorption of FA and the mRNA expression of the jejunal and cecal PCFT and RFC genes were not affected by increasing dietary FA supplementation.

FUTURE STUDIES

Enhancing the folate content of egg provides an opportunity to increase the level of this key nutrient to the general population in particular those deemed marginal or insufficient with folate. In general, the supplementation of FA in the laying hen diet constitutes the efforts to enrich folate in eggs. The present research demonstrates the important role of FA absorption at the level of the intestine as a critical regulatory point for the deposition of dietary FA into the chicken egg. However, several other points of folate metabolism may be involved in this regulation.

1. Dietary regulation of intestinal folate transporter

At the intestinal level, our present work in line with the work of others (Said et al., 2000; Ashokkumar et al., 2007; Qiu et al., 2007; Dev et al., 2010) demonstrated that the concentration of FA in the diet is associated with changes in the rate of FA uptake. Collectively, these studies revealed that the rates of intestinal FA absorption, increased or decreased respectively, when FA is deficient or in excess in the diet. These indicated that the absorptive capacity for

folate in the intestine is adaptively controlled by the presence of FA, and that intestinal folate transporters are expressed at levels matching the availability of folate. Accordingly, evaluations on the regulatory mechanisms involved in the expressions of folate transporters revealed that transcriptional, post-transcriptional or translational regulations possibly mediate the intestinal folate transport. It is well established that intestinal nutrient transporters may be regulated by the diet. Thus, if the regulatory mechanisms that control the intestinal expression of folate transporters in the laying hen can be manipulated by dietary factors to augment the rate of intestinal folate absorption, the level of folate deposition in eggs may be increased. For instance, vitamin D_3 and its nuclear receptor have been reported to increase the expression and activity of hPCFT (Eloranta et al., 2009). In this study, the effect of vitamin D_3 was found to be mediated by its interaction with the vitamin D_3 receptor in the promoter region of the PCFT gene. Vitamin D_3 receptor is a member of the nuclear receptor family of transcription factors which regulates gene expression by directly interacting with so-called direct repeat-3 motifs within the target promoters, and at the same time serving as a heterodimer with another nuclear receptor, retinoid X receptor-α (Eloranta et al., 2009).

2. Absorption of bacterially synthesized folate in the cecum

From the current study we demonstrated that considerable amount of folate can be absorbed in the cecal everted sacs of the laying hen. This result is consistent with our previous demonstration which indicated the presence of mRNA for both RFC and PCFT in the chickens' large intestine, and therefore, our current work provided evidence that both of these intestinal folate transporters are

functionally active (Jing et al., 2009; 2010). Gut microflora can synthesize considerable amounts of folate, and a significant portion of this folate exists in the lumen in the absorbable monoglutamate form (5-methylTHF; Kim et al., 2004). Previous studies on different animal models have shown that a portion of this folate is absorbed and is incorporated into the various tissues (Rong et al., 1991; Asrar and O'Connor, 2005). Therefore, it would be interesting to know whether bacterially synthesized folates may also offer a complementary source of bioavailable folate to the laying hens. A role for the colonic transport system in human folate nutrition was suggested by a report of a significant association between serum folate levels and consumption of dietary fiber (Houghton et al., 1997). The authors of this report suggested that an increase in fiber intake may promote increased folate biosynthesis by the large intestinal microflora and subsequent absorption of some of this folate. Thus, if the microbial milieu of the large intestine in the laying hen can be manipulated to increase folate production, supplementation of dietary folate may be adjusted in such a way that both sources of folate may contribute to the optimization of folate deposition in eggs.

3. Enterohepatic circulation kinetics of bile folates

The majority of folate (mainly 5-methylTHF) arriving at the liver from the intestine is quickly released for distribution to tissues via the enterohepatic circulation. Steinberg et al. (1984) reported that the recycling of folates via the enterohepatic pathway may account for as much as 50% of the circulating blood folate that ultimately reaches the peripheral tissues. Through this transport route, folate is discharged into the bile and subsequently reabsorbed by the small

intestine before re-entering the systemic circulation. As such, the amount of folate appearing in the blood is directly proportional to the amount of folate released and reabsorbed from the bile. Granting that biliary clearance of folate approaches saturation at high doses of folate, it can be assumed that blood folate concentrations will demonstrate a similar pattern of saturation. Therefore, studies looking at the role of the folate enterohepatic cycle on blood folate concentrations may provide insights on how this important pathway may regulate the extent of folate supply and distribution to different tissues, including the egg.

4. Characterization of egg folate binding protein

In light of the fact that both the blood folate and egg folate concentrations saturate within the same range of dietary FA levels, the system that transfer folate from the blood across the oocyte vitelline membrane into the yolk, does not appear to be contributing to the saturation in egg folate concentration. However, the exact mechanism by which folate is transferred from the blood and into the egg yolk is not totally clear. In the case of related vitamins, like biotin and vitamin B_2 (riboflavin), specific maternal proteins escort these vitamins to the developing oocyte, where each is deposited as a stoichiometric vitamin-protein complex (Vieira et al., 1996; Monaco, 1997). For both vitamins, the amount of binding protein limits the amount of vitamin deposited in the egg (Sherwood et al., 1993). With respect to folate, it is believed that its targeted delivery to the ovary and ultimately to the yolk also involved the production of a folate binding protein (Henderson, 1990). However, this perception was derived from the fact that folate-binding protein exist in other systems and that riboflavin-binding protein is

homologous to milk folate-binding protein (Monaco, 1997). As to whether egg folate binding proteins actually exist and contribute to the ovarian transport of folate in the laying hen needs further elucidation.

