FOLATE ABSORPTION ACROSS THE COLON AND THE MODULATION OF BACTERIAL FOLATE SYNTHESIS BY DIET

By

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ABSTRACT

While assessment of folate requirements has been based only on dietary intakes, folate produced by the colonic microflora can exceed amounts consumed in food. Bacterially synthesized folate is absorbed across the rat and piglet colon. *In vitro* studies suggest, but direct evidence is lacking that folate is absorbed across the intact human colon. If indeed folate is absorbed, the amount synthesized may be susceptible to manipulation by fibre and prebiotics intake. We therefore performed two studies to investigate folate absorption across the colon.

To confirm absorption across the intact human colon, in our first study, 684 nmol (320 µg) $^{13}C_5$-glutamyl-[6S]-5-formyltetrahydrofolate was infused into the cecum of six adults and blood samples were collected. Tandem mass spectrometry confirmed folate absorption across the colon by appearance in plasma of $^{13}C_5$-[6S]-5-methyltetrahydrofolate, at a rate of 0.6±0.2 nmol/h versus 7±1.2 nmol/h after intravenous injection of 172 nmol $^{13}C_5$-5-formyltetrahydrofolate.

Since bifidobacteria are potent folate producers, in our second study we evaluated the influence of bifidogenic oligosaccharides on colonic folate production and host folate status, using a piglet animal model. Piglets (n=12) were randomly assigned a milk-based formula with 5g/L inulin + 5g/L galactooligosaccharides, or 5g/L maltodextrin (control). After 28 days, the weights of colon contents (178 %) and colon tissue (37.9 %) of piglets fed oligosaccharides were greater than controls (*P*=0.0003, *P*=0.0044, respectively). The bacterial load and folate contents in the colons of piglets fed oligosaccharides were greater than controls (*P*=0.0022,

P=0.0218, respectively). Body weights, blood folate status and liver and kidney folate concentrations did not differ.

In conclusion, folate is absorbed across the human colon. Supplementation of the piglet diet with 5g/L inulin and 5g/L galactooligosaccharides increased the amounts of microbial folate, and the weights of colon tissue and contents, but folate concentrations in colon contents, blood and organs were not affected.

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
CFU	colony forming units
DRIs	Dietary Reference Intakes
Eh	Redox potential
FR	Folate receptors
FBP	Folate binding proteins
GCPII	Glutamate carboxypeptidase II
GOS	Galactooligosaccharides
HPLC	High Pressure Liquid chromatography
FAD	flavin adenine dinucleotide
FOS	fructooligosaccharides
GOS	galactooligosaccharides
HPLC	high pressure liquid chromatography
IN-GOS	inulin-galactooligosaccharides;
LC-MS/MS	Tandem mass spectrometry
MTHFR	methylene tetrahydrofolate reductase
PABA	*p*-aminobenzoic acid
PCFT	protein coupled folate carrier
RBC	erythrocyte
RFC	Reduced folate carrier
SCFA	Short chain fatty acid
tHCY	total plasma homocysteine

1 INTRODUCTION

Folate functions as a coenzyme in the oxidation, reduction and transfer of one-carbon groups, in the metabolism of some amino acids (serine, glycine, methionine) and homocysteine, in the synthesis of purines and thymidylate, in the methylation of DNA and histones and in the initiation of protein synthesis (1-3). The greater need for folate in pregnancy and lactation, in fetal and childhood development, and generally when anabolic activity increases, is due to the aforementioned roles of folate in metabolism. For many previous generations, folate deficiency was common. Low dietary intake of folate in humans has been associated with certain developmental defects such as neural tube defects (NTDs), oral clefts and Down syndrome, and low infant birthweight, anemia in pregnancy, cancer, stroke, and neuropsychiatric disorders (1, 4). Since fortification of the North American food supply in 1998, blood folate status has risen and the rate of neural tube and some other birth defects has declined as has the mortality and incidence of stroke (5-9). With these benefits, some possible negative health outcomes of high folate intake have been suggested and concerns have risen about the safety of chronically elevated folate status, unmetabolized folic acid and folate overnutrition for many population groups (10-19).

