FOLATE ABSORPTION ACROSS THE HUMAN COLON: CAPLET STUDY

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science

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2010

Abstract

The purpose of this study was to determine the absorption of $^{13}$C$^5$-
formyltetrahydrofolic acid across the human colon with an intact microflora. Bioavailability was
determined after administration of a pH-dependent acrylic co-polymer coated barium sulphate
caplet containing 855 nmol and measuring the plasma appearance of $^{13}$C$^5$-
methyldtetrahydrofolic acid compared to an intravenous injection of the same compound (214
nmol). Blood samples serially collected after both test doses were analyzed by microbiological
assay and tandem mass spectrometry to determine total folates and ratio of labeled to unlabeled
folates, respectively. Caplet disintegration in the colon was quantitatively assessed by
fluoroscopic imaging for six of nine subjects. The plasma $^{13}$C$^5$-methyltetrahydrofolic acid
appearance rate was $4 \pm 0.9$ nmol/h (intravenous) and $0.35 \pm 0.02$ nmol/h (caplet). Mean
apparent absorption across the colon was 35%. These findings suggest that physiological doses
of natural folate are absorbed across the human colon in the presence of an undisturbed
microbiota.
Acknowledgments

I am so grateful to the following people:

Thank you first and foremost to my supervisor Dr. Deborah L. O’Connor for the incredible opportunity to learn so much about folate. Without her expert insight, feedback, ongoing encouragement and mentoring of juggling life events while doing research, the completion of this thesis would not have been possible. Aneta, I also cannot adequately express my heartfelt thank you for her contagious optimistic outlook, kindness and determination to help in any way shape or form. Thank you to the O’Connor lab, all who have contributed greatly to this thesis: Dubraicka Picchardo for her laboratory skills, Yaseer Shakur, Sarah Farmer, Brenda Hartman, Andrea Nash and Jimmy Yang for their continuous collaboration. Susanne Aufreiter and Ashley Aimone whose successful research paved the way for this study. I would still be at the very beginning without their patience and direction in answering my many questions. Onwards and upwards!

Additional thanks to my masters and examination committee: Drs Paul B Pencharz, TMS Wolever, Bairbre Connolly, Young-In Kim and Wendy Ward for their input and critique of this work. Louisa Kung, graduate program administrator. Drs Christine Pfeiffer, Zia Fazili and Jesse Gregory 3rd, for their valuable and intellectual contributions. Thank you also to: Jamie Lugtu, Frank Martinuzzi, and Surinder Chaudhary at the Toronto Institute of Pharmaceutical Technology for their caplet work, as well as Rudolf Moser and Jacqueline Farner of Merck-Epurova, Switzerland for supplying the labeled folate.

I am indebted to all the phenomenal staff at the Hospital for Sick Children who facilitated this study. In particular research pharmacist Mark Bedford and nurses Karen Chapman, Roberta Gardiner, Castle Clyde and Marcia Wilson. Daksha Jadav and Kassa Beimnet at the Core Laboratory, Albert Aziza and Sally Gopaul-Mattook at the Image Guided Therapy Department. Also, Desta Ramlackhansingh for helping with the Health Canada submission and Jennifer Brady, for food analyses. Thank you to those who participated in this study and their substantial time commitment.

Sincere appreciation to my friends and family, whose inspiration and love mean everything. Rose Turshen-Drybala for knowing me better then even I do myself. Adriel O’Malley, for always believing that I would finish. Eunice Ireland for her prayers. Cathy Ireland, for her overwhelming generosity, quick problem solving during any arising crisis and of course celebration, especially when achieving the “almost” impossible. Grazia and Bob Lakoff, for their endless support. Josh Lakoff, for empowering me to laugh daily, love more and reminding me to strive for simplicity.

Valerie Miller Elliot, who remains the wind beneath my wings.
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List of Abbreviations

AUC – area under the curve
BMI – body-mass index
CBC – complete blood count
DFE – dietary folate equivalent
FR – folate receptor
GCPII – glucose carboxypeptidase II
IV – intravenous
LC-MS/MS – liquid chromatography with tandem mass spectrometry
MTHFR – methylenetetrahydrofolate reductase
NTD – neural tube defect
PABA – para-amino benzoic acid
PCFT – protein-coupled folate carrier
RBC – red blood cell
RFC – reduced folate carrier
SAM – S-adenosylmethionine
THF – tetrahydrofolic acid
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Chapter 1

1. Overall Introduction

Folate, a B-vitamin, is a generic term that describes the family of compounds that share a common structural core: pteroylglutamic acid. In the last twenty years, folate has received significant attention due to its preventative role in reducing the incidence of neural tube defects (NTD) through periconceptional supplementation, with the synthetic form of the vitamin (folic acid). These observations eventually led to the mandatory folic acid fortification of the North American food supply in 1998 (1-3). Despite a subsequent decline (approximately 50%) in the incidence of NTDs after folic acid fortification (4-7), many countries have not adopted this public health initiative over concern that too much folic acid may have adverse effects on some sectors of the population (4, 8-10). There are concerns that folic acid fortification alone or in combination with folic acid supplementation may result in supraphysiological levels of circulating folic acid which in turn modify health risks. Proposed negative health risks include: (1) facilitating the progression of pre-neoplastic cells to cancer, (2) neurological deterioration from delayed diagnosis of vitamin $B_{12}$ deficiency, (3) altered immune function as a result of decreased natural killer cell activity, (4) increased obesity and insulin resistance in children of folic acid supplemented mothers, and (5) a reduction in antifolate drug efficacy used in the treatment of cancer, arthritis, psoriasis, and malaria (4, 8, 10-18). Given the growing body of evidence suggesting the healthful intake of folate for one person may be potentially harmful to another, it is critical to comprehensively examine all “input” sources of folate to set dietary and supplement recommendations that strike the right balance between health benefits and risks.
Current recommendations for dietary folate intake are based solely on oral intake and ignore the potential contribution of microbally synthesized folate (1, 19). The substantial size of the folate depot in the colon has been recognized since the 1950s with quantities known to approach or exceed recommended dietary intakes (20-22). Earlier studies provided evidence to suggest that folate could be absorbed across the colon of rats and pigs (23-27) and colonocytes in vitro (28-30). Recently, Aufreiter et al. demonstrated that folate could indeed be absorbed across the intact human colon (19).

In the study by Aufreiter et al., during routine screening colonoscopy, 684 nmol of $[^{13}\text{C}]$-[6S]-glutamyl-5-formyl-tetrahydrofolic acid (THF) was infused directly into the cecum of six healthy adults to assess folate absorption and labeled folate was shown to be absorbed into plasma at a mean appearance rate of 0.6 ± 0.2 nmol/h. As an invasive bowel cleansing protocol was used prior to colonoscopy including consumption of the standard polyethylene glycol solutions, preparation for colonoscopy significantly disrupted the microbial milieu (31). It remains unknown whether, or not, an administrated test dose delivered specifically to the colon with an intact microflora would be bioavailable for absorption. An intact microflora may influence absorption of the test dose by multiple physiological events such as: competition of labeled versus unlabeled microbial synthesized folate for transport across the colonocytes, utilization of the test dose by folate requiring bacteria, or the test dose becoming trapped within cellular dietary matrices. Further, an acknowledged limitation of the Aufreiter et al. study was that following cecal infusion of the $[^{13}\text{C}]$5-formylTHF test dose, blood samples were collected for only four hours (19). During this time, the appearance of the metabolite of $[^{13}\text{C}_2]$5-formylTHF, $[^{13}\text{C}_2]$5-methylTHF, rose in a linear fashion and did not return to baseline suggesting that absorption of folate, while slow, occurred down the length of the colon.
Because the plasma response did not return to baseline over the course of the study, it was not possible to determine how much of the test dose was absorbed. The authors concluded that in future studies blood sampling should continue well beyond 4 hours.

In order to non-invasively assess the impact of folate absorption across the human colon with an intact microbial milieu, we recently developed a pH-dependent acrylic co-polymer coated caplet to quantitatively deliver a test dose of $^{13}$C-labeled folate past the ileocecal junction (32). In the present study we incorporated a physiological dose of 855 nmol (400 µg) $[^{13}$C$]5$-formylTHF within the barium sulphate core of this previously tested pH-sensitive caplet. In vivo caplet transit and disintegration was monitored by fluoroscopy. The primary purpose of this thesis was to directly evaluate whether, and to what extent, the natural folate vitamer 5-formylTHF administered non-invasively to the colon of healthy adult participants containing an intact microflora can be detected in plasma by the appearance of labeled 5-methylTHF.

Secondly, blood samples were collected serially after the caplet passed the pyloric sphincter for up to 14 hours post-caplet ingestion, at 24 and 48 hours. This timeline was to more accurately reflect the longer colonic transit time and observe the labeled response return to baseline, unlike the previous colonoscopy study. We intended to calculate the area under the curve (AUC) of the oral caplet test dose compared to an intravenous (IV) injection of the same test compound (214 nmol) and thereby determine the percent relative bioavailability. The findings reported in chapter three of this thesis were obtained from nine healthy adults.
Overview of Literature Review

In the literature review that follows, the first section describes the biochemical structure of folate and its critical role in one-carbon metabolism and specifically DNA and protein synthesis (1). In the second section, there is an overview of how folate is absorbed across the small intestine, as well as a discussion of the primary folate transporters: the reduced folate carrier (RFC) and the proton-coupled folate transporter (PCFT) (33). These transporters are also expressed in the colon and the current indirect and direct evidence describing folate absorption across the colon of animals and humans are explored in the context of the physiology of the colon. A comprehensive understanding of the transport mechanisms supporting folate intestinal absorption is key to exploring how our test folate dose delivered past the ileocecal junction could be potentially absorbed across the colon and measured in plasma as labeled 5-methylTHF.

The third and forth section of the literature review, provides an explanation of how various factors, such as dietary components and medications, influence folate bioavailability (34). Additionally, the methods for measuring folate bioavailability using acute and chronic experimental protocols are summarized. The next section explores the complexities of administering a test dose to the colon and the various systems available (e.g. oral caplet ingestion or rectal infusion). Finally, in the sixth section of this comprehensive review, the clinical implications of sub-optimal, adequate and supra-physiological folate status are summarized and include folate reference values for the purpose of interpreting results of the present study.
Chapter 2

2. Review of the Literature

2.1 Folate Biochemistry

2.1.1 Structure and Properties

Since the early 1800s, cases of exacerbated macrocytic anemia, particularly during pregnancy, have been documented (35-37). The identification of folate as an anti-anemic factor was introduced nearly 80 years ago by Lucy Wills, who used yeast extract in the treatment of anemic pregnant women in India (38). The active compound in the yeast was later isolated from four tons of spinach in 1941 and coined folium-Latin for leaf (39). This discovery provided the concrete evidence to explain the success in treating anemia through dietary intervention using various extracts of liver and brewer’s yeast (37-38, 40-42).

The term folate is the generic descriptor used to describe structurally related forms of an essential water-soluble B-vitamin that have a common pteroylglutamic acid core (43-46). Folic acid is the most oxidized and stable form of the vitamin and is the form of folate used for food fortification and in vitamin supplements (Figure 2.1.1). It was first crystallized in 1943 during the determination of essential compounds for *Lactobacillus casei* growth (47). Three years later folic acid was first synthesized in its oxidized form (48) with a molecular weight 441.4 g/mol. Folic acid is made up of three components: the center molecule, para-amino benzoic acid (PABA), is linked both to a pteridine ring by a methylene bridge to form pteroic acid and the carboxyl group of PABA is conjugated to the \( \alpha \)-amino group of a single L-glutamic acid by a peptide bond. Naturally, unmodified dietary sources of folate in plants (green leafy vegetables,
fruits and legumes) and mammalian tissues do not normally contain this synthetic form of the micronutrient (37).

![Diagram of folate derivatives]

**Table 2.1.1**

<table>
<thead>
<tr>
<th>Folate derivatives²</th>
<th>*R-substitute at N-5</th>
<th>Bridging N-5 and N-10 positions</th>
<th>R-substitute at N-10</th>
</tr>
</thead>
<tbody>
<tr>
<td>THF</td>
<td>-H</td>
<td></td>
<td>-H</td>
</tr>
<tr>
<td>5-methylTHF</td>
<td>-CH₃</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5,10-methyleneTHF</td>
<td></td>
<td>-CH₂</td>
<td></td>
</tr>
<tr>
<td>5,10-methenylTHF</td>
<td></td>
<td>=CH⁻</td>
<td></td>
</tr>
<tr>
<td>5-formylTHF</td>
<td>-HCO</td>
<td></td>
<td>-H</td>
</tr>
<tr>
<td>10-formylTHF</td>
<td>-H</td>
<td></td>
<td>-HCO</td>
</tr>
</tbody>
</table>

¹PABA: para-amino benzoic acid
²THF: tetrahydrofolic acid

**Figure 2.1.1** The structure of folic acid and its folate derivatives. Reduced folate “R substitutions” are denoted by the (*) at both the N-5 and N-10 positions of the molecule. Adapted from Linzdon et al. (2008) (5).

Natural sources of folate differ from folic acid by three structural differences. First, all naturally occurring folates are reduced to dihydrofolic acid or tetrahydrofolic acid (THF).

Second, various one-carbon moieties may be covalently linked to the pteridine ring at the N-5,
N-10, or bridging both. The folate coenzyme substitution of one-carbon units include: methyl (CH\textsubscript{3}), methylene (CH\textsubscript{2}), methenyl (CH), forminino (CH=NH), and formyl (CHO)]. Third, natural folates are predominantly in a polyglutamate form with additional glutamate residues linked by a gamma glutamyl bond between the amino group and \(\gamma\)–carboxyl group of the preceding glutamate. The stability of folate is pH-sensitive and differs between synthetic folic acid and natural occurring reduced forms (43). Reduced folate is least stable between pH 4-6 and most stable at pH >8 and pH <2, whereas folic acid appears to exhibit much greater overall stability. Folates are also susceptible to oxidative degradation, which is enhanced in the presence of oxygen, light and heat (approximately 30-50% of the folate is destroyed) (49-51). After processing of foods such as white flour, sugars, candies and liquors, no naturally occurring folate remains (37).

2.1.2 Biochemical Roles

Folates are essential coenzymes in the transfer and utilization of one-carbon units in de novo biosynthesis of thymidylate and purines during intermediary metabolism, and for the methylation of phospholipids, proteins, DNA, and neurotransmitters (Figure 2.1.2) (52). In pyrimidine synthesis, deoxyuridyllic acid is methylated to thymidyllic acid by thymidylate synthetase (EC 2.1.1.45) and 5,10-methyleneTHF is the methyl donor. Folate also plays a role in the metabolism of several amino acids: (1) serine transhydroxymethylase catalyzes the reaction of L-serine with THF to form 5-10-methyleneTHF and glycine, (2) histidine catabolism to glutamic acid involves THF as a coenzyme, and (3) the co-substrate 5-methylTHF donates the
methyl group to convert homocysteine to methionine by methionine synthase with $B_{12}$ and riboflavin cofactors (53). The latter pathway prevents the accumulation of toxic concentrations of homocysteine within cells and provides methionine for the synthesis of $S$-adenosylmethionine, the universal methyl donor required in numerous biological methylation reactions in the body. Homocysteine can also undergo a transsulfuration reaction to form cysteine via cystathionine-$\beta$-synthase and a cystathionine intermediate. Three nutrients in addition to folate are critical to the one-carbon metabolism pathways: riboflavin is a cofactor for methylenetetrahydrofolate reductase (MTHFR); vitamin $B_{12}$ is a cofactor for methionine synthase; and vitamin $B_6$ is a cofactor for interconversion of THF and its 5,10methylene-substitue form, as well as the formation of cysteine in the transsulfuration and re-methylation pathway. The complex interactions among folate, $B_{12}$ and $B_6$ status, also reflect the determination of plasma homocysteine concentrations and importantly disposal of homocysteine (54).
Figure 2.1.2 Folate-dependent one-carbon metabolism pathways. Abbreviations: DHF: dihydrofolic acid; THF: tetrahydrofolic acid; SAH: S-adenosylhomocysteine; SAM: S-adenosylmethionine (a feedback inhibitor of enzyme (4)); TMP: thymidine monophosphate; UMP: uridine monophosphate. Enzymes: (1) thymidylate synthase; (2) serine hydroxymethyltransferase; (3) dihydrofolate reductase; (4) methylenetetrahydrofolate reductase; (5) 5,10-methenyltetrahydrofolate synthetase; (6) methionine synthase; (7) betaine-homocysteine methyltransferase; (8) DNA methyltransferase; (9) SAH hydrolase; (10) cystathionine-β-synthase.

Given folates critical role in nucleotide synthesis and the methylation cycle, the requirement for folate is greatest during anabolic stages of the life-cycle such as during gestation and lactation. Significant chronic folate deficiency will eventually impact cell replication and present in red blood cells (RBC) as megaloblastic anemia due to impaired DNA synthesis (55). Factors known to significantly impact cellular folate metabolism include: vitamin B₁₂ deficiency and the MTHFR 677C→T polymorphism (see Figure 2.1.2). Vitamin B₁₂ deficiency impairs the activity of methionine synthase and inhibits the regeneration of THF. In the case of folate and
vitamin B\textsubscript{12} deficiency, the hematological signs of anemia created by impaired DNA synthesis are resolved with supplemental folic acid alone. Folic acid can be reduced to produce THF by bypassing methionine synthase. However, unless vitamin B\textsubscript{12} is also provided, homocysteine and 5-methylTHF will continue to accumulate within the cell as methionine synthase remains block. This scenario is referred to as the methyl trap (see Figure 2.6) (56).

The MTHFR 677C→T polymorphism lowers enzyme activity of 5,10-methylenetetrahydrofolate reductase and formation of 5-methylTHF from 5,10-methyleneTHF. The subsequent impact of less 5-methylTHF is that there is an alteration in the re-methylation of homocysteine to methionine and in turn the formation of SAM, the primary methyl donor in numerous cellular reactions in the body. (57). There appears to be a varying difference in MTHFR activity for the three possible genotypes, with a reduced activity predominately noted with the homozygous (T/T) allele. A study by Frosst et al. reported the mean MTHFR activity for individuals with the homozygous genotypes to be 30\% that of those with the wild type (C/C) (58). The heterozygotes, in comparison to the wildtype had a mean MTHFR activity of 65\% and the ranges of activities overlapped the wildtype values, unlike the homozygous. Commonly homozygote’s display mild hyperhomocysteinemia when folate intakes are low normal or suboptimal (57). It is believed, then, that the dietary folate requirement is higher in homozygous individuals (59). The prevalence of this polymorphism varies worldwide (0-32\%) (60) and ranges from ≤1\% in Native and American Africans, to ≥20\% in Italians and American Hispanics (61).

The MTHFR polymorphism 677C→T has been associated with impaired DNA methylation during inadequate folate intake due to lowered production of SAM (62). Auxume et al. (2007), studied the influence of a 7 week folate restricted diet (135 μg dietary folate
equivalents (DFE/day) followed by a 7 week folate treatment (400 or 800 μg DFE/day for 7 weeks) on methylation in women with the MTHFR 677C→T genotype. The results found that the homozygous individuals had a significantly lower ($P < 0.05$) global leukocyte methylation pattern versus wildtype and heterozygote’s (63). Further, cross-sectional studies associate a maternal MTHFR 677C→T genotype as a moderate risk factor for NTDs in certain populations, as well as 677C→T homozygosity in infants appears to moderately increase the risk for spina bifida (61).

As a result of the MTHFR 677C→T polymorphism association with different intracellular folate cofactors and subsequent potential affect on DNA methylation reactions, Kim et al. examined this relationship to uracil misincorporation and possible cancer risk using in vitro colon (HCT116) and breast (MDA-MB-435) adenocarcinoma cells (64). There was a unique difference noted in the amount of genomic DNA methylation between the two cell lines. The colon cells had significantly increased methylation under adequate or high folate supply and decreased methylation during inadequate folate supply. In comparison, the breast cells displayed a different phenomenon, where under adequate and high supply there was significantly decreased methylation and no effect when the folate supply was low. The occurrence of uracil misincorporation demonstrated a non significant trend towards higher incidence in breast cells compared to lower incidence in colon cells. Recently, in a population based case-control study, Iacopetta et al. found the MTHFR 677C→T allele to be associated with an increased risk for proximal colon cancer (adjusted odds ratio = 1.29), and a decreased risk for distal cancers (adjusted odds ratio = 0.87) (65). The results obtained from food frequency questionnaires and information on alcohol consumption suggest that the increased risk for proximal cancers was
evident in older individuals (adjusted odds ratio = 1.49), as well as those with a low folate diet (adjusted odds ratio 1.67), or high alcohol intake (adjusted odds ratio 1.90) (65).

2.2 Folate Absorption and Metabolism

2.2.1 Small Intestine Absorption

The human gastrointestinal tract is essentially a hollow muscular tube, divided into the mouth, pharynx, esophagus, stomach, small intestine and large intestine or colon. Since humans are incapable of de novo folate biosynthesis or long-term storage of folate, frequent dietary consumption of folate rich food substrates is required. Dietary folates predominantly exist as polyglutamates, predominately with the heptaglutamate form and are deconjugated to the monoglutamate form by intestinal apical brush border glutamate carboxypeptidase II (GCPII; EC 3.4.17.21) (37, 66-67). The following human tissues have been found to contain GCPII: prostate, small intestine, brain, kidney, liver, spleen and colon, (listed in order of prevalence) (68). In addition to GCPII, possible pancreatic secretions may contribute to the deconjugation process of polyglutamates as observed in vitro using porcine pancreatic pteroylpolyglutamate hydrolase (69). The pH optimum for enzymatic deconjugation is approximately 6–7 (34). Intracellular folates have been found to contain up to ten polyglutamates, while those found in serum are often monoglutamated (37, 70-71).

The bioavailability of folate relies largely on the capacity of intestinal absorption, primarily at the brush border of the jejunum (Figure 2.2.1) (34). Passive diffusion across cell
membranes has been shown after pharmacological doses of folate (72-73), although this is an insufficient mechanism to sustain adequate folate status due to the hydrophilic anionic character of folates that limit passive transport during physiological doses of folate (33). Instead, monoglutamate folate absorption across the brush border membrane of the enterocyte is a multi-step active process involving the proton-coupled folate transporter (PCFT) and reduced folate carrier (RFC). For decades it was recognized that an alternative low-pH optimum carrier-mediated folate mechanism existed, however only three years ago was it identified as PCFT. Both the original RFC, and recently discovered PCFT are expressed at the apical brush-border of enterocytes along the small intestine and colon. The RFC and PCFT have different pH sensitivities, with optimal folate transport activity at pH 7.4 and 5.5, respectively (33). The pH levels of the small intestine ranges from 5.5 to 7.0 and gradually rises to 6.7-7.5 in the terminal ileum (74).