5. Passive diffusion of FA in the small intestine at supraphysiologic levels

High oral doses of supplemental FA in humans have been shown to bypass the normal folate absorption process in the intestine through a nonsaturable transport mechanism involving the passive diffusion of the unaltered vitamin, resulting in the increased levels of both FA and 5-methylTHF in the blood (Lucock et al., 1989). In laying hens, House et al. (2002) speculated a biphasic response pattern in egg folate concentration when they observed that egg folate concentration increased above a plateau value when the level of FA in the diet was increased to 32 mg/kg of diet (highest level tested). They explained that above a critical inclusion level for dietary folate, folate accumulation in the egg yolk may surpass the saturable processes via another mechanism (i.e., noncarrier-mediated transport). Although subsequent study by Hebert et al. (2005) did not find evidence of a similar biphasic response in egg folate concentration; it was evident from their result that plasma folate concentration tended to increase at higher levels of FA supplementation. Our current study (Chapter 6) tended to follow a similar trend (10 mg/kg FA = 124.5 ng/mL vs. 100 mg/kg FA = 146.7 ng/mL). Accordingly, the absorption rate of FA in the jejunal everted sac of the laying hen tended to increase even further at the highest FA concentration in the mucosal medium (100 μM) (Chapter 5). Therefore, by knowing how much passive diffusion of FA across the intestinal cell membrane contributes to the

elevation of blood folate concentrations, and subsequently in the egg folate concentration, transport of folate through this mechanism may be maximized.

CHAPTER NINE

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CHAPTER TEN

APPENDICES

Egg Folate Assay

1. Weigh about 0.5 g of freeze dried egg yolk into glass tubes with lids and record weights. Ensure that a boiling water bath has been prepared beforehand.

2. Add 10 mL ascorbate buffer (pH 7.8) to each tube and top with nitrogen gas. Secure caps and vortex.

3. Place in boiling bath for an hour. Wait 35 min for tubes to cool before centrifuging.

4. Centrifuge at 4000 rpm for 30 min at 4 ^{o}C (Jouan CR3000).

5. Remove supernatant from each tube and place in corresponding volumetric flask (25 mL).

6. Add another 10 mL of ascorbate buffer to each tube. Vortex, add nitrogen, and centrifuge as before.

7. Decant supernatant into corresponding volumetric flask and bring to volume with ascorbate buffer. Mix thoroughly.

8. Filter through Mandel 0.2 μm nylon syringe filters (13 mm).

9. Pipette 200 μL into vials (with glass inserts) for HPLC analysis.

Ascorbate Buffer (pH 7.8)

1. Add 12.11 g trizma base to approximately 800 mL of deionized water and stir.

2. Add 20 g of sodium ascorbate to the Trizma base solution.

3. Adjust pH to 7.8 and bring to volume of 1 L.

4. Make final slight pH adjustments by adding hydrochloric acid (HCl). Store in the fridge.

0.03 M Potassium Phosphate Buffer (pH 2.2), 20% acetonitrile (HPLC Eluent)

1. Add 4.08 g of potassium phosphate monobasic (KH_2PO_4) to about 700 mL of deionized water and stir.

2. Add 200 mL of acetonitrile.

3. Adjust pH to 2.2 using phosphoric acid (85%). Bring to a final volume of 1 L with the deionized water and filter through a 0.22 μm filter.

Folate Standards For Egg Yolk

1. Prepare a standard curve from 5-methyltetrahydrofolate (5-methylTHF) using the following concentrations: 0 nM, 50 nM, 100 nM, 150 nM, 200 nM, 250 nM.

2. Prepare a 50 μM stock of 5-methylTHF. The molecular weight of 5-methylTHF disodium salt is 504.3. The purity of the current lot is 90%. Therefore, weigh 14.0 mg, transfer to a 500 mL volumetric and bring to volume with ascorbate buffer to make a 50 μM solution. Retain 250 mL in a labeled container and serially dilute to make the 1 μM stock. From this stock, prepare the standards. Keep both 50 μM and 1 μM stocks in the fridge.

Homocysteine Assay

1. Pipette 150 μL of prepared standard curve solutions and samples into labeled microfuge tubes. Standards are done in duplicate.

2. Add 20 μL of TCEP solution and vortex gently. Avoid splashing.

3. Allow tubes to stand at room temperature for 30 min.

4. During this period make SBDF solution.

5. After incubation, add 125 μL of 0.6 M perchloric acid to all the tubes and vortex.

6. The added acid causes protein denaturation in samples so the samples must be centrifuged for 10 min and 10,000 rpm.

7. Pipette 50 μL of supernatant into new labeled microcentrifuge tubes. Combine with 100 μL of Solution B and 50 μL of SBDF solution and vortex gently. Avoid splashing.

8. Place microcentrifuge tubes on a heating block for 1 hour at 60 ^{o}C.

9. After 1 hr incubation, remove microcentrifuge tubes and place in the fridge to cool for 20 min.

10. Centrifuge for 5 min at 10,000 rpm.

11. Prepare a sufficient number of HPLC vials with glass inserts to accommodate all standards and samples. Pipette 150 µL of supernatant into the inserts and run the batch on the HPLC.

2 M Potassium Borate Buffer, pH 9.5 (Solution A)

This solution is used to make Solution B, Solution C, and SBDF solution.

1. Make a 2 M potassium hydroxide solution (KOH). Dissolve 112.22 g of KOH in a beaker containing 600-800 mL of deionized water. Transfer to a 1 L graduated cylinder. To get all of the KOH dissolved, ensure that the sides and bottom of the beaker are washed with deionized water and transferred. Allow to cool to room temperature. Bring to volume.

2. Add 123.66 g boric acid to 900 mL of deionized water in a 2 L beaker and 300 mL of the 1 M KOH solution.

3. Mix and heat at low-medium heat for approximately 40 min or until the boric acid goes into solution.

4. Cool the solution (leave at the room temperature) until it can be handled. If the boric acid solution cools too much it will precipitate out of solution; if this happens just remix and reheat. To prevent precipitation do not let the solution completely cool.

5. Using deionized water, bring the final combined boric acid/KOH solution to a volume of 1.3 L.

6. To this solution, add KOH until the pH reaches 9.5.

7. Set aside 1 L of the solution and filter through a 0.22 µm filter.

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

1. Using the surplus Solution A-2 M potassium borate buffer, continue adding KOH until pH 10.5 is reached.

2. Measuring the volume at which pH 10.5 was reached, calculate the amount of EDTA that must be added to make 5 mM EDTA (5 mM EDTA = 1.46 g/L). Add in and mix.

3. Filter the solution through a 0.22 µm filter.

0.1 M Potassium Borate Buffer (pH 9.5, containing 2 mM EDTA) (Solution C)

1. Using Solution A, make a 1:20 dilution (50 mL of Solution A + 950 mL of deionized water).

2. Add 0.585 g EDTA to make 2 mM EDTA (0.585g/L EDTA solution).

3. Filter through a .22 um filter.

0.1 M Potassium Phosphate Buffer (pH 6.5, containing 2% methanol) (Buffer A)

1. Make 1 L of 0.1 M potassium phosphate dibasic (K_2HPO_4) by dissolving 17.418 g in a final volume of 1 L with deionized water.

2. Make 1 L of 0.1 M potassium phosphate monobasic (KH_2PO_4) by dissolving 13.61 g in a final volume of 1 L with deionized water.

3. Turn on and calibrate the pH meter using the pink pH 4.0 buffer as buffer 2. To 1 L of 0.1 M KH_2PO_4 add 0.1 M K_2HPO_4 until the pH is 6.5.

4. Measure the amount of pH 6.5 phosphate solution in mL – this is the "buffer amount", used next. Calculate the amount of methanol to be added in order to have a phosphate buffer pH 6.5, 5% methanol final solution.

5. Use the following formula:

   Buffer amount/0.95 = Final amount.
   Final amount – buffer amount = methanol to be added.
   Add the proper amount of methanol and filter through a 0.22 μm filter.

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

1. Make a 10% solution by adding 100 mg TCEP to 1 mL of deionized water in a microcentrifuge tube – weigh directly into the tube.

2. Vortex until the TCEP is dissolved. Each sample uses 20 μL of TCEP solution so adjust the amount of the solution based on the number of samples. For example, if 30 samples and standards are to be run you need a minimum of 30 x 20 μL = 600 μL of TCEP solution.

0.6 M Perchloric Acid

1. Add about 200 mL deionized water into a 500 mL volumetric flask.

2. Add 25.75 mL of 70% perchloric acid to the flask and mix.

3. Bring to volume.

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

1. Fifty µL of SBDF solution is needed per run. Determine how many samples and standards are to be analyzed. For example, 30 runs will need at least 1.5 mL of SBDF solution. Therefore weigh out between 1.7 and 2.0 mg of SBDF. Try to weigh between 0.2 to 0.5 mg more than the minimum amount of SBDF required into a 16x100 mm glass test tube.

2. Add the appropriate amount of Solution A to make 1 mg/mL.

3. Seal with parafilm and place on a 60 ^{o}C heating block.

4. Incubate for 10 min and vortex. Ensure that all of the SBDF is dissolved.

Homocysteine Standard Solutions

1. Prepare a 2 mM L-cysteine solution. Weigh 24.2 mg of L-cysteine into a weighboat and using a Pasteur pipette, rinse down the grains of L-cysteine from the weighboat into a 100 mL volumetric flask with Solution C.

2. Fill two thirds full with Solution C. Swirl until all of the L-cysteine has dissolved.

3. Bring to volume with solution C. Make the solution homogenous by sealing the top with parafilm and swaying 5 times.

4. Prepare a 2 mM DL-homocysteine solution. Weigh 27.0 mg of DL-homocysteine into a weighboat and, using a Pasteur pipette, rinse down the grains into a 100 mL volumetric flask with Solution C.