After absorption within the enterocyte monoglutamated folates are again polyglutamated by the enzyme folylpoly-\(\gamma\)-glutamate synthetase (EC 6.3.2.17). An important intracellular function of the long polyglutamate folate chain, is intracellular retention (70, 75). Polyglutamation of folates also increases the affinity for enzymes that utilize folate as a cofactor and decreases the affinity for efflux transporters (67, 70, 76). Prior to cellular folates leaving the enterocyte basolateral membrane, gamma-glutamyl hydrolase or conjugase removes the terminal glutamate residue of polyglutamates sequentially (77). Exit into the hepatic portal circulation across the basolateral membrane for transport to the liver and other tissues from the enterocyte is presently not well understood, but is thought to be carried out by a less specific mechanism involving a member of the multidrug-resistance-associated protein family of ATP-binding-cassette exporters (33).
Folates enter systemic circulation via the hepatic portal vein and are partly protein-bound, mainly to albumin (50% of bound folates) as well as to soluble folate binding proteins (75) to be transported to the liver and peripheral tissues or organs. To be metabolically available the synthetic form of folic acid must first be reduced by dihydrofolate reductase, however reduction is easily saturated, and folic acid appears unmetabolized in plasma and urine even after ingestion of 260-280 µg (589-634 nmol) (78). Recently, an analysis of 1215 subjects from the Framingham Offspring Study demonstrated that a 19-base pair deletion polymorphism in dihyrofolate reductase was associated with an increased level of unmetabolized folic acid in plasma concentration (folic acid intake \( \geq 500 \mu g/day \)) and decreased red blood cell folate, compared to the wildtype and heterozygous genotypes (79). Folic acid is thought to be primarily metabolized to 5-methylTHF in the liver (80-81) and not the mucosa, as previously was believed (82). Reduced folates, such as 5-formylTHF are still considered to be converted to 5-methylTHF at physiological doses within the enterocyte prior to transfer into the hepatic portal vein (81-82).

Although no complete tissue analysis in humans has been done to directly calculate the total folate pool, it is estimated that approximately half of total-body folate stores are in the liver (37). In the liver, 73% of absorbed folic acid, and 52% 5-formylTHF from intrinsically labeled spinach were estimated to be retained by the enterocyte and the liver during what is commonly referred to as first-pass splanchnic metabolism (81). The three possible destinations of folates in the liver include: (1) polyglutamate storage, (2) bile secretion or (3) monoglutamate folates can be delivered directly to systemic circulation (33).
Figure 2.2.1 Absorption of folate across the small intestine. Abbreviations: THF, tetrahydrofolic acid. Enzymes: (1) GCPII; (5) folypolyglutamate synthetase; (6) GCPII. Folate Transport: (2) reduced folate carrier (RFC), pH optimum of 7.4 does not function at usual folate dietary intakes in acidic small intestine; (3) Passive diffusion is limited; (4) protein-coupled folate transporter (PCFT), pH optimum of 5.5 is favorable for acidic microclimate of small intestine; (7) multidrug resistance associated proteins; (8) folate receptor (FR). Adapted from Zhao et al. (2009) (33).

*Folate Transporter Distribution and Initial Metabolism*

Because of the critical role of folate in one-carbon transfer reactions, adequate intracellular delivery of folates is essential for cell survival (DNA synthesis and proliferation). The presence of folate transporters and varying distributions throughout the human body is complex. For example, the RFC has been isolated using a multi-tissue mRNA array from 76 human tissues and tumor cell lines (multi-tissue Northern blot) and found to be ubiquitously expressed (83). The varying pH of the two transporters: acidic (PCFT) versus neutral (RFC),
distinguish their expression within cells (Table 2.2) (33). The RFC mediates membrane folate uptake from the neutral pH arterial system by bidirectional anion exchange, with minimal contributions by PCFT, except in tissues where Na\(^+\)/H\(^+\) exchanges produce acidic microenvironments at transport interfaces. The PCFT mediates folate active transport into enterocytes by an inwardly directed proton gradient.

The main circulating plasma form of folate (5-methylTHF) can be transported into and out of cells by RFCs, PCFT, as well as the membrane-bound-folate receptors (e.g. FR α & β), which are linked to tissue cell membranes via glycosylphosphatidylinosital-anchored proteins. The primary mechanism to transport folates into the cytoplasm for FRs is by endocytosis, however their specific physiological roles remain unclear (33). Recently, evidence supports that the PCFT plays a role in FR mediated endocystosis by providing a route for folate export from acidified endosomes into the cytoplasm (84). FRα are expressed primarily in the epithelial cells of the uterus, choroid plexus, placenta, retina (basolateral surface) and kidney (apical surface), whereas FRβ in CD34\(^+\) monocytes, in the spleen, thymus and placenta and not thought to be carriers in intestinal folate transport (33). In addition, FRα and β are also expressed in tumors, however FRα expression has been correlated with histological stage (e.g. brain, colon, endometrium, kidney, lung, mammary gland, myeloid cells of hematopoietic origin, and ovarian) (85-87). The affinities vary between folate forms, and FR α and β have a higher affinity to folic acid (K\(_d\) 1-10 nM) compared to both RFC and PCFT (K\(_i\) 150-200 µM and K\(_m\) ~1 µM respectively). In contrast, PCFT and RFC have a higher affinity to reduced folates than FR α and β (33, 88-89). Research is ongoing and continues to examine how these folate transporters contribute individually and holistically to folate homeostasis.
Table 2.2 Overview of two intestinal folate transport proteins, the reduced folate carrier (RFC) and the proton-coupled folate carrier (PCFT).

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>RFC</th>
<th>PCFT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Localized Distribution</td>
<td>Small intestine, colon, kidney, choroid plexus, liver</td>
<td>Small intestine, colon, kidney, brain, liver, retina, placenta</td>
</tr>
<tr>
<td>Intestinal expression</td>
<td>Small intestine (apical basolateral membrane) and colon (villus tip)</td>
<td>Small &gt; colon Apical basolateral membrane</td>
</tr>
<tr>
<td>Binding affinity for reduced folates(^2):</td>
<td>High: (K_m) 2-7 (\mu)M</td>
<td>High: (K_m) ~ 4 (\mu)M (88-89)</td>
</tr>
<tr>
<td>Binding affinity for folic acid(^2):</td>
<td>Very low: (K_i) ~ 150-200 (\mu)M</td>
<td>Low: (K_m) ~ 1 (\mu)M</td>
</tr>
<tr>
<td>Optimal pH</td>
<td>Neutral: 7.4</td>
<td>Acidic: 5.5</td>
</tr>
<tr>
<td>Other substrates</td>
<td>Thiamine pyrophosphate, methotrexate, PT523(^3), pemetrexed</td>
<td>Methotrexate, PT523, pemetrexed</td>
</tr>
<tr>
<td>Energy source</td>
<td>Bidirectional anionic organic phosphate gradient supplies energy for transportation</td>
<td>Downhill flow of protons is coupled to uphill flow of folates into cells in low pH microenvironment generated by Na(^+)/H(^+) exchangers</td>
</tr>
<tr>
<td>Gene locus</td>
<td>21q22.2-q22.3</td>
<td>17q11.2</td>
</tr>
<tr>
<td>Other characteristics</td>
<td>Lack of sterospecificity</td>
<td>High sterospecificity for 6S-natural folate stereoisomers of 5-methyl- &amp; 5-formyl-THF</td>
</tr>
<tr>
<td></td>
<td>Negligible transport when pH &lt;6-6.5</td>
<td>Some transport of 5-methylTHF at pH 7.4</td>
</tr>
<tr>
<td></td>
<td>Solute family of transporters (SLC19)</td>
<td>Gene (SLC46A1)</td>
</tr>
<tr>
<td></td>
<td>Regulated by transcriptional control, promoter methylisation/architecture, chromatin structure</td>
<td>May play role in folate receptor (FR) mediated endocytosis</td>
</tr>
</tbody>
</table>

\(^1\)Adapted from Zhao et al. (2009) (33), unless otherwise noted.

\(^2\)\(K_m\) and \(K_i\) refer to receptor binding affinity and the corresponding experiment to determine \(K\) (e.g. \(K_i\): using a competitive binding experiment).

\(^3\)[N alpha-(4-amino-4-deoxypteroyl)-N delta-hemi phthaloyl-L-ornithine], a potent monoglutamate antifolate (methotrexate analog)

Several polymorphisms of folate transporters are known to alter health outcomes as expression of folate transporters is critical to adequate folate tissue uptake to support overall folate metabolism. The RFC polymorphism RFC (80A→G) resulting in a histidine to arginine change may contribute to a greater NTD susceptibility (90). Potential defective carrier mechanisms may include lowering intestinal absorption, or interfering with folate transport, or both. Interestingly, Chango et al. reported that individuals who were homozygous RFC 80A→G, as well as MTHFR 677C→T had higher plasma folates compared to the 80A→G and
677C→T using genomic samples from 169 healthy adults (91). Additionally, although the autosomal recessive disorder known as hereditary folate malabsorption is characterized by impaired folate intestinal and central nervous system folate PCFT transport as result of inadequate absorption. Hereditary folate malabsorption has been recently linked to the polymorphism resulting in a homozygous frameshift mutation (c.194dupG), which is hypothesized to result in a truncated protein (p.Cys66LeufsX99) (92-93). Two polymorphisms of FRα A1314G and C1816delC examined by Zhang et al. demonstrated that FR polymorphisms may be a contributing factor to increased gastric cancer risk in the at-risk Chinese compared to controls and varying genotypes (94). Future work examining the numerous phenotypic manifestations of folate transporters is needed and their unique tissue distribution highlight some of the complexities involved in examining both local and overall folate metabolism in the context of health and disease.

2.2.2 Physiology of the Colon

Overview

The physiology of the colon is unique compared to the small intestine. It is characterized by its slower rate of transit, viscous luminal environment, substrate availability and range of intra-luminal pH that influences the proliferation of bacterial species. The colon, which begins at the ileocecal junction and ends at the anus, comprises the longest (1.5 m) and most distal segment of the gastrointestinal tract (95), and has a smaller internal surface area in comparison to the small intestine (96). The small intestine has a substantially larger surface area due to the
presence of villi, with each villus being covered with microvilli (also known as brush border), whereas in the colon villi are absent. In the proximal colon or cecum the microbial fermentation producing short chain fatty acids predominate compared to the distal colon and along with H₂ influence colonic motility, as well as transit time, and colonic blood flow (96-98). The transverse is characterized by rather unique motor patterns that function to either hold material for further fermentation or propel it distally to empty the proximal colon, as well as absorb water and begin feces formation. Finally, the distal colon and rectum serve as a reservoir until evacuation.

**Microflora profile**

Although bacteria are predominant, *Achaea*, yeasts, fungi and protozoa also account for a portion of the gastrointestinal tract microbiota. A normal microflora distribution restricts the growth of pathogenic microorganisms, thereby promoting colonic health and immunity (99). Overall, individual microflora profiles are similar in different human populations, although there is a large degree of variability between human adults (100). Based on the sequencing of rRNA from total colonic bacterial populations, it is estimated that the microbiota is compose of 400 to 1000 phylotypes (mainly obligate anaerobes), with the microflora mass weighing an estimated 1-2 kg and numbering $10^{11}$-$10^{12}$ colony forming units/ml of colon contents (99, 101-102).

To maintain such a large bacterial mass, carbohydrates from host secretions (e.g. sloughing of intestinal cells) or dietary sources sustain the microflora colonies. However, only 2-4% of ingested simple sugars generally reach the colon, as they can be readily absorbed in the small intestine. A substantial portion of dietary fiber in most human foods appears to be digestible by the colonic flora (assuming 15-20 g of dietary fiber per day) and thus this source of carbohydrate could provide the energy needed to maintain the highly populated bacterial mass in
the proximal colon (57). Generally, microorganisms are not affected by minor short-term changes in diet and are fairly stable over long periods (99). Physiological factors such as luminal pH, substrate availability, genetics and transit time may alter the indigenous microflora (99-100).

The evidence describing the effect of age on folate absorption is inconsistent and inconclusive. The assumption that malabsorption of both macro- and micronutrients is enhanced with age, is thought to be related to instead the presence of disease (1, 103). Currently, in the absence of disease, little is known about whether folate absorption may be affected by age (1, 103), however studies have observed that microflora abundance and distribution vary with age. For example, in a study of the comparison of microbial populations from different age groups, elderly people had a more diversified flora than younger adults, and the flora of adults was more diversified than babies (104). A different distribution and abundance of bifidobacteria (a folate producer species), has been quantitatively measured in elderly people compared to adults (105-106) and elderly populations appear to have lower bifidobacteria counts (105).

2.2.3 Bacterial Biosynthesis of Folate

The mechanism of bacterial folate synthesis has been well defined (Figure 2.2.3), and many bacterial species present in the colon are capable of synthesizing folate. The process involves seven direct enzyme catalyzed steps to form tetrahydrofolic acid originating from 6-hydroxymethyl-H2-pterin pyrophosphate (GTP). This pathway involves the following enzymes in sequence: first, GTP cyclohydrolase I to form the pterin ring, 7,8-dihydroneopterin triphosphate pyrophosphorydrolase; second, 7,8-dihydroneopterin aldolase catalyses the
conversion to 6-hydroxymethyl-7,8-dihydropterin; third, a 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase adds pyrophosphate to form 6-hydroxymethyl-7,8-dihydropterin pyrophosphate; fourth, dihydropteroate synthase (EC 2.5.1.15) is commonly exploited by antimicrobial agents and this enzyme joins the pterin ring moiety to para-amino benzoic acid (PABA) to form 7,8-dihydropteroate; fifth, a folylpoly-γ-glutamate synthetase adds glutamates for cellular retention after the sixth enzyme dihydrofolate synthetase (EC 6.3.2.12) catalyzes 7,8-dihydrofolate. Finally dihydrofolate reductase (EC 1.5.1.3) forms 5,6,7,8-THF (107-108).

Figure 2.2.3 Overview of the final pathway steps in the bacterial biosynthesis of tetrahydrofolic acid (THF). Sulfonamide drugs target the enzyme dihydropteroate synthetase (X). Adopted from Bermingham & Derrick, (2002) (108).

Within the colonic bacteria population there are both folate synthesizers, as well as folate consumers. Due to the direct result of bacteria producing folate in milk (20-50 µg folate per
liter), dairy fermentation experiments have identified primary bacteria folate synthesizers and measured the amount of folate production compared to bacteria consumers (109). Streptococcus thermophilus and bifidobacteria are two of many bacterial species able to synthesize folate de novo, while several Lactobacillus strains actually deplete folate levels. In general the predominant forms of folate found in foods and dairy products are THF, 5-methylTHF and 5-formylTHF (110), however the form produced can very depending on the bacterial strain. For example, cultures of L. casei have been shown to produce both mono and diglutamated 10-formylTHF, with the monoglutamated form being more rapidly excreted to the medium (111). Additionally, Crittenden et al. 2003 found the level of folate in fermented milk products, after mixing two folate-producing organisms, to result in an increased vitamin level by more than six-fold. Further, inoculation with both a folate producing and utilizing strains resulted in a minimal overall change in the total folate concentration of the milk medium (112). These studies demonstrate the complex dynamics in estimating overall colonic microflora folate production. The rate of folate biosynthesis by folate producers may be impeded by folate consumers and to date we currently have been unable to estimate the rate of folate biosynthesis within the human colon.

It is still unknown what region of the colon has the greatest folate production. Ahmed et al. (2007) characterized bacterial communities using PCR-denaturing gradient gel electrophoresis and found that there were no significant differences in overall bacterial numbers between the ascending, transverse and descending colon (113). The samples were obtained from patients undergoing emergency surgery required for resection of the colon and therefore these patients received no prophylactic antibiotic treatment or bowel cleansing protocol before the operation that could have altered microbial populations. Looking specifically at bifidobacteria,
known folate producers, the authors found no significant differences in numbers between the proximal colon and different parts of the colon, however the numbers in the proximal colon were significantly higher than in the terminal ileum ($P = 0.006$). These results suggest that potential bifidobacteria folate production may be similar throughout the length of the colon. However, additional studies are required before it can be quantitatively predicted what region of the colon has the greatest folate synthesis, as it is important to consider the multiple strains of folate producing bacteria, as well as consuming bacteria. Lastly, the amount of folate present in one area of the colon may not directly relate to greater absorption, as the physiology of the colon and regulation of absorption is complex and remains under investigation.

### 2.2.4 Folate Uptake by Colonocytes

*Large folate depot*

Microbial synthesis of folates in mammals was first suggested by Mitchell et al. nearly 70 years ago (39). Initial human evidence (likely underestimated due to flaws in early analytical procedures) found that the amount of folate synthesized by the intestinal microflora may exceed dietary intake three to five-fold (20-21). Likewise, current examination of adult colonic evacuants after ingestion of 2 L of the standard polyethylene glycol solutions and collected over 3 hours (approximately 1300-1700 g) from South Africans and Caucasian Americans exceeded the recommended dietary allowance for adults (400 µg/day) with 699 ± 131 and 860 ± 129 µg, respectively (22). Further, analysis of infant fecal folate by Kim et al. (2004) found that approximately half (52.5 ± 30.1%) of the folates were in the absorbable form of folate
(monoglutamate) that is known to be readily absorbed across the small intestine (114). The predominant form was short-chain folate (66 ± 21%) and the predominant form being 5-methylTHF. The total folate, approximately 93.2 ± 92.8 nmol/day (41.1 ± 41.0 µg/day) was approximately 63% of the adequate intake level for infants <5 months of age (65 µg/day) (114). Collectively the results from these studies demonstrate that a substantial pool of folate exists in the large intestine that approaches or exceeds recommended dietary intakes.

Absorption

Data from in vitro experiments with colonic apical membrane vesicles isolated from organ donors and non-transformed human colonic epithelial cell lines consistently demonstrate folate uptake by mechanisms similar to small intestine enterocytes (28-30). Specifically, early in vitro experiments, using purified basolateral membrane vesicles from donated colonic mucosa, reported folate transport across the epithelium to be a pH-dependent carrier-mediated system that was an anion exchange inhibitor-sensitive electroneutral process (28). Organ-cultured biopsy samples from the proximal cecal and distal sigmoid colon mucosa were found to have a carrier that mediated folic acid uptake (30). The process of folate absorption across the colon observed by a normal human colonic cell line (NCM460) was thought to be under regulation of a cAMP-mediated, protein tyrosine kinase pathway, as well as pH dependent (115). In another study examining human colonic mucosal scrapings, a folate carrier showed a relatively low affinity for folate (K_m approximately 8 µm), however a higher maximum velocity in the proximal and slightly lower in the distal colon (29).

Until recently, minimal evidence supported that the substantial pool of folate could be absorbed across the colon. Initial animal studies found that substantial quantities of folate
produced by microorganisms supported normal hematology and growth, although the suggested mechanism of absorption was via coprophagy and subsequent folate absorption across the rat small intestine rather than the colon (23-25). The use of radio-labeled folate and PABA demonstrated that microbial folate is indeed incorporated into animal tissue after its direct injection into the cecum (26-27). Rong et al. (1991) showed that radio-labeled PABA, a precursor of microbially synthesized folate, injected into the cecum of rats was incorporated into the liver as folate despite inhibition of coprophagy (27). Recent studies suggest that the pig is considered to be a more suitable animal model than the rat when comparing to human folate absorption physiology (114). Pigs, like humans, have both intracellular and brush border GCPII in the small intestine, whereas in contrast rats use the intracellular folate hydrolase that is expressed by pancreatic γ-GH (116). Using this animal model, Asrar et al. (2005) reported that 23% of the $[^3H]$ folic acid and 13% of the $[^3H]$ PABA injected into the cecum could be found in the liver, kidney as well as excreted in urine as $[^3H]$ folate. It was estimated that at least 18% of the dietary folate requirement for the piglet could be met by bacterially synthesized folate (26). Given these results, folate is considered to be absorbed across the colon of animals.

The first ever direct evidence of folate absorption across the human colon was recently published using a novel stable isotope tandem mass spectrometry approach in our laboratory. After infusing $[^{13}C]5$-formylTHF (684 nmol) directly into the cecum in adult subjects ($n = 6$) undergoing colonoscopy screening, its metabolite, $[^{13}C_5]5$-methylTHF, was detected in plasma at a rate of $0.6 \pm 0.2$ nmol/hr compared to $7 \pm 1.2$ nmol/h after an IV of the same test compound (172 nmol) (19). The authors concluded that the PCFT and RFC were the potential mechanisms of folate absorption across the colon although these mechanisms are not as well understood compared to the small intestine. The presence of the folate transporters RFC and PCFT have also
been found along the length of the colon (33). The optimal polyglutamate chain length for folate absorption across the colon is thought to be similar to the small intestine, the monoglutamate form. Presently, only the folate monoglutamate form is thought to be bioavailable, however early mammalian studies in dogs (1971) found that folate in the monoglutamate and diglutamate form was permeable to jejunal mucosal cell membranes (117). Once folates are taken up within the colonocyte, it is thought to be metabolized like the small intestine, with intracellular retention, hydrolysis and export. The time lag after cecal infusion (before the first detection of the labeled vitamer, 0.8 ± 0.3 hours) in the Aufreiter et al. study and the plasma appearance of the metabolite of the administered 5-formylTHF, 5-methylTHF, provides initial evidence that colonocytes are also likely capable of folate one-carbon substitutions (at physiological doses of reduced folate) similar to enterocytes (80).

An important consideration of this first human study is whether or not the routine bowel cleansing prior to colonoscopy influenced folate absorption across the colon, due to the disturbance of microbial populations (31). While it is well known that some bacteria synthesize folate, while others consume folate, it is unknown how much folate is bioavailable from a test dose for absorption across the colon, as the test dose could be consumed by bacteria or in competition with the existing folate pool for transport across the colonocyte. It is also important to highlight that this study did not observe and a return to baseline of the labeled folate concentration after blood sampling for four hours (19). Instead, the labeled folate response increased linearly, suggesting that absorption although slower than the small intestine appeared to occur along the length of the colon. Without a return to baseline, the authors were unable to calculate the percent relative bioavailability of the colon infusion test dose compared to an IV test dose of the same compound. Therefore, for a more comprehensive understanding of this
large and intact folate depot on whole-body folate status, as well as colonic health, future investigations with non-invasive techniques and longer blood sampling protocols reflecting colon transit (10 hours to days) are recommended.

**Manipulation of microflora and impact on folate biosynthesis**

Substantial evidence supports that dietary intake may impact folate-producing microorganisms and influence host vitamin status. For example, Krause et al. fed rats diets containing human milk solids which contain a natural source of soluble fiber (oligosaccharides) or diets containing cow milk or goat milk solids (118). The two latter milk solids contain very low amounts of oligosaccharides. Their research demonstrated rats fed human milk solids had higher cecal counts of bifidobacteria (folate secreting bacteria) and higher folate levels compared to those fed milk solids from cow or goat sources. In contrast, a human study examining fecal folate in infants feed formula or human milk, Kim et al. measured an approximately 2-fold greater folate content in formula-fed infants (114). The authors accounted for part of this difference due to the measured higher folate content in formula versus human milk. As well, the differential contribution of unabsorbed folates, and potential differences in human and rat microflora profiles of folate synthesizing versus consuming microorganisms could provide additional explanation of the results.

Further, in humans, there is indirect and circumstantial evidence that diet may influence bacterial biosynthesis of folate (119-120). Houghton et al. (1997) found a positive association (p<0.001) between total dietary fiber (particularly soluble) intake and serum folate concentration in young women (n = 224), even after controlling for folate intake (119). From the results a 1.8% increase in serum folate concentration for each 1-g increase in non-starch polysaccharide intake
was predicted (119). Further, in a randomized controlled study of Type 2 diabetics, Wolever et al. (2000) found that miglitol (an α-glucosidase inhibitor that competitively inhibits carbohydrate digestion for improved glycemic control) affected folate status. Subjects treated with miglitol showed significantly higher serum folate levels versus those taking metformin which promotes glycemic control by improving insulin sensitivity and reducing glucose output (120). It is likely that the higher folate status in these studies resulted from a greater availability of dietary fermentable substrates, important determinants of the quantity and quality of bacteria in the human large intestine.

2.2.5 Folate Elimination

Elimination is the irreversible loss of a drug, nutrient or metabolic product by excretion and metabolism (121). Folate is excreted in bile and urine in both metabolically active and inactive forms. Intact folates may also be absorbed by renal FRα and PCFT within the kidney and transported into circulation for redistribution to tissues by RFCs and organic anion transporters (33, 122). On the other hand, folate is oxidatively broken down in the tissues in a process involving ferritin to PABA, the major urinary catabolite which is formed through the cleavage of the C9-N10 bond and its acetylated derivative p-acetamidobenzoylglutmate (see Figure 2.1.1) (37, 123). Interestingly, with higher intakes of folate, the excretion of intact isotope-labeled folic acid also increases (up to 19% in participants consuming 1950 nmol/day folic acid for twelve weeks) (124) compared to catabolites which were unaffected by increases of folic acid intake of 454-907 nmol/day (125).
The amount of the most oxidized folate form, folic acid, has been estimated to be excreted into the gastrointestinal tract as bile in varying amounts from 227 nmol/day (126) in 1965 to recently as large as approximately 5300 nmol/day (127). In the later and modern study, Lin et al. administrated 0.5 nmol $^{[14}C$ pteroylmonoglutamate (100 nCi radioactivity) plus 79.5 nmol pteroylmonoglutamate in water orally to thirteen adults and used kinetic modeling for folate distribution and metabolism (127). The apparent dose absorption of labeled folate was 79% and folate elimination was accounted for by feces (38% folate and oxidation products) and urine samples (56% $p$-acetamidobenzoylglutamate and 5.7% intact folate). Despite the large mass of folate released as bile, the mass of folate absorbed across the intestine was greater and estimated to be approximately 5982 nmol/day or almost six times the mean participant dietary folate intake of 1046 nmol/day. The authors noted that the folate loss (and its oxidation products) in feces was substantially smaller than the amount absorbed (approximately 415 nmol/day), and about 50% of the participant dietary intake. The role of folate excreted in bile is thought to blunt the between-meal fluctuations in folate supply, maintain folate concentrations and prevent folate deprivation through re-absorption via enterohepatic circulation. Whether folate form may impact excretion rates in bile is uncertain, however it is a substantial consideration as folate form has been recognized to impact absorption kinetics across the small intestine (81). Furthermore study protocol fasting conditions (>16 hours) may initiate a slower bile secretion (the major elimination route for folates) into the gut bringing about an increased total plasma folate response (128-129). Thus, folates excreted in feces may be derived from gastrointestinal secretions such as bile, dietary folate intake due to incomplete bioavailability, folates produced by bacterial biosynthesis, and lysed enterocytes and colonocytes (130).
2.3 Folate Bioavailability

The term bioavailability is used interchangeably to describe both the rate and extent of nutrient input into the systemic circulation. It is commonly denoted as the intact fraction, or percent, of the administered dose absorbed systematically. Folate bioavailability thus applies to the fraction of a given folate dose that is absorbed across the small intestine or colon and can be used for metabolic processes (131). Folate bioavailability varies greatly among individuals and can be altered by disease, genetics, diurnal rhythms, non-folate dietary components, impaired enter hepatic circulation, medications that alter the pH of the intestine or affect the quantity or profile of intestinal microflora (34). Folate bioavailability is also influenced by intestinal absorption, as well as transport, metabolism and the kinetics of its catabolism, retention and excretion.

2.3.1 Dietary Folate Equivalents

Presently the bioavailability of different folate forms, oxidized folic acid and naturally reduced folates, are not well understood. General consensus is that dietary folates (reduced) are less bioavailable compared to synthetic folic acid (oxidized) added to foods or taken in supplemental form (1, 132). The recommended dietary allowance for folate is expressed as dietary folate equivalents (DFE) to account for the observed differences in the bioavailability of naturally-occurring food folates versus synthetic folic acid (1). Folic acid is stated to be
approximately 1.7 times more available than natural occurring food. DFEs, then, are defined as:
natural food folates (µg) + 1.7 x of synthetic folate (µg). This calculation of folate bioavailability
is considered to be rather imprecise and requires further investigation. The relative
bioavailability of folic acid versus naturally occurring sources of folate as defined in the
recommended dietary allowance was largely based on one long term (92 day) metabolic study in
non pregnant women who were divided into three parallel groups receiving varied doses of folic
acid. The estimated relative bioavailability of dietary folate was no more than 50% that of folic
acid (133). Additionally the dietary DFE of 1.7 is supported by evidence reported by Pfeiffer et
al. showing that folic acid added to food was approximately 85% the bioavailability of free folic
acid (34, 132). It is likely that the higher folic acid bioavailability value of 50% is an over-
estimation compared to food folate bioavailability in fruits and vegetables, as data on food folate
bioavailability vary substantially from roughly 10% to 98% (134-136).

2.3.2 Dietary Components

Studies have found multiple factors contributing to observed bioavailability of dietary
folates versus the supplemental folic acid form. For example, Pfeiffer et al. observed an
approximate 15% decrease in folic acid bioavailability when supplements were consumed with a
light breakfast compared to the control without food (132). Gregory et al. hypothesized that
various food components inhibit GCPII and reduce the bioavailability of natural dietary
polyglutamate folates (34). Their in vitro work demonstrated GCPII was inhibited by various extracts of foods such as anionic species of organic acids (e.g. citrate, malate and ascorbate) (69). Additional in vitro work with $^3$H-folic acid has also found that phenolic compounds in tea, wine, and beer may reduce folic acid uptake by Caco-2 cells (137). It is unclear whether these finding in vitro are relevant to the in vivo situation where whole diets are consumed.

In an in vivo study by Melse-Boonstra et al. (2004), GCPII was a rate limiting factor in folate absorption was further supported. The results demonstrated that the bioavailability of polyglutamate folates were approximately 65% reduced versus the monoglutamate forms (131). In this study, 180 adults ingested 323 nmol heptaglutamate folic acid/day and 262 nmol monoglutamate folic acid/day, or a control placebo in caplet form for twelve weeks. The polyglutamate folic acid group had significantly lower mean serum and erythrocyte folate concentrations [6.1 (95% CI: 5.3, 7.0) and 155 (95% CI: 122, 188) nmol/L respectively] than in the monoglutamate folic acid group [11.8 (95% CI: 10.3, 13.3) and 282 (95% CI: 246, 318) nmol/L, respectively] (131). In another in vivo study using mono- and poly- glutamate folates labeled with $^2$H added to a variety of foods including lima beans, orange juice, tomatoes and water as a control, only orange juice caused inhibition of the polyglutamate folate tracer (approximately 33%) (138). However due to the high concentration of folate in orange juice, the bioavailability is still considered reasonably high (138). Additionally, short-term bioavailability of natural folates consumed as a supplement were also noted to be equivalent to folic acid (56, 139), likely because these folates were neither polyglutamated nor trapped within cellular matrices (140).
Folate binding proteins

The affect of folate-binding proteins remain inconclusive. For example folate-binding proteins in milk may enhance labile folate stabilization in the gastrointestinal tract and improve bioavailability (141). However, folate-binding proteins when added to fermented milk were also associated with an increased stomal folate excretion of 16% compared to 9% after the apparent absorption of 5-methylTHF without the addition of folate-binding proteins (142). The binding affinity appears to be sensitive to the folate form with 5-methylTHF being largely present as free folate in the duodenal lumen versus bound folic acid (143). The folate form and varying affinity for folate-binding proteins likely impacts bioavailability and therefore necessitates further investigation in the context of milk as a potential matrix for folate fortification.

Fiber

Dietary intake of fiber can increase or decrease folate bioavailability depending on the mechanism of action. For example, whether fiber is soluble or insoluble can impact the viscosity, as well as the movement of material through the digestive tract. In a short-term trial, dietary fiber was shown to have an inhibitory effect on folate bioavailability when Finglas et al. found lower levels of labeled folate excreted in urine when folic acid was ingested with bran flakes, compared to folic acid ingested with white bread (144). The mechanism for this observation was attributed to the folic acid being trapped within the solid matrix of dietary residues or confined by the chains of soluble dietary fiber, thereby preventing absorption (96). In contrast, French et al. reported that cereal foods with high levels of folate and dietary fiber may be as efficient as folic acid in improving folate status in study participants (145). Indeed, increased fiber intakes
have been indirectly associated with higher serum folate concentrations in both adolescent (119) and post-menopausal women (146). The proposed mechanism was that non-digestible, but fermentable dietary fibers are known to stimulate the growth of endogenous microbial populations (e.g. folate producers). Further research exploring the dynamics of diet, microbial populations, folate, and health implications, are worthy of investigation.

2.3.3 Pharmaceutical Interactions

Many drugs have the potential to alter folate metabolism or have decreased drug efficacy by high levels of folate intake (10, 34). Diuretics can alter renal re-absorption of folate and increase urinary excretion. Sulfanilamide or other antibiotics act as a competitive inhibition of bacterial metabolism and can alter the intestinal pH, as well as colonic biosynthesis of folate. Azulfidine, a drug for treatment of inflammatory bowel disease is a competitive inhibitor of folate absorption. Anti-inflammatory drugs may have antifolate activity. Anticonvulsants for treatment of epilepsy like phenytoin or primidone compete with folate and inhibit the uptake of one another at the intestinal epithelium as well as the brain cell wall. Additionally, the efficacy of chemotherapy drugs (e.g. methotrexate), and antimalarial drugs (e.g. pyrimethamine) may be affected by varying folate levels.

Ethanol intake has been shown to reduce the bioavailability of folate and impact metabolism. Ethanol-associated folate deficiency may be attributed to malabsorption of folate by renal and intestinal uptake due to altered expression, binding and transport kinetics of folate transporters (147). Thereby a greater amount of folate excretion, as well as dietary inadequacy
could be further exacerbating factors. In rats, chronic alcohol ingestion has been associated with a down regulation of RFC expression (148). Further, animal studies demonstrated that regular consumption of ethanol is a known indicator of folate deficiency resulting in subsequent impairment of DNA methylation due to inhibition of folate-mediated methionine synthesis and subsequent production of SAM. As of yet, the role of PCFT has not been fully explored within the context of alcoholism (147). Lastly, chronic smoking is also associated with a lower systemic status of plasma and erythrocyte folate, B_{12} and B_{6} and varying folate form distribution after analysis of buccal cells compared to nonsmokers (149).

2.4 Measuring Folate Bioavailability

The bioavailability of folate has been examined in the context of both acute and chronic protocols using reduced folates, as well as the oxidized folic acid form used in supplements and fortification (136). In acute studies, a single ingestion of a test dose is administered, whereas in chronic studies the effect of long-term (e.g. 4-24 weeks) intervention is measured. Both protocols can involve a cross-over or parallel design and changes in folate plasma (acute) or RBC (chronic) status are measured at various time points. In the chronic study, the inclusion of a placebo group is essential to control for any interventional conditions influencing the study, as well as monitoring of subject compliance. A common limitation of chronic studies using food folates is the impact of the food source as aforementioned (section 2.3.2 Dietary Components). Acute studies are often more attractive for understanding absorption kinetics and metabolism, as
the safe use of stable isotopes allows labeled folates from the test dose to be distinguished from unlabelled body folate stores (8, 19, 81). Although, stable isotopes are particularly attractive, a significant limiting factor is the cost of stable isotopes and analysis using sophisticated equipment such as liquid chromatography tandem mass spectrometry. In addition, the supply of stable isotopes is limited and many are unavailable commercially, thereby requiring investigators to synthesize and purify the needed folate form (150). Recently, the Food Standards Agency assessed the current research and concluded that additional research examining folate bioavailability in whole diets is a priority and suggested this direction for future research (151).

**Stable isotope studies**

The use of protocols with stable isotopes originated in the late 1980s and allows for the acute evaluation of the relative bioavailability of folate derivatives. A stable isotope is an element with similar chemical characteristics and additional neutrons resulting in a heavier atomic mass and allows for high specificity. The discrimination of labeled folate doses in plasma from folates originating in unlabelled body folate pools is possible, as the only source of the labeled folate in the blood and/or urine can be from the administered labeled test dose. Subsequent monitoring of isotope molar ratios after as determined by plasma folate responses, provides quantitative measurements (the maximum concentration reached ($C_{max}$), the time to reach the maximum ($t_{max}$), the rate of plasma folate decrease ($t_{1/2}$) and increase (nmol/h)) of folate absorption across the small intestine (80, 128). Investigators using stable isotopes to determine the percent bioavailability of different forms of folate (level of reduction, carbon substitution, level of polyglutamylation) have come to very different conclusions (81) a (132,
Overall, the use of stable isotopes continues to quantitatively enhance our folate metabolism understanding.

In our previous work, conducted by Aufreiter et al., the primary outcome was to measure the appearance of the tracer 5-methylTHF derived as a metabolite from the administrated test dose 5-formylTHF after a cecal infusion and IV injection (19). Administering an IV injection directly into the systemic pool reflected the conversion of formylTHF to methylTHF, as well as plasma enrichments of these two folate forms reflected the plasma pool retention and hence also the rate of clearance from the plasma pool. After an oral or cecal infusion dose the complexity of determining absolute bioavailability across the intestine is compounded by extensive enterohepatic recycling and splanchnic storage first-pass metabolism. Moreover, the disposition of some of the administered labeled folate into unsaturated tissue pools as well as displacement of unlabelled folates to the plasma upon absorption of the test dose are current limitations in our understanding of estimating bioavailability (19, 34).

\textit{Area under the curve}

Area under the curve (AUC) has been shown to be a reliable estimation used when a sufficient number of blood samples are taken after an acute folate test dose to allow for adequate absorption across the small intestine and observation of plasma concentration returning to baseline (81, 153). The simple numeric estimation of area by the trapezoidal rule is a common method of AUC determination (121). Post-dose plasma AUC is assumed to correspond to the fraction of folate absorbed from a single dose. Data are commonly presented as the percent relative bioavailability by calculating the ratio after an oral dose to an intravenous (IV) dose (34). The equation: \[ \frac{\text{AUC (oral dose)}}{\text{AUC (IV dose)}} \] provides an estimate of the percent relative
bioavailability. If different doses were administered, the AUC values must be adjusted accordingly to equal the same test dose and is only appropriate if the same folate form is administered in both the test (oral) and reference dose (IV). The similarity of absorption rates, bioavailabilities and metabolism properties for different folate forms, such as 5-formylTHF and 5-methylTHF versus folic acid have been questioned (81).

### 2.5 Administering Test Doses to the Colon

**Gastrointestinal transit**

Gastric emptying is multifaceted and relies on caloric content of the meal, particle size, as well as intestinal transit and circadian rhythm. In a study by Goo et al. (1987) gastric-emptying was observed to be longer after a solid meal in the evening versus the morning (154). During fasting, gastric emptying is usually rapid within approximately 1 hour, for both (liquids and solids), although inter-individual variability is considerable. Davis et al. (1986) reported that the gastric emptying time was shorter in adults consuming small caplets (0.3 X 1.8 mm) versus large caplets (either 25 X 9 mm or 8 X 12 mm). Large capsules have a shorter gastric emptying time after a light meal then a heavy meal (155). Shortly after ingestion of a solid meal, the stomach initiates a lag phase, followed by a pattern of low amplitude contractions until all the food is expelled into the small intestine known as the “housekeeper” wave. During fasting, myoelectric and contractile activity migrates down from the lower esophagus to the colon to sweep remnants of the previous meal and prevent stagnation and bacterial overgrowth. The exact ejection time of
a solid particle from the stomach is effected by when it was ingested during the motor activity cycle and where it is located within the stomach (121). Contents of the small intestine remain for approximately 3 hours (155-158). In the small intestine, the variability in transit time is less than gastric emptying time and appears to be unaffected by the physical size of the solids, age or physical activity (96, 121, 155).

The rate of entry of particles into the colon is thought to be controlled at the ileocecal junction (159). Typical colonic motor activity is represented by single non propagated contractions, however the most powerful stimulant of colonic motility has been reported to be waking from sleep and following meals (160). Physical exercise may also enhance colonic response to meals, as well as stress (96). The colon transit time can range from 10-72 hours or days, allowing for a greater time of absorption than that of the small intestine. Furthermore, colon transit times may be affected by drugs, disease, diet, and bacterial populations.

Colon-targeted systems

The colon is a suitable site for the safe and slow absorption of drugs, nutrients or other bioactive materials with the potential to influence both local and systemic effects (96). Research in the past few decades has focused on targeting drugs and delivery systems to the colonic region of the gastrointestinal tract by the need to better treat constipation and diarrhea, infections and local disorders such as inflammatory bowel disease (ulcerative colitis and Crohn’s disease), irritable bowel syndrome and carcinoma. Theoretically, the two administration routes available for testing nutrient or drug absorption in the human large bowel are rectal (perfusions, enemas, and suppositories) or orally in the slow release or targeted forms (caplet).
Currently the predominant route for administering a nutrient test dose is often limited to rectal routes. Enemas, suppositories and perfusions have been methods for measuring colonic uptake of certain B-vitamins (161), as well as various fatty acids (162-164). Rectal infusions have provided valuable insight in the absorptive capacity of the colon, however the findings of these studies should be considered in the context of their limitations. Firstly, the invasive nature of intubation methods may lead to low tolerance and acceptability among participants. Secondly, the site-specificity of rectal infusions is limited to the distal colon and the relative contribution of more proximal regions, with distinct motility and absorption characteristics, to overall nutrient absorption maybe underestimated (96). Thirdly, mechanical stimulation may alter absorption, as it has been found to increase blood flow to the colon (165).

The less invasive caplet approach allows for measurement of folate absorption down the entire colon as opposed to the distal region accessed by enemas and suppositories. Delivery strategies and systems proposed for colon targeting rely on the gastrointestinal characteristics for their functionality: transit time (166-171), luminal pressure (172-174), intra-luminal pH differentials (175-180) or bacterial metabolism of pro-drugs (e.g. fiber that is fermented by microflora to release test dose) (181-183). The latter two systems have specialized coats have been commercialized and used for clinical purposes. In Canada pH-sensitive systems are available commercially for clinical use (184), particularly for the treatment of inflammatory bowel disease. Coated caplets with methacrylic acid co-polymer, Eudragit® L100 and Eudragit® S100 have been combined in ranging ratios to manipulate the release profiles of drugs in vitro within the pH range of 5.5 to 7.0 for pH sensitive colon targeted oral drug delivery (185).

Ensuring nutrient dosage delivery distal to the ileocecal junction is important in the quality of results, especially considering that we know nutrients, such as folate are readily
absorbed across the small intestine. Therefore because micronutrients are often readily absorbed from both the small and colon it is critical for valid and reliable results when designing a vitamin delivery system to determine caplet colon arrival and caplet disintegration times. Gamma-scintigraphy and fluoroscopy are two well-established non-invasive imaging methods that can be used for monitoring a caplet’s transit, as well as provide qualitative or semi-quantitative information on the motor activity of the gastrointestinal tract. Gamma-scintigraphy does not require the use of contrast media, such as barium sulphate suspension like fluoroscopy and instead uses radionuclide tracers (186). Alternatively, although the resolution of fluoroscopy is superior to gamma-scintigraphy, the numbers of gastrointestinal events that can be captured are limited due the risk of radiation exposure. Both imaging methods are non-invasive and the natural physiology of the intestinal environment remains unaltered (96).

Prior to Aimone et al. (2009) incorporating fluoroscopy and a colon-targeted pH-dependent caplet, virtually no caplet formula was designed to dissolve specifically distal to the ileocecal junction and deliver a test nutrient (32). After comparing in vitro and in vivo models, the authors determined that a 3:1 (L100/S100 Eudragit®) acrylic co-polymer coating with a placebo caplet containing a barium sulphate core (for in vivo fluoroscopy monitoring), resulted in caplet consistent delivery of the test dose to the colon. This successful disintegration initiation was found despite the likely high gastrointestinal pH variability among individuals (n = 10). In conclusion this caplet protocol maybe be a suitable delivery system for delivering a test nutrient precursor for quantifying nutrient absorption in the human colon, and in particular folate labeled with a stable isotope (32).
2.6 Folate Health Outcomes

2.6.1 Biochemical Assessment of Folate Status

A number of methods have been developed to determine folate levels in serum and plasma (Table 2.6.1) (187). Folate measurement in serum was first described by microbiological assay in 1959 (188). This microbiological assay today uses *Lactobacillus rhamnosus* (formally known as *L. casei*) for the determination of folates in blood samples (189). Clinical laboratories measure blood folates by radio, immuno, chemiluminescence or competitive protein assays, as well as spectrophotometric, electrochemical and fluorometric methods. Although sensitive to total folate measurements, these methods lack the specificity to distinguish folate forms and agree poorly (190). Recent chromatographic methods are suitable for determining plasma of serum folate species, and their introversions by combining liquid chromatography (LC) with tandem mass spectrometry (MS/MS) provides highly sensitive different parent compounds with the same mass-to-charge ratio to produce distinct daughter fragmentation. High-pressure liquid chromatography methods are often combined with microbiological assays to quantify total folate counts, as LC methods would need to use all known forms of folate, some of which are hard to find and costly to make (187). Because serum folate is labile, proper protection to avoid oxidative cleavage and preserve the reduction of the pterin ring, is accomplished by adding an antioxidant (such as ascorbate), avoiding UV light and storing the serum frozen. In the absence of protection from oxidative destruction, false underestimates of serum folate activity occur and should be considered when interpreting early study results (37).
Table 2.6.1 Commonly used methods for determination of folate status.

<table>
<thead>
<tr>
<th>Microbiological assay&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Radioassay</th>
<th>LC-MS/MS&lt;sup&gt;a,b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Indication</strong></td>
<td>Growth of L. rhamnosus depends on amount of folate in sample. Microtiter plate reader measures absorbance (turbidity) and correlates to folate content (nmol/L).</td>
<td>Competitive binding between sample folates and a known amount of radio labeled folate to specific antibodies or folate binding protein. Radioactivity of unknown samples is determined by comparing the ratios.</td>
</tr>
<tr>
<td><strong>Strength</strong></td>
<td>Measure total folate concentrations &lt;100 pmol/L, equivalent growth response for different folate forms.</td>
<td>Rapid analysis of multiple samples for total folate.</td>
</tr>
<tr>
<td><strong>Limitation</strong></td>
<td>Growth affected by: polyglutamyl chains &gt;3, contamination, presence of antifolates, and pH.</td>
<td>Not selective for bioactive stereoisomer found in food samples. Variable affinity for different folate forms.</td>
</tr>
<tr>
<td><strong>Primary Application</strong> Research Laboratories</td>
<td>Clinical Laboratories</td>
<td>Research Laboratories</td>
</tr>
</tbody>
</table>

<sup>a</sup> LC-MS/MS; liquid chromatograph coupled to tandem mass spectrometry.  
<sup>b</sup> Methods used in present study. Adapted from Quinlivan et al. (187).

**Reference values**

RBC folate concentration is an indicator of long-term folate status, and is reflective of liver stores (191). The concentration of folate in RBCs may be influenced by genetics as well as adequate amounts of other vitamins which influence one-carbon metabolism (192-193).

Serum/plasma folate concentration reflects recent folate intake and may vary from individual to individual. The monoglumatate form of 5-methylTHF is the predominant species of folate in human circulation (>90%) (50, 190, 194). Low concentrations of 5-formylTHF (190) and 4-α-hydroxy-5-methylTHF have been documented (195) and folic acid may also be present after ingestion of supplements or folic acid fortified foods (78, 190).