5. Fill two thirds full with Solution C. Swirl until all of the DL-homocysteine has dissolved.

6. Bring to volume with solution C. Make the solution homogenous by sealing the top with parafilm and swaying 5 times.

7. Prepare the 100% standard. A common 100% standard is 40 µM homocysteine and 400 µM cysteine. To prepare 5 mL combine 100 µL 2 mM homocysteine and 1000 µL 2 mM cysteine with 3900 µL Solution C in a 16x100 mm test tube. Since it is difficult to exactly weigh 24.2 mg of cysteine and 27.0 mg of homocysteine, it is easier to obtain weights that are close and then adjust the volumes to make the 100% solution accordingly.

8. Using 16x100 mm test tubes, make the following dilutions:

#5 100%, already prepared
#4 75 % = 750 uL of 100% + 250 uL Solution C
#3 50 % = 500 uL of 100% + 500 uL Solution C
#2 25 % = 250 uL of 100% + 750 uL Solution C
#1 0 % = 1000 uL of Solution C

Dihydrofolate Reductase (DHFR) Assay

Principle of the Assay

The assay is based on the ability of DHFR to catalyze the reversible NADPH-dependent reduction of dihydrofolic acid (DHF) to tetrahydrofolic acid (THF).

$$DHF + NADPH + H^+ \longleftrightarrow THF + NADP^+$$

At pH 7.5, the equilibrium of the reaction lies relatively far to the right, and the direction goes essentially to completion in the forward reaction. The reaction progress is monitored by the decrease in absorbance at 340 nm.

1. Turn on the spectrophotometer and set the parameters to 340 nm and 22 ^{o}C, kinetic program (reading every 15 sec for 2.5 min)

2. Prepare the enzyme blanks by adding 974 µL assay buffer (1x), 20 µL tissue extract, 6 µL 10 mM NADPH for Blank 1 and 975 µL assay buffer (1x), 20 µL tissue extract, 5 µL 10 mM dihydrofolic acid (DHF) for Blank 2 in a microcentrifuge tube, and mix well.

3. Transfer the content of the tubes to a 1 mL quartz cuvette.

4. Cover the cuvette with parafilm and mix by inversion.

5. Start the kinetics program immediately by measuring the decrease in ΔOD (change in absorbance) during the 2.5 min.

6. Pipette 969 µL assay buffer (1x) and 20 µL tissue extract to a microcentrifuge tube, and mix well.

7. Transfer the content of the tube to a 1 mL quartz cuvette.

8. Add 6 µL of 10 mM NADPH solution.

9. Cover the cuvette with parafilm and mix by inversion.

10. Add 5 µL 10 mM DHF just before starting the reaction (DHF is the substrate of the reaction).

11. Cover the cuvette with parafilm, mix by inversion and immediately insert the cuvette into the spectrophotometer.

12. Start the kinetics program immediately. The absorbance at 340 nm will decrease (due to decrease in NADPH concentration).

13. Follow the same reaction scheme with all the other tissue samples.

Activity Calculation:

$$\text{Activity} = \frac{(\Delta OD/min_{sample} - (\Delta OD/min_{blank}) \times d}{12.3 \times V \times g \text{ of tissue}}$$

$\Delta OD/min_{blank}$: $\Delta OD/min$ for the blank, from the spectrophotometer readings
$\Delta OD/min_{sample}$: $\Delta OD/min$ for the reaction, from the spectrophotometer readings
12.3: extinction coefficient for the dihydrofolate reductase reaction at 340 nm
V: enzyme volume in mL (the volume of enzyme used in the assay)
d: the dilution factor of the enzyme sample

Assay Buffer (1x)

1. Dilute the assay buffer (10x) ten fold in deionized water (i.e. add 5 ml assay buffer 10x to 45 ml water). Keep at room temperature.

10 mM Dihydrofolic acid (DHF)

1. Prepare a 10 mM stock solution at pH 7.5 by the addition of 2.2 ml assay buffer 10x to the DHF bottle (i.e. add 2.2 ml assay buffer (10x) to 10 mg powder), and mix well.

2. Aliquot the 10 mM DHF stock solution and store at -20 ^{o}C. The solution is stable for 5 days at -20 ^{o}C. Unused thawed solutions should be discarded the same day.

10 mM NADPH stock solution

1. Prepare a 10 mL suspension buffer by adding 0.2 mL assay buffer (10x) to 9.8 mL water.

2. Add 3 mL of the suspension buffer to the NADPH bottle. Mix well and divide this 10 mM NADPH stock solution into working aliquots and store at -20 ^{o}C. The solutiom is stable for at least one month at -20 ^{o}C.

Serine Hydroxymethyltransferase (SHMT) Assay

Principle of the Assay

Based on the reaction THF + serine ↔ glycine + N^5,N^{10}-[^{14}C]methylene-THF, the label from [^{14}C]serine is transferred by SHMT to tetrahydrofolate (THF) to form N^5,N^{10}-[^{14}C]methylene-THF. The labeled THF derivative is bound to DEAE-cellulose paper. By washing the paper, the unreacted labeled serine is removed and the remaining radioactivity is counted.

1. On the cellulose paper, 3x3 cm of squares are drawn using pencil and then soaked in 2.5 mM EDTA, pH 7.6 for 1 hr. After drying, the paper can be kept for several days. This procedure is undertaken before the day of assay.

2. Add 25 μL of tissue extract in duplicates to microcentrifuge tubes. Add 25 μL of homogenizing buffer to 2 tubes to serve as blanks.

3. Add 50 μL of reaction cocktail.

4. Add 25 μL of serine. (Note: The final reaction mixture contains 0.4 mM serine, 2 mM THF, 2.5 mM EDTA, 1 mM 2-mercaptoethanol, and 0.25 mM PLP and 25 μL of tissue extract).

5. Vortex.

6. Load to the heating block at 37 °C and heat for 10 minutes.

7. Reaction is stopped by streaking a 25 μL aliquot onto a labeled square of DE-81 filter paper (3x3 cm squares). Do not puncture a hole and not be too close to the rim.

8. Wash the paper with running distilled water at 2-3 L/min for 20 min.

9. The paper is dried for several hours and then cut into squares.

10. Count the radioactivity of the paper using a liquid scintillation counter.

0.25 M sucrose and 10 mM Tris HCl (pH 8) (homogenizing buffer)

1. Weigh and dissolve 85.58 g sucrose, 1.21 g Trizma base to a beaker. Add water up to almost 1 L.

2. Adjust pH to 8 using several drops of 6 N hydrochloric acid (HCl) (4.92 mL HCl + 5.08 mL water). Add water up to 1 liter.

50 mM Tris and 1 mM 2-mercaptoethanol (pH 8) (working buffer)

1. Weigh 6.06 g Trizma base to a beaker. Add 70 μL of 2-mercaptoethanol and water.

2. Adjust pH to 8 using 6N HCl. Add water up to 1 L. Store in the fridge.

1.6 mM ^{14}C Serine, L-[^{14}C(U)]-Serine, (50 μCi)

1. Weigh 42.04 mg serine to make 250 mL of 1.6 mM serine solution using the 50 mM Tris buffer containing 1 mM 2-mercaptoethanol (pH 8.0).

2. Take 2.5 mL of the solution to a scintillation vial and add 100 μL of [U-^{14}C] serine into it. Count and store in the fridge (This is based on that 25 μL of serine with [U-^{14}C] labelling is added to the reaction system, which provides ~270,000 cpm). When 3-^{14}C serine is used, the counting of 25 μL of serine should give 90,000 cpm/25 μL.

8 mM Tetrahydrofolic acid (THF)

1. Dissolve 25 mg THF in 7 mL of 50 mM Tris buffer containing 1 mM 2-mercaptoethanol (pH 8.0). This is loaded into microcentrifuge vials and stored at -80 °C.

2.5 mM EDTA (pH 7.6) (for soaking paper)

1. Weigh 730.6 mg EDTA and dissolve in water with medium-high heating.

2. Adjust pH with sodium hydroxide (NaOH) solution to pH 7.6 (one pellet of NaOH is dissolved in a tiny beaker). One or several drops are added.

65 mM EDTA (pH 8)

1. Add about 30 mL of the Tris buffer to a beaker and heat. Weigh 949.8 mg EDTA and transfer to the beaker.

2. Adjust pH to 8.0 using pellets of NaOH. Cool down and top up with the buffer to 50 mL.

6.5 mM Pyridoxal Phosphate (PLP)

1. Add 1.61 mg PLP to a microcentrifuge tube and dissolve with 1 mL of the Tris buffer.

Reaction cocktail containing 4.0 mM THF, 5 mM EDTA, 1 mM 2-mercaptoethanol, and 0.50 mM PLP

1. Depending on the total number of samples, the volume of reaction mixture is adjusted.

2. For example, in 24-26 samples, around 1.3 mL of the mixture is needed. Mix 650 µL of 8 mM THF solution and 100 µL of 65 mM EDTA to a microcentrifuge tube.

3. Add 100 µL of 6.5 mM PLP and 450 µL of the Tris buffer to make 1.3 mL of the mixture.

5, 10-Methylene Tetrahydrofolate Reductase (5, 10-MTHFR) Assay

Principle of the Assay

Under physiologic conditions, the catalytic reduction of 5,10-methyleneTHF to 5-methylTHF by 5,10-MTHFR is considered to be essentially non-reversible (i.e., the oxidation of 5-methylTHF back to 5,10-methyleneTHF) although there is evidence from studies of this enzyme in human leukemia cells that this pathway may be reversible. However, under *in vitro* condition the oxidation of [^{14}C]5-methylTHF to [^{14}C]5,10-methyleneTHF using menadione as the electron acceptor is possible ([^{14}C]5-methylTHF + menadione $\rightarrow$ [^{14}C]5,10-methyleneTHF + menadiol). Since 5,10-methyleneTHF readily dissociates into formaldehyde (HCHO) and THF, the [^{14}C]HCHO generated by this process can be trapped by complexing it with dimedone, and its radioactivity measured.

1. On labeled microcentrifuge tubes, add 300 µL of the reaction cocktail in duplicates.

2. Add 50 µL 0.24 mM 5-methylTHF and 50 µL 42 mM menadione to each individual tube.

3. Add 200 µL tissue extract to initiate the reaction. (Note: The final reaction mixture contains 0.18 M potassium phosphate buffer (pH 6.8), 1.15 mM EDTA (pH 7.0), 11.5 mM ascorbic acid, 54 µM FAD, 20 µM 5-methylTHF, 3.5 mM menadione, and 200 µL tissue extract).