Measurements of RBC and serum/plasma folate levels are very useful indicators to diagnose folate deficiency as well as to estimate additional folate supplement requirements to
reach specific cutoff levels associated with reducing adverse health outcomes (12). Adequate folate status is often indicated in from 11.33-36.25 nmol/L (or 5 to 16 ng/ml) of serum folate (37). Nutritional folate deficiency is defined by inadequate amounts of the biologically active vitamin in one or more intracellular systems to sustain normal biochemical functions (37). Folate deficiency can result from a gamut of physiological conditions such as dietary insufficiency, drug interactions, anabolic states, or malabsorption due to liver disease or limited GCPII. A risk of folate deficiency has been associated with obesity (196-197). In a multivariate analysis of postmenopausal women, those who were obese and overweight had a 22% and 12% lower serum folate concentration, respectively, compared to normal weight women ($P$-trend = 0.02) (196). A potential methodological consideration is that these subjects were also consumed varying intakes of alcohol (none, 1 drink, or 2 drinks per day), although these moderate doses of alcohol did not significantly affect serum folate concentrations (198). Potential physiological reasons for these findings could be increased intracellular uptake and/or retention, as well as greater renal excretion (196).

According to Herbert (1999) there are four stages in the development of folate deficiency starting with an initial decrease in serum folate below 6.8 nmol/L (RBC is above 453.3 nmol/L) (37). Second, further folate depletion lowers RBC folate below 360 nmol/mL. Next, folate-deficient erythropoiesis occurs by defective DNA synthesis and granulocyte nuclear hypersegmentation. Finally, clinical folate deficiency is manifested by gross macroovalocytosis (anemia and increased mean corpuscular volume) (37). Routinely, a RBC cutoff value of 305 nmol/L is indicative of folate deficiency (1). Several serum/plasma folate levels have been indicated to have adverse health outcomes. If levels are <7nmol/L and left untreated megaloblastic anemia is common (199). The considered level needed to avert high blood
Homocysteine levels associated with cardiovascular disease is $\geq 10$ nmol/L is (200). Levels $>16$ nmol/L (RBC $>906$ nmol) is associated with a reduction in NTD occurrence for “normal” populations (201). Although, supraphysiological levels of folate have been measured post-fortification, specific levels associated with adverse effects due to very high folate intakes remains unclear, however in the elderly with low vitamin B$_{12}$ status, a high serum folate value $>59$ nmol/l has been associated with anemia (OR: 3.1; 95% CI: 1.5, 6.6) and cognitive impairment (OR: 2.6; 95% CI: 1.1, 6.1) (202-204).

*Homocysteine*

The intracellular concentration of homocysteine is inversely related to plasma folate concentrations (205-206). Changes in plasma folate concentrations between 2 nmol/L to 15 nmol/L likely influence methylation potentials, however there is no convenient marker to determine the status of nucleic acid synthesis (207). Low folate levels resulting in DNA abnormalities (impaired synthesis, repair, and methylation) are believed to especially affect rapidly proliferating cells. Hence, clinical manifestations of folate deficiency appear first in the hematopoietic system, followed by the epithelial cells surfaces and the gonads. Low folate status and a corresponding elevated homocysteine has been associated to be a risk factor for stroke (208) and adverse pregnancy outcomes (e.g. neural tube defects) (209).
2.6.2 Nutritional Recommendations

The recommended dietary allowance for folate is 400, 600 and 500 µg/day DFE for adults, pregnant and lactating mothers respectively. These recommendations are based on the amount of folate to maintain normal red blood cell concentrations (1). The upper tolerable intake for folic acid (1000 µg/day for adults) does not include naturally occurring folate and was based on a systemic review of the literature. The upper limit value was set to protect against the possibility that high intakes of supplemental folic acid could mask vitamin B_{12} deficiency and thereby safely avoid potential adverse neurological effects in deficient individuals (1, 210). For Canadian women, Health Canada currently recommends a daily multivitamin containing 0.4 mg of folic acid in addition to a healthy diet for women capable of becoming pregnant, to reduce the risk of NTDs (211). The Canadian Society of Obstetricians and Gynecologists recommends varying folic acid intakes based on individual health risks (212). For women planning a pregnancy that have no personal health risks and consume a diet of folate-rich foods and regularly consume a folic containing supplement, a multivitamin containing 0.4-1.0 mg folic acid is recommended 3 months prior to conception and throughout pregnancy and postpartum period. However, if a woman has known health risks, such as previous NTD pregnancy, epilepsy, insulin dependent diabetes, or obesity with BMI >35 kg/m^2, 5 mg of folic acid daily 3 months prior to conception and continuing until 12 weeks is suggested and then decreasing the dose to 0.4-1.0 mg for the remainder of pregnancy. Further, women who have a poor history of compliance with medications, variable diet, no consistent birth control or possible substance use are recommended to take 5 mg folic acid supplement.
Post-fortification, the research literature shows an increase in women who have RBC folate concentrations that approach or exceed 906 nmol/L (9), (213), an indicator of a reduced risk for NTDs (201). In example, one study by Houghton et al. found that during the 3rd trimester well-educated Canadian women had RBC folate concentrations ≥3000 nmol/L (56). However, many women are at a higher risk of inadequate intake, and Sherwood et al. reported one-third of pregnant and lactating women were unlikely to meet their folate requirements from diet alone after analysis of three day weighed food records collected at 36 gestation, and 4 and 16 weeks of lactation (213). Additionally, in comparison of two National Health and Nutrition Examination Surveys (from 1988-1994 and 1999-2000), only 10% of women of childbearing age reached the mean erythrocyte folate concentration >906 nmol/L, supporting current recommendation that women of childbearing age should take folic acid supplements (214). Environmental contributors that can increase a woman’s risk of inadequate folate intake, in addition to not taking a folic acid supplement include food insecurity, lower socio-economic status, and dietary restrictions (215).

2.6.3 Folate Deficiency

Considerable epidemiological and experimental evidence links adequate B-vitamin levels to multiple health outcomes, with the greatest evidence supporting a reduced risk for NTDs. Adequate folate is particularly critical in life stages with increased cell division such as during pregnancy and growth (216). A significant, although small (6%) decline in the prevalence of orofacial clefts (217) and cardiovascular anomalies coincides with the implementation of folic acid fortification (4). A reduction in trisomies 13, 18 and 21 has been conflicting, and whether
abnormal maternal folate metabolism has been linked to an increased risk of Down Syndrome requires further investigation (218-219). Moreover, folic acid supplementation may protect against placental abruption, as well as low infant birth weight (220) and preeclampsia (221). In addition to the affect of folate on women and their offspring, the effect of paternal periconceptional supplementation and folate gene polymorphisms are under current under investigation. In one study, a lower overall frequencies of sperm aneuploidy in healthy men consuming >700 µg of folate/day was reported (222). In addition, research in elderly populations have found adequate folate to be associated with the prevention of stroke (223). Furthermore folate deficiency influences neurodegenerative and neuropsychiatric diseases and thus folate is commonly an adjunct to antidepressant pharmacotherapy (224). To date, the role of adequate folate status and its health benefits continues to an area of growing research.

Neural tube defects

NTDs are the second most common congenital malformation following congenital heart defects. The incidence of NTDs is 1 per 1000 births, namely spina bifida and anencephaly (45). The first association between folate deficiency and NTD incidence was published in 1965 (225), however it was not until compelling results from a double-blind, placebo-controlled study in the early 1990s that switched the focus from nutritional improvement to pharmacological prevention (226). In this large randomized trial of 1817 women who had a previous NTD-affected pregnancy, a 72% reduction in NTD re-occurrence risk was demonstrated after periconceptional folic acid supplementation with 4 mg/day (72). Another large randomized controlled trial provided additional evidence revealing that supplementation with 0.8 mg folic acid as part of a multi-vitamin supplement reduced the first occurrence of NTDs by 93% (73). These two studies
and additional retrospective case-control studies found a reduced risk of NTDs in women taking a supplement containing 0.360-0.8 mg/day, in addition to 0.2-0.3 mg/day of natural folate dietary sources. In light of this, the general recommendation for women planning a pregnancy include taking 0.4 mg of folic acid per day at least one month before conception and during the first trimester of pregnancy (1, 45). A recent Canadian survey (n = 6421) suggests that 57.7% of women report consuming folic acid during the periconceptional period (90.1% took it daily), however almost all women used folic acid during the first three months of pregnancy (89.7%) (227).

Folate is essential during the first few weeks of pregnancy for normal development of the brain, skull and spine of the fetus; and later for the mother’s expanding blood volume and growing fetus tissue. As the neural tube closes during the 1st three to four weeks of pregnancy, many women are unaware of their pregnancy during this critical time of neural tube development. Thus, routine folic acid supplementation of women planning a pregnancy or capable of becoming pregnant is an important public health strategy in reducing the risk of NTDs (1). As approximately half of pregnancies are unplanned, Canada, along with the U.S. mandated a folate fortification policy in 1998 inline with the substantial evidence linking adequate folate intake to a reduction in NTD risk (2-3). The folic acid addition of 1.5 μg/g white wheat flour and 2.0 μg/g enriched pasta was estimated to increase the daily intake of folate by women who habitually ingested low amounts of folate by about 100 μg a day (228). It is important to consider that in addition to folate, several other factors increase the risk of NTDs. As highlighted by Heseker et al., genetics and environmental exposures have been associated with NTDs (229). As well as drugs, alcohol, assisted reproductive technology, maternal vitamin B₁₂ deficiency and obesity are also factors influencing NTD risk (229-230).
Fortification

As anticipated, the RBC concentrations of reproductive-aged women increased from pre- and post-fortification. For example in Canada, the RBC of reproductive-aged women increased from 625 to 818 nmol/L in Newfoundland (9). These increased circulating folate blood levels were associated with the reported significant reduction in NTDs after fortification of the food supply. A landmark Canadian study by De Wals et al. (2007) examined women (n = 2446) from seven provinces and examined the number of live births, still births and pregnancy terminations because of fetal anomalies from 1993-2002 (6). The prevalence of NTDs decreased from 1.58 per 1000 births pre-fortification to 0.86 per 1000 births post-fortification (46% reduction, 95% confidence interval, 40-51). This observed reduction was greatest in geographical areas where the baseline was high. In the individual province of Ontario, Ray et al. (2002) and Gucciardi et al. (2002) after analysis of records from antenatal screening, hospital discharge and therapeutic abortions found a 48% and 47% reduction in NTDs respectively (231) (232). In Quebec, a 32% lowering of NTDs was noted (233), similarly Nova Scotia found a 54% decrease from 1991-2000 (234), and Newfoundland showed a significant reduction from 4.67 (pre-fortification) to 1.01 (post-fortification) per 1,0000 total births (235).

It is important to consider that NTD prevalence is not a reliable estimate for the incidence of NTDs, as often only live and still births are recorded for the prevalence of NTDs, and over the past years there have been an increase in pregnancy terminations which are often classified in a nonspecific manner (45). Overall, the effects of folic acid fortification and supplementation are undisputed and recognized to be protective against adverse pregnancy outcomes and multiple birth defects (12). Fortification has been viewed as highly successful in achieving its primary
objective with fortification preventing up to 50% of NTDS in studies with complete ascertainment of diagnosed prenatal NTD cases (236).

*Stroke*

One-carbon folate metabolism and the methylation of homocysteine to methionine have been linked to vascular disease. As such, numerous observational studies have measured high homocysteine levels in individuals with atherosclerotic disease (e.g. cerebral stroke and myocardial infarction) (237). However, it is noteworthy that no beneficial effects from homocysteine-lowering therapy with B-vitamins has been demonstrated in patients with established coronary heart disease as indicated by four recent secondary intervention trials (VISP, HOPE-2, NORVIT and CHAOS-2) (238). The mechanism of folic acid supplementation and subsequent lowering of the neurotoxic effects of high plasma homocysteine levels is currently associated only with the risk of stroke (239). Comparing different vitamin interventions and homocysteine lowering response (which also depend on renal function); folate has the greatest effect (240), followed to a lesser extent by vitamin B₁₂ (241), vitamin B₆ (242) and riboflavin (243).

In a recent population-based study it was found that a temporal decline in stroke-related mortality coincided with folic acid fortification in North America (244). Notably, no change was noted in England or Wales where fortification is not mandatory. Further, a meta-analysis of clinical trials shows that supplementation with folic acid reduced the risk of stroke by 18% overall, by 29% in trials with a treatment duration of >36 months and by 25% in subjects with no history of stroke (245). Additionally, reanalyze of data from 2 secondary intervention trials in cardiovascular patients (Hope-2 and VISP) (246-247), revealed a reduced risk of stroke
following high-dose vitamin therapy (2.5 mg of folic acid, 25-50 mg of vitamin B$_6$, and 0.4-1 mg of vitamin B$_{12}$) in patients without vitamin B$_{12}$ deficiency and normal kidney function (223). As stroke is presently the only vascular disease demonstrating a relationship with homocysteine levels, additional epidemiological studies are required to determine whether high homocysteine levels represent a risk “indicator” rather than “factor” for individual vascular diseases (237).

Cancer

As a basic component of cell metabolism that is important for replication, cell division, growth and survival, folate plays a critical role in cancer prevention. Inadequate blood folate concentration is inversely related to the misincorporation of uracil into DNA (inefficient biosynthesis of thymidine, a folate-requiring step) leading to two pathways implicated in cancer: increased chromosomal breaking and abnormal methylation reactions (248). In normal tissues, folate deficiency has been associated with leukemia and neuroblastoma, as well as other malignancies including cancer of the breast, lung, cervix, lung, pancreas, stomach, and colon (5, 249-250). Furthermore, epidemiological evidence and multiple published reviews associate a low-folate diet with an increased risk of colorectal cancer, and under these circumstances are in favor of folate supplementation as a preventative measure (251-253).
2.6.4 High Intake of Folic Acid

Despite the decline in NTDs following folic acid fortification, concerns about the chronic exposure to high levels of folic acid remain. Many countries have questioned the safety of fortifying the food supply for the whole population as emerging evidence identifies the positive and potential adverse effects of high folic acid intake such as masking of B\textsubscript{12} deficiency. Folate plays a pivotal role in normal cell replication and is also influenced by disease states such as cancer growth, psoriasis and parasite life cycles which depend upon this nutrient (10, 254). Many anti-cancer, anti-malaria and antibiotic drugs have anti-folate properties (255-257) and higher levels of folic acid intake, such as experienced post-fortification may jeopardize their efficacy. Results from animal studies suggest that depending on the dose and timing of folate intervention, an overly abundant intake of folic acid in the presence of a pre-existing neoplasm may promote tumorigenesis (258-260). These results are supported by epidemiological and clinical studies of folate and cancer, particularly colon cancer (260-262).

Cognitive impairment

The most widely documented concern of population-based exposure to folic acid is that high doses of folic acid may mask the diagnosis and thereby delay treatment of vitamin B\textsubscript{12} deficiency (1, 263) (**Figure 2.6**). Moreover, supplemental folic acid bypasses methionine synthase (activity is impaired and inhibits regeneration of tetrahydrofolic acid during B\textsubscript{12} deficiency) to resolve hematological signs of anemia macrocytosis created by impaired DNA synthesis (56). Cognitive impairment in the elderly may be worsened by high serum folate levels greater then 59 nmol/L (14). Neuropathies due to undiagnosed vitamin B\textsubscript{12} may be exacerbated
without the presence of megaloblastic anemia and result in clinical features such as dementia, psychiatric disorders, paresthesia or sensory loss (264). There has been substantial concern of a significant portion of the U.S. population, in particular older supplement users, who are exposed to amounts of folic acid exceeding the upper tolerable intake (265). Post-fortification analysis of the United States population observed 38% of the elderly with supraphysiological serum folate concentrations (203). The primary cause of vitamin B\textsubscript{12} deficiency in older people is malabsorption of food-bound vitamin B\textsubscript{12} (266-267). In The United Kingdom, approximately 6% of those aged ≥60 have plasma B\textsubscript{12} <148 pmol/L (deficient) and roughly 20% have levels ranging from 148-221 pmol/L (marginal status) (267). Inadequate vitamin B\textsubscript{12} intakes is more commonly associated with low consumption of animal-source foods in younger adults in developed countries, as well as by all ages living in poverty worldwide (267). Further public health strategies are needed to examine potential adverse interactions with folic acid and improve vitamin B\textsubscript{12} status.
2.6.4.1 Cancer

Initial evidence that high supplemental or fortified folic acid intake might enhance carcinogenesis in the presence of a neoplastic lesion dates back to the 1940s (268-269). Sidney Farber termed the “acceleration phenomenon” to describe the effects on tumor growth after a large dose of folic acid was administered to leukemia patients (268). The physiological mechanism supporting cancer growth in the presence of folate is thought to be due to the high
rate of proliferation in neoplastic cells, therefore requiring additional quantities of folate to maintain DNA synthesis. In particular, the risk of colorectal cancer appears to have a “U-shaped curve” as demonstrated by animal models and the timing and dose of folate supplementation is pivotal to the protective or promoting effect (Table 2.6.2) (239). Using mouse models with a genetic predisposition to colon cancer (e.g. Apc\textsuperscript{Min/+}), increasing folate intake appears to be only protective prior to the establishment of neoplastic foci (258). Once cancerous cells are present in the same model, a folate-deficient status may be protective, however additional folic acid supplementation furthers tumor growth (258-259). Therefore, in normal tissue, folate deficiency and supraphysiological folate doses of supplementation are thought to be a risk for carcinogenesis, whereas modest folate supplementation suppresses cancer. However, in established neoplasms, folate deficiency inhibits cancer progression and in contrast folate supplementation has a promoting effect (270).

<table>
<thead>
<tr>
<th>Normal Tissue Cancer Risk:</th>
<th>Folate deficiency</th>
<th>Folate supplementation</th>
<th>Supraphysiological folate status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mechanism:</td>
<td>↑ Impaired DNA repair of strand breaks and increased mutagenesis</td>
<td>↓ Increase mucosal genomic DNA methylation and reverse pre-existing hypomethylation</td>
<td>↑ Enhanced development of cancer (refer to \textsuperscript{a})</td>
</tr>
<tr>
<td>Neoplasm Cancer Risk:</td>
<td>↑ Mutagenesis inhibited by optimal DNA repair, stability, integrity and prevention of aberrant methylation</td>
<td>↑ \textsuperscript{a} Proliferation and growth of cells with increased nucleotide precursors and altered methylation resulting in gene inactivation of tumor suppressor genes</td>
<td>↑ Enhanced progression of cancer (refer to \textsuperscript{b})</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Adopted from Kim et al. (270).
Currently colorectal cancer is the second leading cause of death from cancer in Canadian men and women combined (271). Folic acid fortification has been associated with a brief increase in colorectal cancer immediately after the fortification of the food supply with in Canada and the United States prior to 1998 (243). Within the past year, Hirsch et al. (2009) reported an apparent increase in colorectal cancer in Chile since the introduction of mandatory fortification with 220 g of folic acid per 100 g of white flour. The rates of cancer risk increased by 162% in people aged 45 to 64 and by 190% in people aged 65-79 with high intakes of folic acid (272). In the Aspirin/Folate Polyp Prevention randomized control trial, the administration of 1 mg folic acid/daily for six years in individuals with previously removed adenomas, did not result in a decreased adenoma recurrence, rather an increased number of adenomas was recorded, as well as a higher incidence of advanced lesions and non-colorectal cancers (261). Results from the same randomized clinical trial estimated the probability of prostate cancer diagnosis over a ten year period to be 9.7% in the folic acid supplement group compared to 3.3% in the placebo group (273). On the other hand, a multi-centre, double-blind randomized control trial \( n = 853 \) reported no significant effect of folic acid intervention on the incidence of developing colorectal adenoma recurrence (274). Although this latter study used gave half the folic acid dose (500 \( \mu g/day \)) and had a shorter follow-up (three years) compared to the Aspirin/Folate Polyp Prevention Study. As further research examines how folate modulates carcinogenesis, it is imperative to include various times periods of observation to capture the progression of cancer, as well as to consider gene-folate interactions and micronutrients that are critical cofactors for several pivotal enzymes of the cellular functions of one-carbon metabolism (B\(_6\), B\(_{12}\), and riboflavin) (275-279). This ‘dual modulatory’ effect of folate and cancer, coined by Kim et al.
(240), is poignant when considering guidelines, as the healthful intake of folate for one person, maybe potentially harmful to another (241).

*Post-fortification*

Unmetabolized folic acid has been detected in supplement users (8, 13), as well as in adults and infants from countries with voluntary fortification (280). This is likely due to supplements or voluntary fortification of foods. Nevertheless, the phenomenon of unmetabolized folic acid, along with the large occurrence of supra-physiological serum levels of folate in populations is a substantial evolutionary change from previous centuries of folate intake. In animals, high folic acid-rich diets can influence DNA and histone methylation resulting in phenotypic changes in subsequent generations (281). For example, maternal diets containing high folic acid content influenced varying epigenetic effects of phenotypic agouti mice offspring coat color by varying cystosine residue methylation in the transposon promoter area of the gene (282). Alarming evidence suggests that high folic acid levels in pregnant women were associated with an increased risk of insulin resistance and obesity in their children (15). There may be direct adverse effects from unmetabolized folic acid in blood, which was found to be inversely related to natural killer cell cytotoxicity, a component of the nonspecific immune response (13). Another, study by Ashokkumar et al. reported an in vitro reduction in $[^3]$H-folic acid uptake by Caco-2 cells (from a human colon carcinoma cell line) in media designed to mimic folate over-supplementation compared to folate-sufficient conditions (283). The reduction in labeled folic acid uptake was associated with a decline in both mRNA levels of RFC and PCFT in Caco-2 cells, as well as decreased RFC protein. This study demonstrates short-term adaptability; however, the potential harmful effects long-term changes remain unknown.
As folate intakes post folic fortification of the food supply rise, folate blood values are considered supraphysiological in many segments of the general population (6-7, 231, 284-285). The mean level of serum folate before and after fortification in the United States increased from 12.0 to 29.7 nmol/L (12). In Canada, a retrospective review of adult mean RBC concentrations reported an increase from 680 to 852 nmol/L post-fortification of the food supply, however there was no measurement of supplement use (286). In a review from the United States pre-and post-fortification, the largest contributors of folate in the American diet were found to be in bread, rolls and crackers made from fortified flour (214). Routinely, industries over-fortify enriched grain foods to protect against shelf-life decomposition by amounts estimated by some to be twice the mandated amount (287). Ten years post-fortification an analysis of commonly purchased folic acid-fortified foods revealed that neither the food label or database (Canadian Nutrient File 2007b) values accurately reflect the actual amount of folate which was approximately 50% higher than what was mandated (288).

Although, mass fortification has been considered a successful intervention by the lower prevalence of NTDs (12), further examining the distinction between folate sources is pertinent. For example, in a recent study published examining folic acid source and usual intake in American adults from the National Health and Nutrition Examination Survey 2003-2006, Yang et al. (2010) reported that half of those who use supplements >400 µg folic acid/day had intakes that exceeded the upper intake level (289). However, the authors observed that at current fortification levels, only 2.7% of individuals consuming folic acid supplements ≤400 µg/day or not consuming supplements had intakes above the upper intake level. Therefore, clearly closer monitoring of the nutrient content of fortified foods, as well as supplement use is one of the
critical steps in moving forward and achieving the optimal balance between health benefits and risks.
Chapter 3

3. Folate Absorption across the Human Colon: Caplet Study

3.1 Data Chapter Introduction

3.1.1 Rationale

The B-vitamin folate is most recognized for its role in nucleotide biosynthesis and repair. Adequate folate is critical for the re-methylation of homocysteine and production of the essential amino acid methionine, a precursor for SAM, the primary methyl donor in the body. As such, folate is critically required during anabolic stages of the life cycle as well as to maintain healthy cell replication (1). Interestingly, folate intake and optimal health appears to have a “U-shaped” relationship where inadequate and excessive intakes are associated with undesirable consequences (270). For example, low folate status is directly associated with an increased risk of NTDs. This observation lead to mandatory folic acid fortification of the North American food supply in 1998 (1). At the same time many individuals have folic acid intakes that greatly exceed requirements which may increase their risk of colon cancer when pre-existing pre-neoplastic lesions are present (270, 272, 275).