4. In two separate microcentrifuge tubes, add 200 µL of homogenizing buffer to serve as blanks.

5. Vortex.

6. Incubate the reaction mixture for 1 hr at 41 ºC.

7. Terminate the incubation by adding 10 µL of 1 M carrier formaldehyde, 200 µL of 50 µM dimedone, and 100 µL of 3 M potassium acetate (pH 4.5).

8. Heat the reaction mixture at 95 ºC for 15 min, after which let it cool on ice for approximately 10 min.

9. Add 3 mL toluene to the reaction mixture and vortex vigorously for 15 sec.

10. Centrifuge at low speed (3000 rpm for 5 min) and take aliquot of 2 mL of the toluene phase.

11. Count the radioactivity of the toluene phase using a liquid scintillation counter.

<u>0.1 M Potassium Phosphate Buffer (pH 7.4) (homogenizing buffer)</u>

1. Prepare 0.1 M potassium phosphate dibasic (K_2HPO_4) by weighing and dissolving 8.70 g K_2HPO_4 to a final volume of 500 mL water.

2. Prepare 0.1 M potassium phosphate monobasic (KH_2PO_4) by weighing and dissolving 6.80 g KH_2PO_4 to a final volume of 500 mL water.

3. Place the 500 mL 0.1 M KH_2PO_4 into a 1 L beaker with a magnetic stirrer and insert a pH electrode. Add the 0.1 M KH_2PO_4 slowly to adjust the pH to 7.4.

<u>0.72 M Potassium Phosphate Buffer (pH 6.8) (working buffer)</u>

1. Prepare 0.72 M potassium phosphate dibasic (K_2HPO_4) by weighing and dissolving 62.64 g K_2HPO_4 to a final volume of 500 mL water.

2. Prepare 0.72 M potassium phosphate monobasic (KH_2PO_4) by weighing and dissolving 48.99 g KH_2PO_4 to a final volume of 500 mL water.

3. Place the 500 mL 0.72 M KH_2PO_4 into a 1 L beaker with a magnetic stirrer and insert a pH electrode. Add the 0.72 KH_2PO_4 slowly to adjust the pH to 6.8.

<u>11.5 mM EDTA (pH 7.0)</u>

1. Weigh 336.1 mg EDTA and dissolve in 100 mL of 0.72 M potassium phosphate buffer (pH 6.8) with medium-high heating.

2. Adjust pH with sodium hydroxide (NaOH) solution to 7. One or several drops are added.

<u>0.115 M Ascorbic Acid</u>

1. Weigh 2.03 g of L-ascorbic acid and dissolve in 100 mL of 0.72 M potassium phosphate buffer (pH 6.8).

1.08 mM Flavin Adenine Dinucleotide (FAD) (Disodium Salt Hydrate)

1. Weigh 22.4 mg of FAD (disodium salt hydrate) and dissolve in 25 mL of 0.72 M potassium phosphate buffer (pH 6.8). This is loaded into microcentrifuge vials and stored at -20 ºC.

0.24 mM [^{14}C]5-methylTHF (50 mCi/mmol, barium salt)

1. Weigh 3.03 mg 5-methylTHF (disodium salt) and dissolve in 25 mL of 0.72 M potassium phosphate buffer (pH 6.8). This serves as the stock solution and should be stored at -80 ºC when not in use.

2. Take 5 mL of the stock solution to a scintillation vial and add 100 µL of [^{14}C] 5-methylTHF and count the radioactivity in the liquid scintillation counter. Adjust the radioactivity to provide 800,000-900,000 cpm/50 µL of 0.24 mM [^{14}C] 5-methylTHF in the reaction mixture.

42 mM Menadione (menadione sodium bisulfite)

1. Weigh 1.16 g of menadione sodium bisulfite and dissolve in 100 mL of 0.72 M potassium phosphate buffer (pH 6.8).

1 M Formaldehyde

1. Measure 300.3 µL of formaldehyde and dissolve in 10 mL of water.

50 µM Dimedone

1. Weigh 7.01 mg dimedone and dissolve in l L of ethanol.

3 M Potassium Acetate Buffer (pH 4.5)

1. Weigh and dissolve 14.7 g of potassium acetate to 20 mL water and slowly add glacial acetic acid.

2. Adjust pH to 4.5 by adding water or glacial acetic acid to make 50 mL.

Reaction cocktail containing 0.36 M potassium phosphate (pH 6.8), 2.3 mM EDTA (pH 7.0), 23 mM ascorbic acid and 108 µM FAD

1. Depending on the total number of samples, the volume of the reaction mixture is adjusted.

2. For example, in 24-26 samples, 7.8 mL of the mixture is needed. Mix 1.56 mL of 11.5 mM EDTA and 1.56 mL of 0.115 M ascorbic acid in a 16x100 test tube.

3. Add 780 µL of 1.08 mM FAD and 3.9 mL of 0.72 M potassium phosphate buffer (pH 6.8) to make 7.8 mL of the mixture.

Methionine Synthase Assay

Principle of the Assay

Methionine synthase enzyme catalyzes the methyl group transfer from 5-methyltetrahydrofolate (5-methyTHF) to homocysteine (Hcy) to give methionine and tetrahydrofolate (THF). This assay is established to monitor the transfer of a ^{14}C-methyl group from the N^5-position of 5-methylTHF to Hcy to produce ^{14}C-methionine.

1. 100 µL aliquots of the reaction mixture are transferred to microcentrifuge tubes in duplicates.

2. Add 100 µL of tissue extract. (Note: The final incubation mixture contains 20 µM cyanocobalamin, 58 mM D, L-dithiothreitol, 0.5 mM S-adenosylmethionine, 15 mM D, L-homocysteine, 14 mM β-mercaptoethanol, 1 mM 5-methylTHF with 0.25 µCi of [5-^{14}C]5-methylTHF, 175 mM phosphate buffer (pH 7.3) and 100 µL tissue extract).

3. Top the samples with nitrogen and capped and incubate in 41 ºC heating block for 15 min.

4. Add 400 µL of ice cold deionized water to stop the reaction and immediately place the samples on ice.

5. Add 100 µL of homogenizing buffer to 2 tubes to serve as blanks. Blanks were treated identically to the samples except that the cold water was added prior to the supernatants and blanks were applied directly to the column with no incubation.

6. Drain the entire reaction mixture on top of AG1-X8 (200-400 mesh) chromatography column.

7. Wash the column 3x with 500 µL of deionized water and collect about 2.1 ml of total effluent in scintillation vials.

8. Count the radioactivity using a liquid scintillation counter.

0.05 M Potassium Phosphate Buffer (pH 7.3) (homogenizing buffer)

1. Prepare 0.05 M potassium phosphate monobasic (KH_2PO_4) solution by weighing and dissolving 3.4 g KH_2PO_4 to a final volume of 500 mL of water.

2. Prepare 0.05 M potassium phosphate dibasic (K_2HPO_4) solution by weighing and dissolving 4.35 g K_2HPO_4 to a final volume of 500 mL of water.

3. Place the 500 mL 0.05 M KH_2PO_4 into a 1 L beaker with a magnetic stirrer and insert a pH electrode. Add the 0.05 M KH_2PO_4 slowly to adjust the pH to 7.3.

Reaction cocktail containing 175 mM phosphate buffer (pH 7.3), 20 µM cyanocobalamin, 58 mM D, L-dithiothreitol, 0.5 mM S-adenosylmethionine, 15 mM D, L-homocysteine, 14 mM β-mercaptoethanol, and 1 mM 5-methylTHF

1. In approximately 50 mL of 0.05 potassium phosphate buffer (pH 7.3) dissolve 3 mg cyanocobalamin, 233 mg D, L-homocysteine, and 112.9 µL β-mercaptoethanol. Bring the reaction mixture to final volume of 100 mL using the 0.05 potassium phosphate buffer (pH 7.3).

2. Weigh 2 mg of S-adenosylmethionine directly into a small beaker. Using a pipette, add 4 mL of the reaction mixture prepared in Step #1.

3. Weigh 89.4 mg dithiothreitol into a weigh-boat and wash it into the same small beaker using 4 mL of the reaction mixture prepared in Step #1. Cover and set aside.

4. Add 1.981 g sodium ascorbate to approximately 70 mL of deionized water. Bring to a final volume of 100 mL using deionized water to make a 0.1 M sodium ascorbate buffer.

5. Weigh 4.98 mg 5-methylTHF (calcium salt) into a microcentrifuge tube and add 1 mL of 0.1 M sodium ascorbate. Vortex solution.

6. Add the entire 1 mL contents of the eppendorf to the 8 mL reaction mixture solution set aside in Step #3. Bring the complete reaction mixture to a final volume of 10 mL using the solution prepared in Step #1.

Intestinal Everted Sac Method

1. Fast the chicken overnight.

2. On the day of the experiment, kill the fasted chicken by cervical dislocation.

3. From their abdomen, remove the whole digestive tract and isolate the duodenum, jejunum, ileum, and cecum immediately. Rinse each intestinal section free of their digestive contents using oxygenated physiological saline.

4. Cut approximately 4 cm segment from each region and evert immediately by a glass rod.

5. Tie each length of the everted segment on both ends by a thread ligature. Make sure that one end is not fully tighten by the ligature to allow the tuberculin syringe to infuse oxygenated 1 mL Krebs-Ringer bicarbonate buffer supplemented with 20 mM HEPES (pH 6.0), 10 mM glucose, and 15 mM sodium ascorbate.

6. After infusion, tighten both ends of the segment to form the intestinal sac.

7. Transfer the intestinal sac to a 50 mL conical flask and incubate in 20 mL of Krebs-Ringer-bicarbonate buffer solution (mucosal medium) at 41°C and a shaking rate of 100 strokes/min.

8. Throughout the incubation period, oxygenate the intestinal sac continuously with 95% O_2-5% CO_2 gas.

9. Add trace amounts of 100,000 cpm/50 µL of [^{3}H]FA (250 µCi), 20,000 cpm/50 µL of 1.25 µM [1,2-^{14}C]polyethylene glycol 4000 (0.7 mCi); as a non-absorbable marker, and unlabeled folic acid (to adjust folic acid concentration) in the mucosal medium.

10. Incubate an intestinal sac without adding labeled folic acid in the mucosal medium for each measurement to deduct the background folic acid uptake.