For decades, we have known that a large pool of folate exists in the colon (20-21, 39, 114). Using modern folate state-of-the-art quantification techniques, we reported that the amount of folate in colonic evacuants of humans from two different geographic locations (Africa and North America) significantly exceed the adult dietary allowance (400 µg/day) (22). We recently demonstrated that folate can be absorbed across the human colon. Specifically, after cecal infusion of $[^{13}\text{C}]$-[6S]-glutamyl-5-formylTHF 684 nmol, we observed the presence of $^{13}\text{C}_5$-
methylTHF in plasma at a rate of 0.6 ± 0.2 nmol per hour. (19). The primary mechanism of folate absorption across the colon is thought to be similar to the small intestine via two pH-sensitive folate transporters also expressed along the length of the colon (proton-coupled folate transporter and reduced folate carrier) (33). Given the size of the depot of folate in the colon and the “U-shaped” relationship between folate intake and health outcomes, we propose that a more comprehensive understanding of the role of colonic folate in colonocytes, whole body homeostasis and folate metabolism is required.

A limitation of this first study was that following cecal infusion of the $[^{13}\text{C}]5$-formylTHF test dose, blood samples were collected for only four hours. During this time, the appearance of the metabolite of $[^{13}\text{C}]5$-formylTHF, $[^{13}\text{C}]5$-methylTHF, rose in a linear fashion and did not return to baseline suggesting that absorption of folate, while slow, occurred down the length of the colon. It was concluded that future studies follow the plasma response for a longer period of time, reflective of the transit time of the colon, to observe the test dose response return to baseline. This would facilitate a percent relative bioavailability calculation comparing the AUC of the colon dose versus IV test dose of the same compound. A potential confounding factor that may have influenced the rate of the labeled folate plasma appearance in the study by Aufreiter et al. is the work-up procedures in preparation for colonoscopy. These routine bowel cleansing protocols remove most colonic contents and significantly disrupt the concentration and profile of the colonic microbiota (31). While it is well known that many bacteria in the colon secrete large quantities folate into the lumen, how much of a test dose could make its way through the physical barrier of undisturbed intestinal contents and localized competing folate pool for transport to colonocytes for absorption or be consumed by folate requiring bacteria is unknown.
In order to non-invasively deliver a folate test dose to the colon we developed and tested different formulations of caplet coatings to enable us to quantitatively deliver a physiological dose of labeled 5-formylTHF to the colon. The placebo caplet was composed of a barium sulphate core to facilitate fluoroscopic monitoring of caplet disintegration and to ensure that the test dose was not available prior to the ileocecal junction (32). The caplet coating consisted of two different pH-dependent acrylic co-polymer products in a 3:1 ratio, (Eudragit® L100 and Eudragit® L100 S100) producing a pH threshold of 7.0-7.5 consistent to the pH of the colon ranging from >5.2 in the proximal colon to >7 in the distal colon (97).

Using these specially designed caplets, the objective of the present study was to non-invasively evaluate colonic absorption of folate with an intact microbiota after quantitative delivery of stable isotope labeled folate directly to the colon. Eight hundred and fifty-five nmol (400 µg) of $^{13}$C5-formylTHF was mixed with the barium sulphate core of these caplets and coated as described above. In vivo monitoring of caplet gastrointestinal transit and disintegration were performed via hourly fluoroscopic imaging. After a washout period of 3 or more weeks, each subject received an IV injection of the same folate compound (214 nmol). Blood samples were collected before and after each treatment for determination of total folate content by microbiological assay and for the presence of labeled and unlabeled folates by tandem mass spectrometry. The caplet blood sampling time period was extended to reflect total transit time in the colon with samples taken up to 14 hours after caplet ingestion, at 24 and 48 hours post-ingestion. This was to observe a return to baseline of the labeled folate response and calculate the AUC to determine the percent relative bioavailability of the caplet and IV test doses.
3.1.2 Hypothesis

A significant fraction of a physiological dose of $[^{13}\text{C}]-[6\text{S}]-\text{glutamyl-5-}
\text{formyltetrahydrofolic acid}$ will be absorbed across the colon of healthy adults containing an
intact microflora.

3.1.3 Objectives

1. To non-invasively determine whether a physiological dose of folate 855 nmol of $[^{13}\text{C}]-[6\text{S}]-
\text{glutamyl-5-formyltetrahydrofolic acid}$ is absorbed across the intact colon containing an
undisturbed microflora.

2. To compare the appearance and kinetics of the metabolite $[^{13}\text{C}_5]-\text{5-methyltetrahydrofolic acid}$, from two physiological doses of $[^{13}\text{C}]-[6\text{S}]-\text{glutamyl-5-formyltetrahydrofolic acid}$, after oral and
intravenous administration (214 nmol), with a suitable washout period.

3. To extend blood sample collections to reflect total transit time in the colon in order to
calculate the area under the curve produced by the appearance of $[^{13}\text{C}_5]-\text{5-methyltetrahydrofolic acid}$. This will enable determination of the percent relative bioavailability of folate the colon
after an oral dose in relation to an intravenous dose of the same compound.
3.2 Methods and Materials

3.2.1 Study population

For this observational and open-label study, healthy adults between the ages of 18 and 65 years of age were recruited between September and October 2008 by public advertisement at the University of Toronto. No priori sample size calculation was conducted as this was an exploratory study. Individuals completed three documents during the screening interview: an eligibility checklist with the study coordinator (a registered nurse), written consent (Appendix 1), and a health history questionnaire to determine general health status. At the time that subjects gave written consent, a healthcare provider not related to the study was present to witness the informed consent process and provide a signature. The study was approved by the human research ethics board at the Hospital for Sick Children and by the Therapeutic Products Directorate, Health Canada.

Exclusion criteria included a verbally reported history of any chronic disease, gastrointestinal surgery (e.g. polyp removal) or medication known to influence folate metabolism. These medications include: primidone, metformin, sulfasalazine, triamterene, dilantin, phenytoin, methotrexate or nonsteroidal anti-inflammatory drugs. Potential subjects were also excluded if they had a medical condition or were taking medication that affected intestinal motility or pH (e.g. anti-acids, laxatives, or antibiotics). Individuals who consumed >1 alcoholic drink/day or were habitual or current smokers (last 6 months), or envisioned difficulty swallowing large pills or providing venous blood samples were excluded. To ensure subjects were generally healthy, and to minimize potential inter-subject differences in how folate was metabolized, individuals provided a screening venous blood sample to confirm normal RBC
folate concentration, blood chemistries (serum electrolytes and complete blood counts), as well as vitamin B\textsubscript{12} and B\textsubscript{6} status, and that the subject was not homozygous for the MTHFR 677C $\rightarrow$ T genotype. Females were asked to provide a screening pregnancy test (Clearview hcG II, Wampole Laboratories, Princeton NJ) and only those with a negative pregnancy test, and were not planning a pregnancy, breastfeeding or using high-dose estrogen in the form of oral contraceptives/hormone replacement therapy were included. Subjects were asked to refrain from using vitamin and or/mineral supplements $\geq$ 2 weeks before study initiation and to inform the study coordinator if laxatives, antibiotics or other medications and supplements were used during this time period. Subjects were asked a second time prior to initiating both study periods whether or not they had used supplements or vitamins within the 2 weeks prior to initiating the study period. Lastly, subjects were asked to avoid alcohol within 24 hours of caplet or IV folate test doses.

Anthropometric measurements (height, weight, and waist circumference) were determined according to standardized procedures (52) using a digital stadiometer ($\pm$ 0.01 cm, Hightronic Model #235, Quick Medical, Snoqualmie WA), calibrated beam scale ($\pm$ 100 g, Detecto, Webb City MO) and measuring tape ($\pm$ 0.01 cm, Heart & Stroke Foundation, Canada). RBC folate concentration was determined by microbial assay (290). Blood chemistries, vitamin B\textsubscript{12} and B\textsubscript{6} concentration and the genetic test for the MTHFR 677C $\rightarrow$ T allele were analyzed in the Core Laboratory Facilities at the Hospital for Sick Children (Toronto ON). The electrolytes sodium, chloride and potassium were measured using slides that contained multilayered, analytical elements coated on a polyester support that uses direct potentiometry for measurement of ions (Vitros® Chemistry Products: ref. Na + 837 9034, Cl- 844 5207, K+1616200 Johnson & Johnson, Rochester NY). Complete blood count (CBC), was determined by CELL–DYN
Sapphire hematology analyzer Multi-Angle Polarized Scatter Separation Abbott Sapphires, Saint-Laurent QC) with the corresponding co-efficients of variation (CV): WBC ≤2.7%, RBC ≤1.5%, HGB ≤1.0% MCV ≤1.0%, PLT ≤4.0%.

Vitamin B\textsubscript{12} was measured using solid phase competitive chemiluminescent enzyme immunoassay involving an automated alkaline denaturation procedure (IMMULITE\textsuperscript{®} 2500 Vitamin B12 kit and Analyzer Equipment, Diagnostics Products Corporation, Los Angeles CA). Quality control solutions targeted three levels of concentrations; low 164 pmol/L, medium 295 pmol/L, and high 522 pmol/L and had an inter-assay CV <10%. Pyridoxal-5-phosphate was measured by high-performance liquid chromatography with on-line derivatization and fluorescence detection with an inter-assay CV <10% (291). All subjects were screened for the 5,10-methylenetetrahydrofolate reductase (MTHFR) \textsubscript{677C →T} polymorphism by the method described previously (292). DNA extracted from the buffy coat of centrifuged whole blood and was treated with Taq Gold DNA Polymerase (5 u/µL) (Perkin Elmer Waltham MA) and analyzed with the following equipment: Spectrophotometer, DU640 (Beckman Coulter, Brea CA) and PTC 200 Thermal Cycler (MJ Research Incorporated, Taunton MA).

3.2.2 Test Compound and Dose Formulations

The isotopically labeled test compound, \textsuperscript{[13C]-[6S]-glutamyl-5-formyltetrahydrofolic acid} was synthesized by Merck Eprova AG (Schaffhausen, Switzerland) and purchased in powdered form as previously described (19). The chemical purity of the test compound was confirmed by HPLC and infrared spectroscopy to be 98% pure. This test compound was dissolved in
physiologic saline (pH 7.0; 74 µg folate/mL) under aseptic conditions to prepare the intravenous (IV) test dose of 214 nmol (100 µg). The test compound was also incorporated into barium caplets for oral delivery. Caplet cores were coated with a pH-dependent coating to quantitatively deliver the test folate dose to the colon as described previously (32).

Except where indicated, all ingredients to prepare caplets were purchased from Sigma (Oakville ON). All caplets used in the present study were produced in the Good Manufacturing Practices compliant production facility of the Toronto Institute of Pharmaceutical Technology (Toronto ON). Caplets were composed of 64% (w/w) barium sulfate blended using a high-shear mixer with the binding agent, polyvinyl pyrillidone K90 (7% w/w). The caplet cores consisted of predominately barium sulphate, a radio-opaque substance to allow monitoring of gastrointestinal transit via serial fluoroscopic imaging of the abdomen. During wet granulation the barium sulfate, the small test folate dose (0.02% w/w) was dissolved in purified water and incorporated homogenously as part of the caplet core granules. Wet granules were dried at 30°C until optimum moisture content was achieved (1.5-2.0%). Granules were screened through US Mesh # 20 and #30, then blended with a diluent (microcrystalline cellulose), as well as a superdisintegrant (sodium starch glycolate; JRS Pharma, Patterson NY). These ingredients accounted for 13% and 4% of the final target caplet weight of 2220 mg respectively. The blend was compressed on a rotary tablet press (Minipress II; GlobePharma Inc., New Brunswick NJ) using convex caplet tooling (19.1 mm X 9.7 mm).

Caplets were coated in a conventional pan coating system using the pH-dependent acrylic co-polymer products Eudragit® L100 and S100 (Evonik Industries AG, Essen, Germany) in a 3:1 ratio. As shown in a previous study, this combination of Eudragit products was found to most efficacious in ensuring caplets did not disintegrate prior to the ileocecal junction (32). The co-
polymer coating was prepared by first dispersing Eudragit® L100 (4% w/w) and S100 (1% w/w) in water and then adding a plasticizer (triethylcitrate) (4% w/w), glidant (talc) (2% w/w) and a neutralizing agent (potassium hydroxide) (0.1% w/w). The final aqueous mixture was filtered to eliminate sediment or agglomerates, and then sprayed on to the barium sulphate caplet cores (LDCS-5 Hicoater, Vector Corporation, Marion IL) at an atomizing pressure of 22-24 psi to achieve a target increase in caplet weight of 12.6% and a final caplet weight of approximately 2500 mg. The coated caplets were dried at 40°C, and both IV and caplet test doses were stored at 4 °C and shielded from light in the Research Pharmacy at the Hospital for Sick Children (Toronto ON).

The label claim of ± 10% of the target folate concentration for both test dose routes of administration (oral and IV) were confirmed by microbial assay. Four crushed caplets were dissolved in 10 volumes of 0.1 M potassium phosphate buffer containing 0.05% sodium ascorbate (pH 7.5). The folate content was measured to contain a mean of 393.2 µg total folate ± 63.5 µg (SD). For IV injection, the folate concentration of 1.35 mL aliquots of the test solution was also within ± 10% of the 100 µg target (99.6 µg total folate). The sterility of the IV aliquots was confirmed to contain less then 12.5 endotoxin unit/mL, and met United States Pharmacopeia and National Formulary (USP-29-NF24) requirements for bacterial endotoxins using a validated Limulus amebocyte lysate (LAL) test (test <85>) (Nucrotechnics Inc, Scarborough ON). The IV solution was used within 1 month of preparation. The physiological dose of the IV injection as a quarter of the caplet dose was the same as previously reported by Aufreiter et al (19). This dose is supported by the plasma apparent absorption of 38% (190 nmol) from a similar dose (500 nmol) administered orally to the small intestine, which is comparable to our 214 nmol dose (81).
3.2.3 Study Protocol

Overnight fasting blood samples were collected from each subject at approximately 0800 hours and an IV injection of 214 nmol \[^{13}\text{C}\]5-formyltetrahydrofolic acid in 1.35 mL sterile saline, was administered. Blood samples (5 mL) were collected 15 min post-injection and at 30-min intervals thereafter for 4 h, via an indwelling catheter inserted in the arm not used for injection of the test dose. The IV and caplet treatments were separated by three or more weeks.

On the morning before the day the caplet was ingested, a fasting baseline venipuncture blood sample (5 mL) was collected. At approximately 0600 hours the next day, subjects consumed a single caplet containing 855 nmol \[^{13}\text{C}\]5-formylTHF with as much water as desired and a standard breakfast (32). Approximately 2 hours later, following a negative urine pregnancy test for females, the intestinal transit and disintegration profile of each caplet was monitored using a fluoroscope (Infinix, Toshiba America Medical Systems Inc., Tustin CA). Subsequent abdominal images were taken at approximately 60 minute intervals by a qualified radiation technologist in the Image Guided Therapy Unit (Department of Radiology, Hospital for Sick Children) as described by Aimone et al (32). Imaging concluded at 1800 hours or earlier if complete caplet disintegration was observed. Each image required an average of 2 to 3 seconds of fluoroscopic exposure, and delivered an approximate radiation entrance dose of 20 mRem per image. In total subjects received on average 127 ± 22 (SD) mRem exposure which is comparable to one tenth of the effective dose for an adult abdominal X-ray. In the event that the caplet had not completely dissolved by 1800 hours, the next stool was collected. Between imaging sessions, subjects moved around freely, however were asked to abstain from vigorous exercise.
Peripheral blood samples (5 mL) were collected hourly via an indwelling catheter after the caplet had left the stomach and until 2000 hours. Subjects returned at approximately 24 and 48 hours post caplet ingestion to provide one additional blood sample (5 mL) at each time. Standardized beverages and snacks previously determined to be low in folate content (19), were available after both oral (14 hours) and IV (4 hours) test dose administration.

3.2.4 Quantification of Caplet Transit and Disintegration Times

Caplet transit and disintegration times were determined by a radiologist (Bairbre Connolly) and a study coordinator (Ashley Aimone) based on fluoroscopic images (32). Gastric emptying and colon arrival time for each trial was calculated as the amount of time that it took, after ingestion, for the caplet to pass through the pyloric sphincter and the ileocecal junction, respectively. Small intestinal transit time was determined by subtracting gastric emptying time from colon arrival time. Caplet disintegration initiation time was defined as the amount of time that elapsed after caplet ingestion, when clear erosion of the caplet edges could be observed on fluoroscopy. Complete disintegration was recorded as the time post caplet ingestion, when a solid caplet core was no longer discernable. Initial and complete disintegration times were also used to calculate the total time that it took for each caplet to disintegrate (duration of total caplet disintegration = complete disintegration time – disintegration initiation time). Finally, the total time from caplet administration to complete disintegration was denoted as total transit time, or the time that elapsed between caplet ingestion and the last fluoroscopic image obtained at the end of the study day.
3.2.5 Biochemical and Mass Spectrometry Analyses

   Blood samples for folate analyses were collected into EDTA-treated tubes, wrapped in foil and processed and frozen within 2 hours. After measurement of the hematocrit, 100 µL aliquots of whole blood samples were diluted 10-fold with ascorbic acid and deionized water (1%wt:vol) and incubated at 37 °C for 30 min to allow pteroylpoly-glutamate hydrolase (EC 3.4.19.9) to convert polyglutamated folates to an assayable form. Remaining whole blood was centrifuged (1500 X g for 20 min at 4 °C) and plasma was collected and stored with added sodium ascorbate (1% wt:vol) to prevent the oxidation of folate. All samples for later determination of RBC folate were stored at -80 °C. The total folate content was determined by the standard microbial assay using the test organism *Lactobacillus rhamnosus* (ATCC7649; American Type Tissue Culture Collection, Manassas, VA) (290). RBC folate was calculated by subtracting plasma total folate from whole-blood folate with correction for hematocrit. The accuracy and reproducibility of these assays were assessed by using a whole-blood folate standard with a certified value (29.5 nmol/L, whole blood 95/528; National Institute of Biological Standards and Control, Hertfordshire, United Kingdom). Analysis of the whole-blood control standard in our laboratory during this experiment yielded a folate content of 29.7 ± 1.8 nmol/L, with an interassay CV of 6%.

   Plasma enrichment of the IV injection and caplet administered test dose [\(^{13}\)C]5-formylTHF and its metabolite [\(^{13}\)C\(_5\)]5-methyltetrahydrofolic acid were determined by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) at the Centers for Disease Control and Prevention (Atlanta, GA) as described in detail previously (190). The limits of detection for measuring 5-methylTHF and 5-formylTHF were determined using a signal-to-noise
ratio of 3. Signal peaks were determined to be background noise if the measured value was less than the limit of detection. No correction for natural abundance of isotopes was necessary because the contribution at \((M+5)\) due to the presence of \(^{13}\text{C}\), \(^{15}\text{N}\), \(^2\text{H}\) and \(^{18}\text{O}\) in the unlabeled species is negligible. The measured area ratio is therefore equivalent to the molar ratio. When the area ratio is calculated as labeled divided by total folate \([M+5]\) divided by sum of \([M+5]\) and \([M+0]\), it is equivalent to the enrichment level. Plasma samples collected directly after IV injection showed >37% enrichment for \([^{13}\text{C}_5]5\)-formyltetrahydrofolic acid. Samples collected after cecal disintegration showed <30% enrichment.

### 3.2.6 Determination of Plasma Folate Response

In addition to reporting our plasma folate response data as molar ratios of 5-formylTHF and 5-methylTHF, we quantified the plasma response and pharmacokinetic data as described previously to nmol/folate/person (19). This provided the lexicon most familiar to readers without a background in stable isotopes (e.g. sum of peak areas and nmol/folate/person). The following important caveats were considered: (1) The sum of all peak areas were calculated by adding the labeled \((M+5)\) and unlabeled \((M+0)\) for 5-formyl- and 5-methylTHF, and the peak areas for 5-formylTHF were adjusted (divided by 2.3) to account for the differences in the LC-MS/MS signal between 5-formyl- and 5-methyltetrahydrofolic acid. (2) Using the total plasma folate concentration (nmol/L) determined by microbial assay for each subject (e.g. either caplet or IV injection) the following equation was used to quantify the total amount of labeled \((M+5)\) 5-
formyl- and 5-methylTHF: where $X$ is the concentration (nmol/L) of labeled 5-formyl- or 5-methylTHF.

$$X = \frac{(M+5) \text{ peak area} \times \text{total plasma folate determined by microbial assay (nmol/L)}}{([M+5] + [M+0] \text{ peak areas 5-formylTHF}) + ([M+5] + [M+0] \text{ peak areas 5-methylTHF})}$$

(3) The baseline plasma folate concentration determined by microbial assay for each subject and treatment (e.g. either caplet or IV injection) was used to normalize the molar ratio and microbial assay results. (4) Lastly to convert nmol/L to nmol/person, and thereby express our data on a whole-body basis, we calculated each individual subject’s total plasma volume by correcting for hematocrit and using the whole blood volume values 75 mL/kg for males and 66.5 mL/kg for females (293).

3.2.7 Dietary Intake and Analyses

On the day that the IV injection was given or the caplet was ingested subjects were provided with beverages and snacks that were confirmed to be low in folate content by direct analyses in our laboratory (19) (294). Dietary intake records were kept on these days and analyzed for caloric and select macronutrient content using the Canadian Nutrient File (Health Canada 2007b) or food packaging labels. The standard breakfast designed by Aimone et al. (2009) was low in energy and residue to minimize the delay in gastric emptying that is known to occur following the consumption of larger meals and dietary fiber (295-296). The breakfast consisted of 500 mL puffed rice cereal and 250 mL non-dairy rice beverage (approximately 940
kJ, 85% carbohydrates, 5% protein, 10% fat, and 0% dietary fiber). Subjects were not permitted to consume any other foods after the standard breakfast while the caplet remained in the stomach. Once gastric emptying had occurred, all other meals and snacks were provided ad libitum (e.g. rice cakes and crackers, oat cookies, apple, apple-sauce and cucumber). Lunch consisted of rice with chicken or tofu, bean sprouts and teriyaki sauce; and the dinner option was beef or low folate containing vegetarian chili. Available drinks were apple juice, grape juice or water and was consumed ad libitum through out the two study days.

Each participant received written and oral instructions on how to record their food intakes for 48 hours after caplet ingestion using common household measurements (297). Subjects were also asked to limit foods high in folate as described by an administered list detailing high folate containing foods (e.g. fortified foods). To estimate food portions, subjects were instructed to use household measurements. A dietician entered the food data into a food composition data-base (Esha Food Processor® SQL version 10.2.0, Salem OR) to determine energy, macronutrient and folate intake.

3.2.8 Statistical Analysis

We used SAS for Windows (Version 9.1; SAS Institute Inc, Cary, NC) to generate descriptive statistics (e.g., mean, SEM). Changes in the total plasma folate concentration described by microbial assay or molar ratios of either 5-formyl- or 5-methylTHF, over time were analyzed by repeated measures ANOVA (PROC MIXED) with sample as the main effect and quadratic sample or cubic sample as necessary. The statistical model for these analyses included
baseline RBC folate concentration. The apparent plasma half-life (one-phase exponential decrease over time) of $^{13}\text{C}_5\text{5}$-formylTHF after IV injection was determined by using the slope of the descending portion of each plasma response curve with GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA). The rate of appearance of $^{13}\text{C}_5\text{5}$-methylTHF in plasma over time after caplet ingestion was determined from the linear slope of the ascending portion of each plasma response curve. The energy and macronutrient intakes of each subject, as well as sex and BMI were compared to his/her corresponding caplet transit and caplet disintegration profile, using a mixed repeated-measures ANOVA (PROC MIXED), to determine if there was an effect on in vivo caplet disintegration. Area under the curve (AUC) was also determined by GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA).
3.3 Results

3.3.1 Subject Characteristics

A total of twenty subjects responded to the study recruitment poster (Figure 3.3.1). Eight were unable to attend the screening interview and of the twelve that were able to attend, eleven provided blood samples to determine their eligibility for the study. One potential subject was excluded because she was homozygous for the MTHFR 677C→T allele, and 1 withdrew due to other time commitments. All nine remaining participants received both caplet and IV injection test doses and completed blood sample collection, however only six subjects were included for caplet analyses as the caplets did not disintegrate in three subjects.

Figure 3.3.1 Study sample participation flow chart.

1THF: tetrahydrofolic acid.
2Included in statistical analyses.
3Caplet disintegration determined by fluoroscopy.
4Excluded from caplet statistical analyses as caplet did not complete disintegration and there was no absorption (data for these participants were included in the statistical analyses for the IV injection).
5Absorption was indicated by detection of $[^{13}C]5$-methylTHFin plasma.
Subject characteristics are summarized in Table 3.3.1. Three females and six males were included. The age range was 22-26 years. RBC concentrations were varied, but were well above the cutoff of 360 nmol/L associated with tissue folate depletion (52). The mean vitamin B$_{12}$ status for all six participants was also above the cutoff indicative of deficiency ($\geq$150 pmol/L) (298). In analysis of plasma vitamin B$_{6}$ concentration, one subject’s value was three standard deviations greater than the mean (368 nmol/L) for all subjects and was omitted from the analysis of the data in Table 3.3.1. Remaining vitamin B$_{6}$ concentrations were above the cut-off value of $\geq$20 nmol/L (299). Four subjects, including the subject with the outlying vitamin B$_{6}$ value consumed supplements containing folic-acid and vitamin B$_{6}$ before enrollment, and all participants discontinued supplementation $\geq$2 weeks before the study intervention. The individual body mass index (BMI) of all the subjects was $<$30 kg/m$^2$. During the serial blood sample collection 4 h after IV injection and 14 h after caplet ingestion, mean (± SD) dietary intakes of folate were 12 ± 1.2 µg DFE and 47.9 ± 8 µg DFE, respectively.
Table 3.3.1 Subject characteristics

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Value¹</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (F/M)</td>
<td>(3/6)</td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>25 ± 1</td>
<td>(22-26)²</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>73.2 ± 17.2</td>
<td>(47.8-103.6)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.6 ± 3.6</td>
<td>(18.8-29.9)</td>
</tr>
<tr>
<td>Plasma volume (L)⁴</td>
<td>2.91 ± 0.7</td>
<td>(1.87-4.07)</td>
</tr>
<tr>
<td>MTHFR 677C→T³</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red blood cell folate (nmol/L)</td>
<td>1108 ± 437</td>
<td>(604-1988)</td>
</tr>
<tr>
<td>Total plasma folate (nmol/L)</td>
<td>41.7 ± 17.7</td>
<td>(14.9-68.5)</td>
</tr>
<tr>
<td>B₁₂ (pmol/L)</td>
<td>331 ± 188</td>
<td>(151-583)</td>
</tr>
<tr>
<td>B₆ (nmol/L)⁵</td>
<td>53 ± 17</td>
<td>(34-82)</td>
</tr>
<tr>
<td>Ethnicity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Middle Eastern</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Chinese</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>South Asian</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Dietary intake of folate during blood sampling³</td>
<td></td>
<td></td>
</tr>
<tr>
<td>After intravenous injection (µg DFE)</td>
<td>12 ±1.2</td>
<td>(7.3-25)</td>
</tr>
<tr>
<td>After caplet ingestion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-14 hrs (µg DFE)</td>
<td>47.9 ± 8.0</td>
<td>(2.47-65.9)</td>
</tr>
<tr>
<td>15-24 hrs ( µg DFE)</td>
<td>48.0 ± 19.4</td>
<td>(0-162.3)</td>
</tr>
<tr>
<td>25-48 hrs ( µg DFE)</td>
<td>327.3 ± 74.9</td>
<td>(26.6-677.2)</td>
</tr>
</tbody>
</table>

¹(n = 9), unless otherwise indicated.
²Mean ± SD; range in parentheses.
³BMI: body mass index; MTHFR: 5,10-methylenetetrahydrofolate reductase; DFE: dietary folate equivalents.
⁴Whole blood volume was calculated by using an estimate of 66.5 ml/kg for women and 75 ml/kg for men (293).
Plasma volume was estimated by subtraction of the hematocrit value from the whole blood volume.
⁵(n =8)

3.3.2 Caplet Transit and Disintegration

Based on our results for fluoroscopic imaging it did not appear that any caplet began to disintegrate prior to the ileocecal junction (Table 3.3.2). Subjects G, H and I, were excluded from further caplet analyses as the consumed caplet did not disintegrate. Fluoroscopy imaging of
stool samples collected from subject H and I the following morning indicated complete
disintegration by the presence of residual trails of barium sulphate (subject G was unable to
provide a stool sample). There was no detection of the labeled test dose in the plasma of these
three subjects.

Table 3.3.2 In vivo transit and disintegration times of pH-dependent acrylic co-polymer coated
caplets as determined by fluoroscopy.

<table>
<thead>
<tr>
<th>Transit time (min)</th>
<th>Subject</th>
<th>Mean&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>Gastric emptying</td>
<td>117</td>
<td>301</td>
</tr>
<tr>
<td>Small intestinal transit</td>
<td>240</td>
<td>200</td>
</tr>
<tr>
<td>Colon arrival</td>
<td>357</td>
<td>501</td>
</tr>
<tr>
<td>Caplet disintegration initiation&lt;sup&gt;2&lt;/sup&gt;</td>
<td>280</td>
<td>501</td>
</tr>
<tr>
<td>Caplet disintegration duration&lt;sup&gt;3&lt;/sup&gt;</td>
<td>315</td>
<td>116</td>
</tr>
<tr>
<td>Total caplet transit&lt;sup&gt;4&lt;/sup&gt;</td>
<td>594</td>
<td>616</td>
</tr>
</tbody>
</table>

<sup>1</sup>Values are expressed as means ± SD.
<sup>2</sup>Total time from caplet administration to the first signs of coating disruption.
<sup>3</sup>Total time from the first signs of caplet coating disruption to complete disintegration.
<sup>4</sup>Total time from the first signs of caplet coating disruption to complete disintegration or final image.
<sup>5</sup>ND: not detected.
3.3.3 Pharmacokinetics

A summary of the pharmacokinetic data after IV injection and caplet disintegration is shown in Table 3.3.3.1 and Table 3.3.3.2, respectively. The maximal concentration ($C_{\text{max}}$) of $[^{13}\text{C}_5]$5-formyltetrahydrofolic acid after IV injection was $17 \pm 1.9$ nmol (mean $\pm$ SEM). The labeled 5-formylTHF decreased from its maximum concentration value with a half-life ($t_{1/2}$) of $0.3 \pm 0.1$ h. Subject F had the greatest half-life, suggestive of a slow metabolic rate for converting the injected 5-formyl dose to 5-methyl ($t_{1/2} = 0.645$ h). The mean $C_{\text{max}}$ $[^{13}\text{C}_5]$5-methylTHF was $7.5 \pm 1$ nmol per person.

**Table 3.3.3.1** Pharmacokinetic data after IV injection of 214 nmol $[^{13}\text{C}]$5-formyltetrahydrofolic acid

<table>
<thead>
<tr>
<th>Subject</th>
<th>5-formyltetrahydrofolic acid</th>
<th>5-methyltetrahydrofolic acid</th>
<th>Rate of appearance $^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$C_{\text{max}}$ nmol</td>
<td>$t_{1/2}$ h</td>
<td>$C_{\text{max}}$ nmol</td>
</tr>
<tr>
<td>A</td>
<td>25.1</td>
<td>0.298</td>
<td>8.47</td>
</tr>
<tr>
<td>B</td>
<td>10.8</td>
<td>0.234</td>
<td>5.35</td>
</tr>
<tr>
<td>C</td>
<td>11.5</td>
<td>0.261</td>
<td>6.48</td>
</tr>
<tr>
<td>D</td>
<td>16</td>
<td>0.263</td>
<td>5.13</td>
</tr>
<tr>
<td>E</td>
<td>16.1</td>
<td>0.244</td>
<td>6.77</td>
</tr>
<tr>
<td>F</td>
<td>20.4</td>
<td>0.645</td>
<td>12.5</td>
</tr>
<tr>
<td>G</td>
<td>22.2</td>
<td>0.22</td>
<td>7.03</td>
</tr>
<tr>
<td>H</td>
<td>9.1</td>
<td>0.212</td>
<td>3.8</td>
</tr>
<tr>
<td>I</td>
<td>20.6</td>
<td>0.275</td>
<td>11.6</td>
</tr>
<tr>
<td>Mean $\pm$ SEM</td>
<td>17 $\pm$ 1.9</td>
<td>0.3 $\pm$ 0.1</td>
<td>7.5 $\pm$ 1</td>
</tr>
</tbody>
</table>

$^1$ $C_{\text{max}}$: maximal concentration; $t_{1/2}$: apparent plasma half-life.

$^2$ Determined from the ascending slope over time of the concentration of labeled $[^{13}\text{C}_5]$5-methyltetrahydrofolic acid in plasma ($n = 9$).
After caplet disintegration, there was no observed \(^{13}\text{C}_5\)5-formylTHF found in plasma of any subject. However, its metabolite, \(^{13}\text{C}_5\)5-methylTHF, was detected in the plasma of 5 of 6 subjects where caplets disintegrated. For these 6 subjects, the mean delay after caplet injection before \(^{13}\text{C}_5\)5-methylTHF appeared in plasma was 5.5 ± 1.6 h and it appeared at a mean rate of 0.3 ± 0.05. The labeled 5-methylTHF plasma response reached a mean \(C_{\text{max}}\) 2.8 ± 0.6 nmol per person at 6.5 ± 2 h after caplet ingestion. Of the six caplets that disintegrated, half of the caplets initiated disintegration at the ileocecal junction and the other half in the cecum. Caplet disintegration completed in the ascending colon for 4 subjects and in the cecum and transverse colon for one subject. A value of zero was used for subject F where the labeled 5-methylTHF response was undetected in plasma samples after caplet disintegration.

Table 3.3.3.2 Pharmacokinetic data after caplet disintegration of 855 nmol \(^{13}\text{C}_5\)-formyltetrahydrofolic acid\(^1\).

<table>
<thead>
<tr>
<th>Subject</th>
<th>Plasma volume</th>
<th>Sex</th>
<th>(t_{\text{delay}})</th>
<th>(C_{\text{max}})</th>
<th>(t_{\text{max}})</th>
<th>Rate of appearance(^2)</th>
<th>Caplet disintegration initiation location</th>
<th>Caplet disintegration completion location</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2.21 L</td>
<td>F</td>
<td>5</td>
<td>4.5</td>
<td>6</td>
<td>0.75 ± 0.03</td>
<td>ICJ</td>
<td>Cecum</td>
</tr>
<tr>
<td>B</td>
<td>4.07 M</td>
<td>8</td>
<td>4.9</td>
<td>10</td>
<td>0.46 ± 0.2</td>
<td>Cecum</td>
<td>Ascending</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>1.87 F</td>
<td>4</td>
<td>2.3</td>
<td>5.5</td>
<td>0.4 ± 0.03</td>
<td>IJC</td>
<td>Ascending</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>3.54 M</td>
<td>9</td>
<td>3.4</td>
<td>11</td>
<td>0.26 ± 0.09</td>
<td>Cecum</td>
<td>Ascending</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>2.38 F</td>
<td>7</td>
<td>1.7</td>
<td>7</td>
<td>0.24</td>
<td>ICJ</td>
<td>Ascending</td>
<td></td>
</tr>
<tr>
<td>F(^4)</td>
<td>2.95 M</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>Cecum</td>
<td>Transverse</td>
<td></td>
</tr>
<tr>
<td>Mean ±</td>
<td>2.84 ±</td>
<td>5.5 ±</td>
<td>2.8 ±</td>
<td>6.5</td>
<td>0.35 ± 0.02</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SEM</td>
<td>0.3 ±</td>
<td>1.6</td>
<td>0.6</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^{1}\)\(t_{\text{delay}}\): time lag before detection of \(^{13}\text{C}_5\)5-methyltetrahydrofolic acid in plasma after caplet ingestion (baseline);
\(C_{\text{max}}\): maximal concentration; \(t_{\text{max}}\): time of \(C_{\text{max}}\); ND: not detected.

\(^{2}\)Determined from the ascending slope for the concentration of labeled \(^{13}\text{C}_5\)5-methyltetrahydrofolic acid in plasma of 6 subjects with complete caplet disintegration.

\(^{3}\)ICJ: ileocecal junction.

\(^{4}\)A zero value was used for subject F because \(^{13}\text{C}_5\)5-methyltetrahydrofolic acid was undetected in plasma after caplet ingestion.
3.3.4 Plasma Labeled Folate Response

The molar ratios (M + 5/M + 0) after $^\text{[13C}_5\text{]}$5-formylTHF by IV injection and caplet disintegration are described in Figure 3.3.4.1 and Figure 3.3.4.2 respectively. After IV injection there was a statistically significant change in the molar ratios for $^\text{[13C}_5\text{]}$5-formyl- and $^\text{[13C}_5\text{]}$5-methylTHF ($P < 0.0001$). Similarly, after caplet disintegration, there was a statistically significant change for $^\text{[13C}_5\text{]}$5-methylTHF ($P = 0.0321$). In Figure 3.3.4.2 (B), subjects B and D had a slower appearance of 5-methylTHF compared to subjects A, C and E. As the caplet gastrointestinal transit was similar for all six subjects, the slower appearance in two of the subject is unknown, however perhaps related to slower absorption or some delay in the conversion of the 5-formylTHF test dose to its metabolite 5-methylTHF.

![Figure 3.3.4.1](image1.png)

![Figure 3.3.4.2](image2.png)

**Figure 3.3.4.1** Molar ratios of (M+5):(M+0) for labeled 5-formylTHF (A) and 5-methylTHF (B) after IV injection of $^\text{[13C]}$5-formylTHF in 9 subjects. Note that y axes differ between panels. The change in the molar ratio for both 5-formyl- and 5-methylTHF was statistically significant ($P < 0.0001$, repeated-measures ANOVA). THF: tetrahydrofolic acid.
Figure 3.3.4.2 The mean (A) and individual (B) molar ratios of (M+5) to (M+0) for 5-methylTHF after caplet disintegration of $^{13}$C5-formylTHF. (A), the change in the mean molar ratio was statistically significant ($P = 0.0321$, repeated-measures ANOVA). (B), the individual molar ratios and the time of the last fluoroscopy image without observed caplet disintegration is indicated by an arrow (↓). Blood samples in both graphs (A) and (B) show $n = 6$, unless otherwise indicated ($^a n = 5$, $^b n = 4$ and $^c n = 3$). THF: tetrahydrofolic acid.

We converted each individual LC-MS/MS (M + 5) and (M + 0) peak areas for $^{13}$C5-formyl- and $^{13}$C5-methylTHF to nmol/person after both test dose administrations, as shown in Figure 3.3.4.3. After the IV injection, there was an immediate appearance of labeled 5-formylTHF to a maximum mean of 17 ± 0.8 nmol (SEM), which was followed by a rapid decline to baseline value at the end of the 4 h study period. The labeled 5-methylTHF response was
slightly delayed and reached a maximum mean of $9 \pm 0.6$ nmol per person, which was followed by a small decrease to approximately $5$ nmol per person and then maintained a mean incremental increase of approximately $6$ nmol per person throughout the remainder of the study period. The unlabeled 5-formylTHF slowly increased from $1.3 \pm 0.5$ to $\pm 2.2 0.5$ nmol per person over the duration of the 4-hour sample collection period. The change in labeled 5-formyl-, 5-methylTHF, and unlabeled 5-formylTHF over time was statistically significant ($P < 0.0001$, $P = 0.0032$, $P = 0.0391$, respectively).

After caplet ingestion the labeled 5-methylTHF showed an approximately linear increase to a maximum value of $1.96$ nmol per person at $11$ h post-ingestion, and continued to decrease over the remaining sample collection period until $14$ h post-caplet ingestion. By the $24$ hour blood sample collection, the labeled 5-methylTHF plasma concentrations had returned to baseline in all six subjects. As shown previously in both the small intestine and colon, we saw a significant displacement of unlabeled folates in response to absorption of $[^{13}\text{C}]$5-formylTHF (19, 80). The unlabeled 5-formylTHF slowly increased from $2.4 \pm 1.1$ to $6.4 \pm 2$ at $10$ h and returned to $3 \pm 2.6$ nmol per person (by the end of the $14$ h sample collection). The change in labeled 5-methylTHF, and unlabeled 5-formylTHF during the $14$ h blood sample collection after caplet ingestion were statistically significant ($P = 0.0055$ and $P = 0.0062$), as well as over the approximate $48$ hour post-caplet ingestion ($P = 0.0185$ and $P = 0.0367$), respectively.
Figure 3.3.4.3 The mean (± SEM) plasma folate concentrations of labeled 5-formyl- (○, M+5) and 5-methylTHF (▲, M+5) and unlabeled 5-formylTHF (▼, M+0) after administration of [13C]5-formylTHF (A) by IV injection and (B) caplet ingestion. Note that the y axes differ between panels. The change in 5-formylTHF after (A) IV injection was statistically significant ($P < 0.0001$) and there was no response measured after caplet ingestion. The change in labeled 5-methylTHF was statistically significant after (A) IV injection ($P = 0.0032$) and (B) caplet ingestion ($P = 0.0062$). The change in unlabeled 5-formylTHF was statistically significant after (A) IV injection ($P = 0.0391$) and (B) caplet ingestion ($P = 0.0367$). Repeated measures ANOVA statistical analyses were used to determine the change in the amount of plasma THF per person after test doses over 48 h post-caplet ingestion; (A) $n = 9$ (B) $n = 6$ unless otherwise indicated $^a n = 5$, $^b n = 4$ and $^c n = 3$. THF: tetrahydrofolic acid.
3.3.5 Area under the Curve and Calculation of Apparent Absorption

Our original objective was to calculate the area under the curve (AUC) of the plasma labeled 5-methyl response after the oral and IV test doses. By comparing the oral caplet AUC to the IV AUC we intended to determine the percent relative bioavailability. However, due to blood sampling of only four hours, there was no observed return to baseline of $[^{13}\text{C}_3]5$-methylTHF after IV injection of 214 nmol $[^{13}\text{C}]5$-formylTHF (Figure 3.3.4.1 (B) and 3.3.4.3 (A)) and in fact the line appeared to plateau. As such we were unable to confidently calculate the IV AUC using linear regression analysis of the last three to five points and therefore elected to calculate the apparent absorption of the oral dose caplet dose 855 nmol $[^{13}\text{C}]5$-formylTHF. We conducted mathematical modeling of the labeled 5-methylTHF plasma response as described by Wright et al. (2005) for the small intestine (81) using the following three equations:

\begin{align*}
(1) \quad C &= \frac{M}{V \cdot T \cdot k} \cdot X (1 - e^{k(t-t_{\text{lag}})}) \quad (0 < t < t_{\text{max}}) \\
(2) \quad C &= \frac{M}{V \cdot T \cdot k} \cdot X (1 - e^{-k^{-1} \cdot e^{-k(t-t_{\text{max}})}}) \quad (t > t_{\text{max}}) \\
(3) \quad \text{Apparent absorption} &= \frac{M}{\text{dose}_{\text{oral}}}
\end{align*}

The absorption time period (T) is defined as the time to peak plasma labeled 5-methylTHF concentration ($t_{\text{max}}$) minus the time during which the plasma-labeled enrichment initially remains at baseline ($t_{\text{lag}}$). We used a $t_{\text{lag}}$ as 1 hour before the first appearance of labeled 5-methylTHF. The rate constant of elimination from the plasma compartment to body tissues and/or excretion was $k$. The apparent volume of distribution for folate in the individual plasma was $V$ and the plasma labeled folate concentration was $C$. We solved for $M$, the mass of dose that is
absorbed for each individual by summing all the samples taken before $t_{\text{max}}$ and after the peak value. The mean apparent absorption percent of 855 dose$_{\text{oral}}$ was $35 \pm 0.2$. This estimate of folate absorption does not consider the fraction of newly absorbed folate that may be metabolized by the colonocytes or sequestered by the liver.
3.4 Discussion

3.4.1 Folate Absorption across the Colon

The results of this study provide the first direct evidence of folate absorption across the colon with an intact microflora. A physiological dose (855 nmol) of the naturally occurring folate, \(^{13}\text{C}\)5-formylTHF, was delivered to the colon of nine healthy adults, using caplets coated with an acrylic co-polymer pH-dependent coating. Fluoroscopic imaging confirmed that the caplets were quantitatively delivered to the colon in six subjects. In these six subjects, the mean rate of absorption was 0.35 ± 0.02 nmol/h (SEM) as indicated by the plasma appearance of \(^{13}\text{C}_5\)5-methylTHF. In comparison, after an IV injection of the same test compound (214 nmol), the rise in labeled 5-methylTHF was 4 ± 1 nmol/h (n = 9).

Our observation that folate absorption occurs across the colon is consistent with our recently published work where the apparent rate of absorption of \(^{13}\text{C}\)5-formylTHF infused into the cecum during colonoscopy was also approximately 10% of the IV dose. In the latter study, the rate of appearance of labeled 5-methylTHF in plasma was 7 ± 1.2 nmol/h after IV injection (172 nmol) and 0.6 ± 0.2 nmol/h after cecal infusion (684 nmol) of the test compound (n = 6) (19). The range of \(^{13}\text{C}_5\)-methylTHF appearance in plasma in the present study after IV injection (2-11 nmol/h) was similar to our previous study (3-10.87 nmol/h) and mean values did not differ statistically (P = 0.1008, unpaired t-test) (19). Likewise the rate of appearance of \(^{13}\text{C}_5\)5-methylTHF in plasma after administration of the colon-targeted test dose overlapped between the caplet (present) and cecal infusion (previous) studies: 0.2-0.8 nmol/h versus 0.4-1.5 nmol/h, respectively. As expected, the mean \(^{13}\text{C}_5\)5-methylTHF appearance rates after administering the test dose to the colon did not differ between the two studies. (P = 0.2419, unpaired t-test).
Considering the very similar labeled 5-methylTHF appearance rate between both the present caplet and previous colonoscopy studies, it appears that perhaps the presence or absence of an intact microflora does not significantly influence folate absorption across the colon.

The rate of labeled 5-methylTHF appearance (0.35 nmol/h) in the present study was determined using the time of caplet ingestion (baseline) as time zero (Figure 3.3.4.2 (B)). Given that we used specially coated caplets to delay the release of the test dose until the colon, using the time of caplet ingestion as "time zero" may have inappropriately resulted in an underestimate of the rate of appearance of $^{13}$C$_5$-methylTHF. If we use instead the most recent fluoroscopic image where caplet disintegration had not yet commenced as time zero, the appearance rate of $^{13}$C$_5$-methylTHF was $0.9 \pm 0.1$ nmol/h (SEM). Again, this value was not statistically different from our previous study where $^{13}$C5-formylTHF was infused directly into the cecum (0.6 nmol/hr) ($P = 0.2094$).