11. At the end of incubation, remove the sac from the conical flask and briefly wash with ice-cold saline to stop the uptake process.

12. Drain the sac content (serosal fluid) into individual scintillation vials and count for radioactivity.

13. Determine the uptake in the mucosal tissue of the sac by counting the radioactivity after solubilization of the tissue sample using 2 mL of Soluene-350 as a tissue solubilizer.

14. Calculate the mucosal to serosal uptake of folic acid by adding the uptake in the mucosal tissue and the serosal transfer express in per 100 g of wet tissue weight of the intestinal segment.

Krebs-Ringer-bicarbonate buffer (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 10 mM glucose)

Krebs-Ringer Bicarbonate (10x) (stock solutions) (should be stored in refrigerator)

10x Salt Stock

1. Weigh and dissolve 68.96 g sodium chloride (NaCl), 3.50 g potassium chloride (KCl), 1.40 g magnesium sulphate ($MgSO_4$), and 1.63 g potassium phosphate monobasic (KH_2PO_4) in 1 L of deionized water.

10x Calcium Stock

1. Weigh and dissolve 2.78 g calcium chloride ($CaCl_2$) (anhydrous) in 1 L of deionized water.

10x Bicarbonate Stock

1. Weigh and dissolve 21.0 g sodium bicarbonate ($NaHCO_3$) in 1 L of deionized water.

Krebs-Ringer Bicarbonate (working solution)

1. Add 100 mL of salt stock + 200 mL deionized water. Then, add 100 mL of 10x calcium stock + 200 mL deionized water. Finally, add 100 mL of 10x bicarbonate stock. Prepare the solution daily and discard at the end of the experiment.

2. Add 1.8 g of α-D-Glucose.

3. Add 3.90 g 2-(N-morpholino)ethanesulfonic acid (MES) or 4.77 g 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) to provide 20 mM concentration of each and fill with deionized water to 1 L total volume. When the effect of pH is examined, 20 mM MES is use for pH below 5.5 and 20 mM HEPES is use for pH 6.0 and above. Adjust the pH using 1 N HCl or 1 N NaOH

200 µM Folic Acid (stock solution)

1. Weigh and dissolve 44.2 mg folic acid to a final volume of 500 mL Kreb-Ringer-Bicarbonate buffer. From this stock, prepare the different folic acic concentrations required for the experiment. Stock solutions when not in use should be stored at -80 ºC.

1.25 µM PEG

1. To make 50 ml of 1.25 mM PEG, weigh 250 mg PEG (FW=4000) and dilute to a final volume using Kreb-Ringer-Bicarbonate buffer. Stock solutions when not in use should be stored at -80 ºC.

Labeled Folic Acid (^{3}H-folic acid) (250 µCi) and Polyethylene Glycol (^{14}C-PEG) (0.7 mCi)

1. Dissolve labeled folic acid (^{3}H-folic acid) and polyethylene glycol (^{14}C-PEG) in their corresponding unlabeled solutions. Count the radioactivity of each isotope in the liquid scintillation counter so that 50 µL of ^{3}H-folic acid provides 100,000 cpm; and 50 µL of ^{14}C-PEG provides 20,000 cpm when added to 20 mL mucosal medium (Wingate et al., 1972; Helman and Barbezat, 1978).

Cell Viability (Trypan Blue Assay)

1. Scrape the mucosa of the intestinal everted sac with a glass slide to harvest the mucosal cells.

2. Add a volume of Dulbecco Ca^{2+} and Mg^{2+} free PBS (Sigma Aldrich) equal to about 10 times the volume of the harvested cells. Agitate the tube to suspend the cell debris.

3. Allow the fragments to settle to the bottom of the tube and then decant the supernatant fluid.

4. Suspend the cell fragments in an equal volume of Dulbecco Ca^{2+} and Mg^{2+} free PBS.

5. Centrifuge a 1 mL aliquot of the cell suspension for 5 min at 100 x g and discard the supernatant.

6. Resuspend the cell pellet (~ 50 µL) in 1 mL of Dulbecco Ca^{2+} and Mg^{2+} free PBS solution to obtain a cell suspension containing approximately 1×10^{6} cells/mL.

7. Mix 15 µL of 0.4% trypan blue and 15 µL of cell suspension (dilution of cells). Pipette up and down several times to ensure a uniform cell suspension using the same pipette tip.

8. Allow mixture to incubate for approximately 3 min at room temperature. (Cells should be counted within 3 to 5 min of mixing with trypan blue, as longer incubation periods will lead to cell death and reduced viability counts.

9. Apply a drop of the trypan blue/cell mixture to a hemacytometer.

10. Load the hemacytometer. Place the coverslip over the counting chambers. Use only the coverslip provided by the manufacturer of the chamber. Load both counting chambers with the diluted cell suspension. Approximately 10 µL will be required per chamber. Place the pipette tip at the edge of the coverslip, and allow the cell suspension to fill the space by capillary action. Fill the entire volume of the chamber, but do not overfill.

11. Count the unstained (viable) and stained (nonviable) cells separately in the hemacytometer. View the cells under a microscope at 100x magnification. The cells should be visible above the grid of the counting chamber.

12. Calculate the percentage of viable cells as follows:

    viable cells (%) = total number of viable cells per mL of aliquot ÷ total number of cells per mL of aliquot x 100