It is also important to consider that we did not observe absorption of folate across the colon in all subjects in either the present or previous study. In both studies, one of six subjects did not display a labeled 5-methyl response. Herein the subject that did not have a labeled response is designated as subject F (Figure 3.3.4.2 (B)). As there were no outstanding characteristics that distinguished the two subjects with no absorption from the other ten participants, in both the caplet and colonoscopy studies, further investigations are necessary.

The rate of folate absorption of $^{13}$C5-formylTHF, whether delivered to the colon via caplet ingestion or infusion during colonoscopy, was considerably lower than that reported in the literature for the small intestine (80-81). Extrapolating from the data published by Wright et al (80-81) in which the appearance of $^{13}$C5-methylTHF in plasma was monitored after an oral dose of 431–569 nmol $^{13}$C5-formylTHF, we estimated the rate of folate absorption across the
small intestine to be 34 nmol/h (80-81). It is important to acknowledge, however, that whereas the rate of folate absorption across the colon appears to be much slower than across the small intestine, the transit time in the small intestine (3 ± 1 hours) is considerably shorter than in the colon (10–72 hours). This transit time difference provides a greater opportunity for absorption to occur in the distal portion of the gastrointestinal tract versus the small intestine (39–42).

The primary objective of the IV injection was to determine the percent relative bioavailability by comparing the AUC of the caplet dose compared to that of the IV AUC. As shown in Figure 3.3.4.1 (B), the (M+5/M+0) molar ratio for 5-methylTHF after IV injection did not return to baseline during the 4 hour blood collection period and, in fact appeared to plateau. As such, it was impossible to confidently calculate an IV AUC for \(^{13}\text{C}_5\)/5-methylTHF and we were unable to determine the percent relative bioavailability of the caplet test dose compared to the IV test dose. However, by using the mathematical model presented by Wright et al. (2005), we determined the apparent absorption of the caplet test dose across the colonic epithelium, to be 35 ± 0.2% (81). This was estimated by first calculating the mass of the test dose absorbed for each blood sample using the plasma concentration, the volume of plasma distribution (389 ml/kg body weight) (300), the rate constant of elimination from the plasma compartment (e.g. excretion and/or uptake by body tissues), and the time period for absorption. We then divided the individual total mass of the test dose that was absorbed by the test dose 855 nmol and calculated the mean apparent absorption as 35%. In comparison, the small intestine apparent absorption was similar to Wright et al. who reported an apparent absorption of 38 ± 6% following an approximate 500 nmol test dose of the same folate form (\(^{13}\text{C}_5\)/formylTHF) (81).

The 35% apparent rate of absorption is the first ever reported estimation of what percentage of a test dose administered to the human colon can be absorbed into systemic
circulation as measured by serial plasma analysis. By applying this novel apparent rate to an estimation of the total folate content in the colon, we are able to gauge the potential impact on this pool of folate on whole body metabolism. After using state-of-the-art folate extraction techniques we recently reported the folate content of colonic evacuants from South African and Caucasian Americans, to be $699 \pm 131 \mu g$ and $860 \pm 129 \mu g$ respectively (22). These amounts of folate are well in excess of the test dose administered in the present study and exceed the adult recommended dietary allowance (400 µg/day). Applying our estimated apparent absorption of 35%, we hypothesize that approximately 245 - 301 µg of colonic folate evacuant could be potentially absorbed each day.

It is important to consider that the 35% rate of apparent absorption of the test dose does not account for the amount of folate sequestered by the liver by first-pass splanchnic metabolism (81, 301-303). We know that a substantial portion of the absorbed dose goes into the portal vein to be delivered to the liver, yet we are unable to account for this physiological phenomenon or the labelled fraction of newly absorbed folate that may be retained within the colonocytes. Further, our understanding is limited in regard to how much of the test dose was utilized by microflora or evacuated within feces. Upon examination of the labeled 5-formylTHF response, the present study did not observe a labeled 5-formylTHF plasma response after folate absorption across the colon like the colonoscopy study (19) and only the appearance of labeled 5-methylTHF was measured in both experiments. We believe that the distribution of the two solute carriers, RFC and PCFT, with an optimal pH of 7.4 and 5.5, respectively (33, 92, 304), may have contributed to these findings. In particular, we hypothesize that the use of proton-pump inhibitor therapy in three of the colonoscopy subjects, with the observed appearance of the test dose 5-formylTHF directly in plasma, inhibited the expression of PCFT and thereby altered
folate absorption across the colon. A recent in vivo study of a subset of patients receiving proton-pump inhibitor therapy found an approximate 50% reduction in duodenal PCFT mRNA expression. These results support that the PCFT transporter activity can be modulated by additional drugs, such as proton-pump inhibitor therapy, as these findings suggest that the PCFT is directly influenced by the proton-gradient to drive transport activity and that the transcriptional regulation of this transporter also appears to be directly or indirectly regulated by the use of this drug. The authors also observed the variability of PCFT expression directly in patient biopsies and noted a 25-fold variation in duodenal PCFT, as well as a significantly reduced levels in the ileum and colon in comparison to the duodenum (305).

In addition to medication, multiple physiological factors may influence the distribution of folate transporters. For example, high levels of folate exposure may down regulate folate intestinal absorption (283). Ashokkumar et al. (2007) found that after in vitro long-term folate supplementation resulted in significantly lower folate uptake by intestinal and renal epithelial cells compared to sufficient supplementation exposure (283). This reduction in folate uptake was associated with a significant decrease in the RFC protein and mRNA levels and the activity of PCFT promoter. Additionally, it was also linked to lower mRNA levels of the PCFT/heme carrier protein 1 and FR (283). In both the current caplet and previous colonoscopy studies, subjects had a mean supraphysiological RBC folate value >1100 nmol/L (19). Both the mean RBC and plasma folate levels measured in these participants are reflective of reported folate levels in a post-fortification era (285). As there are only presently these two studies demonstrating direct in vivo folate absorption across the colon, the effects of individual subject characteristics, such as supraphysiological folate status on the number of transporters along the length of the colon, and the potential influence on bioavailability are unknown.
The results herein from the caplet study are comparable to our previous colonoscopy work, despite several differences between study populations. The ages of participants in the present study (25 ± 1 (SD) years) are significantly younger than the previous study (56 ± 2 years) ($P < 0.0001$, unpaired t-test). In the current study, none of the six subjects had a BMI >30, whereas three older subjects in the colonoscopy study were considered obese with a BMI >30 (19). Adiposity has been shown to affect folate metabolism (196-197) and the effects of age are inconclusive in the absence of disease (1, 103). Although the effect on the conversion between 5-formyl to 5-methyl is unknown, the apparent plasma clearance (“plasma half-life”) of the injected dose of labeled 5-formylTHF (0.3 ± 0.1 h and 0.3 ± 0.4 h, respectively) was not statistically different ($P = 1.0000$) (19). This preliminary data suggests a potential similarity in the metabolism of 5-formylTHF between the two distinct subject populations.

In the present study, the absence of invasive pre-colonoscopy mechanical bowel cleansing facilitated investigation of folate absorption across the colonic epithelium containing an unaltered microflora distribution. The standard polyethylene glycol solution protocol used in the colonoscopy study for optimal intra-luminal visualization is known to lower bacterial content, as well as clear out dietary remnants (31). Although the mean rate of plasma appearance of labeled 5-methylTHF after caplet disintegration was similar to the colonoscopy study, we do not yet fully understand how the specific roles of mucosal-associated and intra-luminal bacterial populations affect folate absorption. The proximal colon is associated with the greatest amount of bacteria per gram of intestinal contents in healthy individuals ($10^{11}$-$10^{12}$ g$^{-1}$ versus $10^3$-$10^5$ g$^{-1}$ in the small intestine) (99). The effects of bacterial consumption of our test dose may have influenced the rate of absorption in the present study. For example, certain strains of bacteria are folate consumers (Lactobacillus), while others are high folate synthesizers (Streptococcus
thermophilus) (109). Furthermore, the presumed continuous supply of folate from microbial synthesis did not appear to have competed for folate transport across the colonocytes with our administrated test dose as the plasma rate of labeled folate was similar to the previous colonoscopy study. Additionally, we do not know what the total bioavailability of bacterially synthesized folate is, and whether or not folates that are polyglutamated and/or trapped within cellular matrices in the colon are absorbed.

The colon-targeted pH-dependent caplets were an effective non-invasive means to study the absorption of folate across the colon (0 of 9 caplets began dispersal of the test dose prior to the ileocecal junction). We monitored the slow caplet transit and caplet disintegration by fluoroscopic imaging and the test dose was delivered along the length of the proximal colon, rather than in one specific location. All six caplets that disintegrated were observed to be completely dispersed in the cecum, ascending or transverse colon and the opportunity for absorption of the test dose in more distal regions of the colon was not observed. The caplet disintegration results are consistent with the pH threshold of the coating formulation (pH 7.0-7.5) resembling that of the terminal ileum pH 6.7-7.5 (74) and cecum 5.5-7.5 (306). The proximal colon can have a pH as low as 5.2 due to microbial short-chain fatty acid synthesis, whereas this slowly rises to 6.4-6.9 in the midcolon and up to >7 in the distal colon (97). Finally, the caplet transit and disintegration times did not statistically differ between the results previously published using the same acrylic co-polymer pH-dependent coating with a placebo caplet in regards to gastric emptying, small intestinal transit, colon arrival, caplet disintegration initiation and caplet disintegration duration (unpaired t-test) (32). The total transit time in the present study was significantly greater than the placebo study ($P = 0.0385$, unpaired t-test), however this may
be due to the smaller sample size or the fact that we did not observe complete disintegration in three out of the nine caplets.

Our observation that folate absorption occurs across the colon after disintegration of a caplet distal to the ileum and after a cecal infusion of a physiological dose of natural folate is consistent with earlier animal work (23-27) and in vitro work (28-30). For example, we previously delivered labeled $[^3]H$ PABA, a precursor of bacterially synthesized folate, to the cecum of piglets. After analysis of extracted labeled folates from the kidney, liver and urine, we estimated that approximately 18% of the pigs dietary requirement could be met by folate absorption across the colon (26). Similarly, Rong et al injected $[^3]H$ PABA into the cecum of rats and observed that bacterially synthesized tritiated folate was incorporated into the liver of rats despite the prevention of coprophagy.

Due to the close proximity of bacterial folate biosynthesis to colonocytes, the role that this folate pool may have on the local colonocytes health, and colorectal cancer specifically, requires further investigation. In an observational pre-fortification study in Canadian adolescents, we reported that dietary fiber was positively associated with serum folate concentration ($n = 224$), even after statistically controlling for folate intake ($P < 0.001$). We estimated that for each gram of dietary fiber ingested, serum folate increased by 1.8% (119). The proposed mechanism for this observation was that an increase in fermentable substrate (e.g. fiber) supported an increase in total bacterial load and hence microbial folate biosynthesis. However, if colonocytes can use bacterially synthesized folate, and the size of this pool can be modified by diet, perhaps this could explain why the association between folate intake and colorectal risk is stronger for dietary folate than for total folate (307). The dietary folate relative risk of high compared with low intake is 0.75; 95% CI: 0.64, 0.89, whereas the total folate (including natural food and
supplemental folate sources) relative risk of high compared with low intake is 0.95; 95% CI: 0.81, 2.22 (307). Natural sources of folate are typically excellent sources of fiber (e.g. leafy green vegetables and legumes).

To date, the complex relationship of folate intake and optimal health is “U-shaped” where inadequate intakes and high intakes exceeding requirements are linked to colorectal cancer (1, 260, 270). These studies have only examined this relationship in the context of oral intake and the additional natural folate source described in the present study (absorption of folate across the colon) has not been explored. Furthermore, despite a subsequent decline (approximately 50%) in the incidence of NTDs after folic acid fortification (4-7), there is increasing concern over supraphysiological levels of circulating folic acid modifying health risks in the population at large (4, 8, 10-18). The safe and effective use of folic acid interventions deserves to be optimized, particularly in the presence of recent calls to increase folic acid fortification levels to further prevent 25% of NTDs suspected to be folate related (308-309). From an epidemiological perspective, knowing the minimum dose that is able to reduce NTD incidence may help to minimize existing conflicts around folic acid interventions and make them more acceptable and justifiable for population-wide coverage (201). Knowledge gained from this research, provides evidence of an additional natural folate source not currently considered in folate nutritional recommendations and supports future investigations to enhance public policies for the healthful intake of folate. This is particularly useful as current studies suggest that the level of folate intake that is safe and healthy for one person may be potentially harmful to another.
3.4.2 Strengths and Limitations

Strengths

The present study is the first exploratory experiment to measure folate across the colon in healthy human adults with an intact microflora. By using a non-invasive colon-targeted caplet containing a stable isotope labeled folate and a barium sulphate core, we observed disintegration and delivery of the test dose to the colon by fluoroscopic images. These images were analyzed by the same radiologist (Bairbre Connolly) in the Image Guided Therapy department of the hospital and had a 95% concordance rate with a second independent review by a study coordinator (Ashley Aimone) not involved in the study. All nine subjects participated in both the caplet and IV treatments, which provided a more reliable comparative analysis to be made between folate metabolisms after two different routes of administering a test dose. The ethnic diversity of the participating subjects may have prevented biases in gastrointestinal characteristics due to food preferences (310). In our study, blood sampling for >4 hours reflected the transit time of the colon and facilitated an apparent absorption calculation.

Limitations

Due to the exploratory nature of this study, our sample size was small, and we appreciate that the generalizability to the larger population is limited. Additionally, the restricted age group may not be reflective of overall folate metabolism and absorption in the adult population or in a non folic acid fortified country. At the end of the blood collection phase after IV injection, the $^{13}$C$_5$-methylTHF concentration reached a plateau and it appears that a longer blood sample phase greater than 4 hours is required to accurately calculate the AUC. As such, we were unable
to calculate the percent relative bioavailability of the caplet dose compared to the IV test dose as intended. Therefore, we calculated the apparent absorption across the colon as 35% of the test dose (855 nmol) using mathematical modeling previously described for the small intestine (81).

The fluoroscopic analysis of in vivo caplet transit and disintegration times may have been subject to errors of accuracy and precision, since the caplet’s location in the gastrointestinal tract was determined only by the intra-luminal air accumulations and bony landmarks. Further, the frequency of imaging (one image per hour) may have led to overestimated measurements of transit and disintegration times, as observations of gastrointestinal events such as colon arrival or caplet disintegration initiation may have been delayed. For example, we detected the labeled folate plasma response to be related to the first fluoroscopic image indicating disintegration of the outer edge of the caplet (Figure 3.3.4.2 (B)). In the present study, folate was added during wet granulation and dispersed over a powder blend of a large amount of the excipient barium sulfate during granulation. This allowed for a high level of homogeneity and the target amount of test dose was accurately incorporated. The test dose was not added to the center of the caplet, as the amount of error was predicted to be greater than 10% of the label claim (400 µg/caplet) due to the small test dose in microgram amounts which would result in inherent losses during dispensing and transferring the materials between equipment. Perhaps additional blood sampling such as every 30 minutes would provide a more accurate approximation of the first plasma detection of the labeled test dose.

Despite the success of six caplets, caplet disintegration was not observed in three subjects. We did not detect the labeled test dose at all in the plasma of these individuals. At the end of the study day, these caplets appeared in the ascending colon and transverse colon at the time of the final image. We were able to collect 2 stool samples from 3 of the individuals with no
observed caplet disintegration and complete caplet disintegration was recorded (as seen by barium sulphate dispersal within the stool sample). Similarly, in the placebo trial by Aimone et al. (2009), complete disintegration in two out of ten caplets was not observed (32). We recommend that additional fluoroscopic imaging for greater than twelve hours post-caplet ingestion is necessary with the current caplet administration protocol to allow us to include caplets with significantly extended total transit times in future analyses. The ingestion of a breakfast meal with caplet administration may have contributed to these delays in total transit time, since the presence of food has been shown to increase the gastric emptying time of large insoluble particles (121).

The high inter-subject variability of caplet disintegration initiation and completion could also be related to host-related intrinsic factors such as genetics, transit times, viscosity, metabolic activity, and bacterial load influencing the pH through production of short chain fatty acids in the terminal ileum and colon. As well, gastrointestinal motility determines not only the rate of delivery to the absorptive sites but also residence time, and the extent of mixing (32). Lastly, the study participants were not randomized to complete the caplet and IV trials, thus potentially introducing a type of intervention or exposure bias. All subjects were analyzed and interpreted with the assumption that each subject served as his/her own control and a mandatory washout period of at least three weeks was scheduled between trials to minimize the effect of a stable isotope carry-over effect.
3.4.3 Conclusion

The results of the present study provide the first evidence that a physiological concentration of folate can be absorbed across the human colon of healthy adults with an intact microflora. The estimated apparent absorption of $[^{13}\text{C}_3]5$-methylTHF after 855 nmol of $[^{13}\text{C}]5$-formylTHF was 35%. Caplets containing a pH-dependent acrylic co-polymer coating and barium sulphate core are an effective model for the non-invasive delivery of test nutrients to the colon. Measuring the labeled 5-methylTHF plasma appearance for 24 hours post-caplet ingestion was an appropriate time period to observe the labeled 5-methylTHF return to baseline. The physiological significance of the large depot of folate on the folate status of humans and the health of colonocytes warrants future investigation.

3.4.4 Future studies

The results of this study support our previous work in that folate can be absorbed across the colon after a cecal infusion (19) and that a placebo colon-targeting caplet formulation is a non-invasive delivery method to administer test nutrients past the ileocecal junction (32). While we know that folate is absorbed across the human colon containing an intact microflora, the contributions of bacterial biosynthesis specifically to total colonic folate needs to be confirmed. We estimate that >50% of total colonic contents are monoglutamated (114), the form known to be readily absorbed across the small intestine and now supported in this current research to be absorbed across the colon. However, we do not fully understand what proportion of colonic
folates are attributed to endogenous bile losses, bacterial biosynthesis or dietary origin escaped from small intestinal absorption. Further it is presently unknown whether potentially polyglutamated and/or trapped folates within cellular matrices are hydrolyzed by GCPII found in the colon for subsequent absorption across the colon (68).

Animal models are a potential methodology for distinguishing bacterial folate synthesis from other colonic folate pools. For example, by providing piglets with exposure to different folate content and fermentation substrates, the folate content from urine and fecal analysis, and segments of the colon, as well as liver and kidney could be compared for total folate content. The randomized piglet diets could perhaps include: (1) 0 mg/kg folate, (2) 0 mg/kg + anti-folate drug, to decrease bacteria proliferation, and (3) high folate and fiber, to enhance microflora folate biosynthesis. Additionally, the contribution of colonic folate from bile excretion that is not re-absorbed by the small intestine could be estimated using a labeled folate infused into the bile duct and collecting serial cecal samples for LC-MS/MS analysis. Further, in other animal research we could observe how inadequate, optimal and supraphysiological folate intakes could affect colonic folate absorption and expression of the two folate solute transporters (RFC and PCFT). Afterwards the level of mRNA RFC and PCFT and their respective protons from mucosal samples could be measured using real-time PCR and Western Blot analyses.

Lastly, the exploration of manipulating the microbial milieu to increase folate status through altered diet or probiotics is important to determine the effect on folate concentration in colonocytes and on blood folate is a potential consideration. The implications of a non-invasive pH-dependent caplet to deliver test nutrients to the human colon, such as probiotics or folate precursors (PABA labeled with a stable isotope) will help to differentiate bacterially synthesized folate from total colonic folate and the bioavailability as detected by plasma folate. Overall,
future studies will provide a more comprehensive understanding of how the “input side” of folate nutrition impacts the colonic depot of natural folate. This will help to set dietary recommendations that strike the right balance between benefits and risks in order to optimize whole body folate homeostasis.
Chapter 4

4. References

1. Institute of Medicine. Dietary reference intakes for thiamin, riboflavin, niacin, vitamin
   B6, folate, vitamin B12, pantothentic acid, biotin, and choline, 10th ed. Washington DC:
   Food Regulatory IaIA, Health Canada (Ed.) 1998; 3029-33.
3. Food and Drug Administration. Food Standards: amendment of standards of identity for
   2006;1352-61.
5. Lindzon G, O'Connor DL. Folate during reproduction: the Canadian experience with folic
7. Honein MA, Paulozzi LJ, Mathews TJ, Erickson JD, Wong L-YC. Impact of folic acid
   fortification of the US food supply on the occurrence of neural tube defects. JAMA
   2001;285:2981-86.
8. Wright JA, Dainty JR, Finglas PM. Folic acid metabolism in human subjects revisited:
   potential implications for proposed mandatory folic acid fortification in the UK. Br J Nutr
   folic acid for the primary prevention of neural tube defects. BMC Pregnancy and
    2008;87:517-33.
11. Mason JB, Cole BF, Baron JA, Kim YI, Smith AD. Folic acid fortification and cancer
    2009;67:235-44.
    associated with reduced natural killer cell cytotoxicity among postmenopausal women. J
    Nutr 2006;136:189-94.
14. Morris MS, Jackques PF, Rosenberg IH, Selhub J. Folate and vitamin B12 status in
    relation to anemia, macrocytosis, and cognitive impairment among older Americans in
    during pregnancy and insulin resistance in the offspring: the Pune maternal nutrition
42. Waisman HA, Elvehem CA. The role of biotin and "folic acid" in the nutrition of the rhesus monkey. The Journal of Nutrition 1943;26:361-75.
56. Houghton LA, Sherwood KL, Pawlosky R, Ito S, O'Connor DL. [6S]-5-
Methyltetrahydrofolate is at least as effective as folic acid in preventing a decline in
60. Wilcken B, Bamforth F, Li Z, et al. Geographical and ethnic variation of the 677C>T
allele of 5,10 methylenetetrahydrofolate reductase (MTHFR): findings from over 7000
61. Botto LD, Yang Q. 5,10-Methylenetetrahydrofolate reductase gene variants and
677C>T polymorphism affects DNA methylation in response to controlled folate intake
63. Axume J, Smith SS, Pogribny IP, Moriarty DJ, Caudill MA. The MTHFR 677TT
genotype and folate intake interact to lower global leukocyte DNA methylation in young
64. Sohn KJ, Jang H, Campan M, et al. The methylenetetrahydrofolate reductase C677T
mutation induces cell-specific changes in genomic DNA methylation and uracil
miscorporation: a possible molecular basis for site-specific cancer risk modification. Int
C677T and Delta DNMT3B C-149T polymorphisms confer different risks for right- and
67. McGuire JJ, Bertino JR. Enzymatic synthesis and function of folypolyglutamates. Mol
68. Devlin AM, Ling EH, Peerson JM, et al. Glutamate carboxypeptidase II: a polymorphism
associated with lower levels of serum folate and hyperhomocysteinemia. Human
Molecular Genetics 2000;9:2837-44.
69. Bhandari S, Gregory JF. Inhibition by selected food components of human and porcine
71. Foo SK, MsSloy RM, Rousseau C, Shane B. Folate derivatives in human cells: studies on
normal and 5,10-methylenetetrahydrofolate reductase deficient fibroblasts. Am J Clin Nutr
1982;112:1600-8.
73. Sirotnak FM, Tolner B. Carrier-mediated membrane transport of folates in mammalian
90. De Marco P, Calevo MG, Moroni A, et al. Reduced folate carrier polymorphism (80A-->
reduced folate carrier gene and its associations with folate status and homocysteinemia.
mutation (p.Cys66LeufsX99) causing hereditary folate malabsorption. Mol Genet Metab
2009.
94. Zhang G, Zhang QY, Miao XP, Lin DX, Lu YY. Polymorphisms and mutations of the
96. Edwards C. Physiology of the colorectal barrier. Advanced Drug Delivery Reviews
97. Lewis SJ, Heaton K. Increasing butyrate concentration in the distal colon by accelerating
98. Topping DL, Clifton PM. Short-chain fatty acids and human colonic function: roles of
resistant starch and nonstarch polysaccharides. Physiological Reviews 2001;81:1031-64.
99. Leser TD, Molback L. Better living through microbial action: the benefits of the
mammalian gastrointestinal microbiota on the host. Environmental Microbiology
2009;11:2194-2206.
100. Mai V, Morris JG, Jr. Colonic bacterial flora: changing understandings in the molecular
Microflora. In: Human Intestinal Microflora in Health and Disease (Hentges, D.J., ed.).
102. O'Keefe JS. Nutrition and colonic health: the critical role of the microbiota. Curr Opin
103. Russell RM. Factors in aging that affect the bioavailability of nutrients. J Nutr
2001;131:1359S-61S.
104. Blaut M, Collin MD, Welling GW, Dore J, van Loo J, de Vos W. Molecular biological
methods for studying the gut microbiota: the EU human gut flora project. Br J Nutr
2002;87:203S-11S.
105. Reuter G. The Lactobacillus and Bifidobacterium microflora of the human intestine:
microflora of Korean adults and seniors, identified by SDS-PAGE of whole cell proteins
and 16S rDNA sequence analysis. Journal of microbiology and biotechnology


Tozer T, Rowland M. Introduction to pharmacokinetics and pharmacodynamics. Baltimore, Maryland: Lippincott Williams & Wilkins, 2006.


235. House JD, March SB, Ratnam MS, Crowley M, Friel JK. Improvements in the status of folate and cobalamin in pregnant Newfoundland women are consistent with observed reductions in the incidence of neural tube defects. Can J Public Health 2006;97:132-5.

247. HOPE. Homocysteine lowering with folic acid and B vitamins in vascular disease. NEJM 2006;354:1567-1577.


281. Cropy JS, Suter CM, Beckman KB, martin DIK. Germ-line epigenetic modification of the murine Avy allele by nutritional supplementation. Proc Natl Acad Sci USA 2006;103:17308-12.


301. Shoveller AK, Brunton JA, Pencharz PB, Ball RO. The methionine requirement is lower in neonatal piglets fed parenterally than in those fed enterally. J Nutr 2003;133:1390-7.
Chapter 5

5. Appendix

CONSENT TO PARTICIPATE IN A RESEARCH STUDY

TITLE OF RESEARCH PROJECT:
Folate Absorption Across the Large Intestine: Capsule Study

Investigators
The Hospital for Sick Children

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Sponsorship: The sponsor of this research is Dr. Deborah L. O’Connor and the Hospital for Sick Children. The study is funded by the National Science and Engineering Council of Canada (NSERC)

INTRODUCTION
You are being asked to take part in a research study. Before agreeing to participate in this study, it is important that you read and understand the following explanation of the proposed study procedures. This form provides all the information we think you will need to know in order to decide whether you wish to participate in the study. It also describes your right to refuse to
participate or withdraw from the study at any time. In order to decide whether you wish to participate in this research study, you should understand enough about its risks and benefits to be able to make an informed decision. This is known as the informed consent process. If you have any questions after you read through this form, ask your questions to a doctor or study personnel. You should not sign this form until you are sure you understand everything described in this form. You may also wish to discuss your participation in this study with your family doctor with respect to your health history and any medications you may be taking, in order to prevent any unnecessary harm to you should you decide to participate in this study. Make sure all your questions have been answered to your satisfaction before signing this document.

BACKGROUND
The nutrient folate is naturally found in your diet when you eat green leafy vegetables, fruits and legumes. It is a B-vitamin used by your body for healthy cell growth and development. This natural dietary reduced form of folate differs from the oxidized folate form called folic acid that is synthetically produced for supplements and fortification of our pasta and white flour in the Canadian food supply since 1998. Adequate blood folate levels have been shown to reduce the incidence of some negative health outcomes such as some birth defects (e.g. neural tube defects and oral clefts), anemia, growth retardation, cancer, and cardiovascular disease.

At the same time, it has been suggested that chronic consumption of high levels of supplemental folic acid, may produce negative health outcomes such as a greater cancer risk, altered immune function and masking of vitamin B12 deficiency. Vitamin B12, found in animal products, maintains blood vessels and the nervous system. A deficiency is common among older people, and sometimes in vegetarians. Microflora (bacteria) in the human large intestine, also called the colon, produce large amounts of folate in the same forms as those found in natural foods, and these forms, unlike folic acid, are not known to have undesirable health outcomes. Bacterial synthesis of folate could contribute a complementary folate source not currently considered in nutritional recommendations (based on oral dietary intakes and vitamin supplements).

PURPOSE OF THE RESEARCH
In our laboratory we are investigating folate absorption across the human large intestine using three observational studies with healthy adult volunteers. To date, we have completed 2 of the 3 studies. The first study delivered a test dose of folate during a routine colonoscopy with a clean bowel (removal of normal microflora populations). In the second study we tested two different types of placebo coated caplet to determine which would ensure quantitative delivery of all of a test folate dose to the colon for this third and final study. Finally, the purpose of our current study is to observe whether the intact human colon (containing normal and undisturbed microflora populations) absorbs folate and to what extent it's used by the body. We will do this by providing a pH-dependent caplet that has a special coating, which is designed to dissolve (or break apart) in the colon and release a small amount of natural folate called leucovorin. This folate contains something called a label. It is different from regular folate only in that it weighs more. The label (stable isotope), found naturally in small amounts in your body, is completely harmless. A fluoroscope (similar to an X-ray machine) will take X-ray like pictures of your midsection to determine if the caplet has reached the ileal-cecal junction (the beginning of your large intestine) and allow us to make sure the caplet dissolves in your colon. We will continue to take hourly pictures until the caplet has reached the end of your large intestine. At least three weeks later we will give you an intravenous (IV) injection of a small amount of the same folate
form used in the caplet dose. These two folate doses will allow us to determine the folate amounts absorbed across your colon. We will ask for blood and urine samples to measure the appearance and gradual disappearance of this labeled folate.

DESCRIPTION OF THE RESEARCH

Study Enrollment: You are being invited to participate in this study because you are a healthy person with normal digestive tract motility and absorption. Ten healthy adult volunteers will be included in this study. The Research Ethics Board at The Hospital for Sick Children (SickKids) has reviewed this study.

Study Procedure: The study will take place at SickKids. If you agree to be in our study after a screening visit, we will need your participation over two study periods for approximately 20 hours. Study Period 1 (4 days) includes ingestion of a folate caplet and Study Period 2 (3 days) administration of an IV folate dose. The second period (IV) will take place 3 weeks or more after the first period (caplet). Please see refer to the timeline on page 7 of this form.

Screening: If you decide to participate, the study nurse will meet with you for an hour to conduct a screening interview before the first study period at the Clinical Investigation Unit (CIU). You will be asked simple questions concerning your past medical history and use of medications, vitamins and minerals to determine eligibility. Your height, weight and waist circumference will also be measured. The nurse will discuss what criteria could result in study withdrawal such as alcohol consumption 24 hours before administration of either the caplet or IV folate dose, or laxative, folate supplement or antibiotic use - 2 weeks before both study periods. In order to ensure that you will be eligible for the study, we will take a screening blood sample (10 mL or 2 teaspoons) to make sure your blood folate status is normal. We will also perform a genetic test to confirm that you do not carry the common variant of an enzyme (“MTHFR”), which can alter the amounts of the forms of folate in your blood (see attached Research Consent for Genetic Testing form which will also require your signature). No additional genetic tests will be performed on your blood.

If you are a female considering participating you will be asked to agree to not plan a pregnancy and take appropriate precautions to prevent a pregnancy during the course of the study. If you are female and able to get pregnant, a urine pregnancy test will be done during the screening visit and the morning of x-ray like imaging (fluoroscope). If the test is positive, you will not be able continue in the study. The results of the pregnancy test are confidential and will be told to you by one of the study nurses in private. Every effort will be made to keep positive pregnancy test results private.

A. Study Period 1

Day 1: The day before the fluoroscope imaging, you will collect a 24-hour urine sample, in a collection bottle supplied by us. We can pick this urine sample up at your home or you can bring it to SickKids. You will also visit the CIU for 30 minutes to provide a blood sample for a baseline folate level (approximately 5 mL or 1 teaspoon). During this visit, you will receive a standardized breakfast and directions for ingestion of the caplet containing 400 µg of folate. This is the same amount of folate found in most vitamin supplements that you can buy in the store. We would ask that you not eat or drink anything (except for water) from 8 pm until caplet ingestion. You will be asked to swallow the caplet the next morning at 6 am, with as much water as needed, and immediately eat a standardized breakfast that we will have provided. A morning call will be made by the study coordinator at 0600 to confirm caplet ingestion.
**Day 2:** This day includes ingesting the caplet at 6 am (followed by a breakfast). You should arrive at the CIU for 7:30 am. Female subjects will be asked to provide urine for a pregnancy test before imaging, the results of which will be confidential. You will need to collect all of your urine on this day in collection bottles provided by us. An indwelling catheter (IV) will be inserted to measure your folate blood level 5 mL (one teaspoon) after the first image has been taken. After fluoroscopic imaging shows that the caplet has left your stomach, 5 mL of blood will be taken via the IV every hour until 8 pm (end of study day). Over the course of the day, a maximum of 12 blood samples will be taken to measure the uptake of folate into your blood. Once we detect the labeled folate in your blood, we will be able to determine how much folate was absorbed. We will provide study-specific meals and snacks throughout the day, starting after the caplet has left the stomach.

**A fluoroscope** is similar to an X-ray machine (with approximately 10 times less radiation exposure) and will take pictures of your midsection to monitor the movement of the caplet through your gastrointestinal tract beginning at 8 am. This will take place at the Image Guided Therapy (IGT) unit at SickKids every hour for a total of 10 images (6 pm) or until the caplet has reached the end of your large intestine (dissolved). The shots will involve taking 2-3 second long videos of your midsection. This will add up to a maximum of 20-30 seconds of fluoroscopy, which is approximately equivalent to 1 chest x-ray. In the event that the caplet has not completely dissolved in your colon by the time the last image is taken, we may ask you to collect stool samples for up to 2 days, which will be further analyzed with the fluoroscope. We will provide stool collection kit(s), as well as instructions for sample delivery.

**Day 3 & 4:** You will need to collect all of your urine for the next two days after fluoroscopic imaging, in collection bottles provided by us. We can pick these urine samples up at your home or you can bring them to us. Also, one blood sample 5 mL (1 teaspoon) will be taken on both of these days at the CIU to measure the folate level in your blood during a 15 minute visit. You may resume using supplements containing folate at the end of Day 4; we all ask again that supplements (containing folate) not be used at least two weeks prior to the second study period (IV folate dose).

**B. Study Period 2 (3 weeks after caplet ingestion)**

**Day 1:** The day before the IV folate administration, you will collect a 24-hour urine sample, in a collection bottle provided by us. We can pick this urine sample up at your home or you can bring it to us the next day at SickKids. The evening before the IV day, you will begin fasting at 8 pm.

**Day 2:** This day includes having an IV injection of folate solution. You will be at the hospital for about 5 hours in the CIU at SickKids beginning at 8 am. There we will insert an IV into each of your arms. For the study, we will inject folate in an IV dose (100 µg of folate dissolved in 1 mL [about a quarter of a teaspoon] of salt solution). This is about 1/4 the amount of folate found in most vitamin supplements that you can buy in the store. We will measure the amount of the label in your blood and urine.

At the time of the injection of folate, and 15 minutes afterwards following with every 30 minutes after the injection for 4 hours, blood samples of 5 mL (one teaspoon) will be taken, to measure the amount of folate remaining in your blood. These will be taken via a second IV in your other arm. A total of ten blood samples will be collected.
All meals and snacks for this day will be also provided like Study Period 1 Day 2. You will need to collect all of your urine on this day, in collection bottles provided by us.

**Day 3:** You will need to collect all of your urine on this day, in collection bottles provided by us. We can pick this urine sample up at your home or you can bring it to us the next day. This is the last day of the study and you may continue with your normal routine of taking supplements containing folate.

**POTENTIAL HARMS**

*Fluoroscopy after ingestion of caplet:* Although the dose of radiation that is given with fluoroscopy is approximately 10 times less than a regular X-ray, there are certain risks associated with exposure to radiation. Routine precautionary measures will be taken to ensure that your radiation exposure is as minimal as possible. Female volunteers will be asked to take appropriate precautions to avoid pregnancy while participating in this study and a urine pregnancy test will be completed the morning prior to initiating fluoroscopy to avoid potential harm to embryo/fetus. Females with nursing infants will also be excluded.

*Consumption of folate caplet, infusion of folate and blood draws:* Although rare, allergic reactions including, skin rash, itching and wheezing are possible side effects of the natural form of folate used in this study. But there may be harms that we do not know about. There may be a small amount of bleeding when blood is taken from a vein. There may also be slight discomfort and bruising or redness at the blood draw site that will usually disappear in a few days.

**POTENTIAL DISCOMFORTS OR INCONVENIENCE**

You may be inconvenienced by having to travel to The Hospital for Sick Children. Study obligations also require you to take time away from your regular schedule.

**POTENTIAL BENEFITS**

*To Individual Subject:* You will not benefit directly from participating in this study. There is a possibility of incidental findings from fluoroscopic imaging, pre-study blood testing and pregnancy tests. The results of these findings are confidential and will be told to you immediately by one of the study nurses or doctors in private. Every effort will be made to keep all incidental findings private. A study team member will discuss the overall results of the research with participants upon request, once the study is completed.

*To Society:* Your participation is appreciated, as the results will be used to better understand if supplemented folate is absorbed across the intact human colon and whether bacterially synthesized folate maybe a relevant complementary source of bioavailable folate.

**CONFIDENTIALITY AND PRIVACY**

The results of the tests described will be used for research purposes only in the context of this study. We would need your permission and signed consent to send the test results to another professional involved in your care.

We will respect your privacy. No information about who you are will be given to anyone or be published without your permission, unless the law makes us do this. For example, the law could make us give information about you in the following circumstances:

- If a child has been abused
• If you have an illness that could spread to others
• If you or someone else talks about suicide (killing themselves), or
• If the court orders us to give them the study papers

Sick Kids Clinical Research Monitors, employees of the granting agency funding the study (National Science and Engineering Council of Canada [NSERC]), or Health Canada Health Products and Food Branch, the regulator of the study may see your health record to check on the study.

By signing this consent form, you agree to let these people look at your records. We will put a copy of this research consent form in your patient health records. We will give you a copy for your files.

The data produced from this study will be stored in a secure, locked location. Only members of the research team (and maybe those individuals described above) will have access to the data. This could include external research team members.

We will analyze your blood and urine samples for total and labeled folate but will keep some of each sample in case we decide later to analyze them for other nutrients (no genetic tests will be done on these samples).

Following completion of the research study, the data will be kept as long as required and then destroyed as required by Sick Kids policy. After completion of the study, the investigators plan to present study results at conferences, seminars and other public forums. Eventually the investigators plan to publish study findings in a research journal. Published study results will not reveal your identity.

REIMBURSEMENT
We will pay for your expenses for being in this study such as meals, babysitters, parking and getting you to and from Sick Kids. If you stop taking part in the study, we will pay you for your expenses for taking part in the study so far. The compensation for both study periods will be in the amount of $600.00, in recognition of your time and effort.

PARTICIPATION
It is your choice to take part in this study. You can stop at any time. The care you or your family gets at SickKids will not be affected in any way by whether you take part in this study.

New information that we get while we are doing this study may affect your decision to take part in this study. If this happens, we will tell you about this new information, and we will ask you again if you still want to participate in the study.

During this study we may create new tests, new medicines, or other things that may be worth some money. Although we may make money from these findings, we cannot give you any of this money now or in the future because you took part in this study.

In some situations, the study doctor or the sponsor may decide to stop the study. This could happen if the treatment given in the study is helping you. If this happens, the study doctor will talk to you about what will happen next.
If you become ill or are harmed because you took part in this study, we will treat you for free. Your signing this consent form does not interfere with your legal rights in any way. The study staff, any people who gave money for the study, or the hospital are still responsible, legally and professionally, for what they do.

CONFLICT OF INTEREST
I, and the other research team members, have no conflict of interest to declare. This means that none of the people involved in this will benefit personally, financially or in some other way from doing this study.

WHO DO I CALL IF I HAVE QUESTIONS OR PROBLEMS?
If you have questions or would like more information about this study, please call Alanna O’Malley at (416) 813-5777 (telephone) or pager: (416) 375-5020, email: alanna.omalley@utoronto.ca
Should you have any questions or concerns during the study or in the event you experience a study-related injury, please contact your study nurse, doctor or any of the study investigators listed on the front page. If you have questions about your rights as a subject in a study or for information on whom to contact in the event of injuries during a study, please call the Research Ethics Manager at 416-813-5718.
TIMELINE FOR PARTICIPATION:
**TWO STUDY PERIODS: 4 DAYS + 3 DAYS**

**Pre-Study Day**
**Clinic Visit: 1hr**
- Informed Consent, Eligibility Questionnaire & Assessment Interview will be completed. We will ask you to provide a blood sample and pregnancy test (female subjects only).

**Study Period 1**
**Caplet**

**Day 1**
**Clinic Visit: 30 min**
- You will receive instructions on fasting after 8 p.m. this day and how to take the test caplet tomorrow morning at 6 am.

**Day 2**
**Clinic Visit: ~12.5 hrs**
- You will take the caplet at 6 am followed by the breakfast we provide. Please arrive at the CIU by 7:30 am. There will be a pregnancy test (female subjects only) before the first fluoroscopic image. This day will consist of 10 hourly images, 10-12 blood samples, and 24-hour urine collection. Meals/snacks will be supplied.

**Day 3**
**Clinic Visit: 15 min**

**Day 4**
**Clinic Visit: 15 min**

**Study Period 2**
**IV**

**Day 1**
**Clinic Visit: ~4.5 hours**
- We will ask that you begin fasting tonight by 8 pm.

**Day 2**
- Please arrive at the CIU by 8:00 am. 2 indwelling catheters will be inserted for an injection of the test folate solution and to collect 10 blood samples. Meals/snacks will be supplied. 24-hour urine will also be collected on this day.

**Day 3**
**- LAST DAY OF STUDY**

*1 blood (5mL) and 24-hour urine samples collected on this day.

**Only 24-hour urine samples collected on this day. We can pick your urine sample up at home or you can bring it to us at SickKids.
STUDY TITLE: FOLATE ABSORPTION ACROSS THE LARGE INTESTINE: CAPSULE STUDY

CONSENT
By signing this form, I agree that:
1) You have explained this study to me. You have answered all my questions.
2) You have explained the possible harms and benefits (if any) of this study.
3) I know what I could do instead of taking part in this study. I understand that I have the right not to take part in the study and the right to stop at any time. My decision about taking part in the study will not affect my (or my child’s) healthcare at Sick Kids.
4) I am free now, and in the future, to ask questions about the study.
5) I have been told that my medical records will be kept private except as described to me.
6) I understand that no information about who I am will be given to anyone or be published without first asking my permission.
7) I have read and understood pages 1 to 8 of this consent form. I agree, or consent, to take part in this study.

__________________________                                      ______________________________
Printed Name of Subject & Age Subject’s Signature & Date

__________________________
Printed Name of person who explained consent

______________________________
Signature & Date

__________________________
Witness

______________________________
Signature & Date
Research Consent for Genetic Testing

(Addendum)

TITLE OF RESEARCH PROJECT:
Folate Absorption Across the Large Intestine: Capsule Study

Investigators
The Hospital for Sick Children

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Study Coordinator

Karen Chapman, RN PhD (c), (416) 813-6121
Research Nurse Manager, The Clinical Investigation Unit

Sponsorship: The sponsor of this research is Dr. Deborah L. O’Connor and the Hospital for Sick Children. The study is funded by the National Science and Engineering Council of Canada (NSERC)

Background
You are being asked to take part in research examining folate absorption across the large intestine (please refer to Consent Form for more details). Part of the screening process involves a genetic test that is described in this form. Before agreeing to participate, it is important that you read and understand the following explanation of the proposed genetic test. If you have any questions after you read through this form, ask
your questions to a doctor or study personnel. Make sure all your questions have been answered to your satisfaction before signing this genetic test consent.

By analyzing 1mL of your screening blood sample, The Hospital For Sick Children Molecular Genetics Laboratory will determine if you have a genetic polymorphism (mutation) in an enzyme called methylenetetrahydrofolate reductase (short form MTHFR). You need this MTHFR enzyme in your body to process the folate from your food and also if you take supplements or vitamins containing folate. Results from this genetic test will help us better understand your blood folate levels.

The full (homozygous TT genotype) MTHFR C677T mutation is common and related to ethnicity. The frequency has been shown to range between 5-20% of those who are of Caucasian, Hispanic and Asian decent, and a few percent of those of African decent. About 50% of people have a milder form of this mutation (heterozygous CT genotype).

If you have the full mutation (homozygous), you will have a lower activity of the MTHFR enzyme in your body. This can affect the way folate is used and may be of concern when you have a low amount of folate in your blood. The full mutation and low levels of folate in your blood can further increase your risk for neural tube defects in a baby during pregnancy (females) and may also increase your risk for certain cancers and heart disease.

No additional genetic tests will be performed on any blood samples collected during the study. When the results are available, the registered nurse that collected your blood will phone you. All information will remain confidential. If you have the full mutation you will not be able to continue participating in the study.

**General Section**

I confirm that the _____________________ has explained the genetic tests that I am [professional's name]

about to have done with respect to homozygous MTHFR C677T polymorphism, and that any questions that I have asked have been answered to my satisfaction. The discomforts, consequences and possible risks associated with these tests have been explained to me. I understand that it is my choice whether or not to have this testing. Results of this test will be explained to me and I understand that this information may be shared, if necessary, with professionals involved in my/my child's medical care, including our family physician. I have been assured that records relating to me or my child and the care that we received will be kept confidential, and that no information will be released or printed that will reveal my or my child's identity without my permission or unless required by law.
I understand that the interpretation of the genetic information will depend in part on the family information that I have given. Differences between family information and the results of genetic tests occur when the parents of a child are different from those reported. Non-paternity may be detected with this testing.

I understand that although genetic testing is usually accurate, as with all testing some inaccuracies may occur. Also genetic testing is ongoing and new research may mean that the interpretation of the test results may change over time. On occasion, in the process of testing for one genetic condition, another genetic alteration may be identified. Such findings would be reported to your health care provider to discuss with you.

I understand that it is my responsibility to notify the Division of Clinical Dietetics of any change of address, and to check with the department for updated genetics information and counseling that I feel I may need, for example in making decisions about a pregnancy.

Closed Consent:
Closed consent means that any tissue or DNA obtained from my child or me will be analyzed and then destroyed. Specifically, I give my consent for a blood/tissue sample to be taken for testing related only to homozygous MTHFR C677T polymorphism; this testing will be undertaken in an accredited clinical service laboratory and/or a research laboratory and that the sample and any DNA extracted from it will be destroyed once the results of the testing are available. I also understand that if I want any further genetic testing to be done in the future, I will need to have another sample taken from my child or me.

Signature: _____________________ Date: ____________________

Witness: _____________________ Date: ____________________

Witness: _____________________ Date: ____________________

If you have questions about this study, please call Alanna O’Malley at (416) 813-5777 (Telephone), or (416) pager: (416) 375-5020, email: alanna.omalley@utoronto.ca
If you have questions about your rights as a subject in a study or for information on whom to contact in the event of injuries during a study, please call the Research Ethics Manager at 416-813-5718.