The folate requirements of humans to date have been based entirely on dietary intakes of folate and its bioavailability in food and supplements (1). However, in the human colon there is a high concentration of folate, produced by the intestinal microflora (5). This depot of folate represents a potential source of the vitamin which may influence whole body folate status. The requirement for folate is higher in tissues with high rates of cell division, such as the colonocyte, and localized folate deficiencies have been observed in normal colonic epithelia of subjects with normal blood folate indices (20-24). Microbial folate seems ideally located to be an important source of this vitamin. In traditional diets and before the advent of industrial processing of foods, the fibre component in the diet was very much higher than it is today. Its

importance to human health today is an acknowledged focus of current study (25-26). Dietary fibre is an important source of energy for the colonic microflora and rapid bacterial fermentation of fibre in the colon increases bacterial biomass (27). Further, feeding specific soluble forms of fibre (*i.e.* prebiotics), such as inulin, oligofructose and galactooligosaccharides, has been shown to increase the number of bifidobacteria in the colon, and bifidobacteria are among the bacteria known to produce folate in humans (28-33). We reason that promoting an increase in bifidobacteria may cause an increase in colonic folate biosynthesis. If folate is absorbed across the colon, this source may be an important influence on whole body folate status and to meet the elevated local requirement of colonocytes.

An understanding of colonic folate absorption would be helpful to set dietary recommendations with the right balance between health benefits and risks but little is known about the bioavailability of colonic microbial folate in humans. Data from animal studies has shown that folate crosses the colon of the rat and the pig (34-35). *In vitro* studies on human colon-derived biopsy scrapings and cell lines have suggested that substantial amounts of colonic folate can be absorbed (36-37) by mechanisms similar to that of the small intestine (36, 38-39). However direct evidence that folate is absorbed across the intact human colon, and at what rate, does not exist.

Our long term goal for this research is to explore the relationship between dietary fibre, the modulation of folate production by the microbial community, and its effects on folate status and colonic health. To date, the effect of dietary modulation on colonic folate production has been tested only in rats by dietary supplementation with various types of fibre (40-42). Rats are also unsuitable for studies of colonic folate absorption because of the difficulty in avoiding coprophagy. Coprophagy could confound experimental results by potentially allowing absorption of microbial folate across the small intestine (43). The piglet is a more suitable

model for human folate metabolism, since it is not coprophagic and absorption across the porcine small intestine is similar to that in humans (44-47). Albeit no information on the site of post-absorptive first-pass folate metabolism is available for the pig, the piglet has been found to be an excellent model for the related areas of gastrointestinal physiology, sugar digestion and infant development (48-51).

To provide a basis for investigating the relationship between diet, human colonic folate production and health, we conducted two studies. For the first study, recognizing a need to verify that folate is absorbed across the intact human colon, we designed a protocol to test such absorption by infusing a solution of labeled folate directly into the human colon. We first verified by colonoscopy that the colon was healthy and intact. A labeled solution of folate (^{13}C-5-formyltetrahydrofolic acid) was then delivered directly to the cecum, using an irrigation catheter inserted in the biopsy channel of the colonoscope, which also allowed us to verify correct placement of the folate dose. We determined colonic absorption of folate by measuring the appearance in plasma of $^{13}C_5$-5-methyltetrahydrofolic acid.

For the second study, we used the piglet to see if we could manipulate the microflora by dietary supplementation with bifidogenic oligosaccharides to increase folate production. In this study, five day old piglets were fed for 28 days on one of two experimental milk-based formula diets: one, supplemented with prebiotic bifidogenic oligosaccharides, the other with a control carbohydrate, maltodextrin. We chose to use a mixture of inulin, a long-chain oligofructose, and galactooligosaccharides, at a dose of 10g/L, because similar oligosaccharide mixtures have been used successfully at a comparable dose, to increase bifidobacteria numbers in human infants (28-29, 52). We examined the effects of these diets on the colonic microflora by enumeration of the colonic aerobic and anaerobic bacterial load, and of bifidobacteria and lactobacilli, and by determination of folate in the colonic contents. We monitored the response

of the piglets by determining folate indices in blood, and folate levels in their liver and kidneys, and by comparing growth in the two groups of piglets.

2 REVIEW OF THE LITERATURE

2.1 FOLATE

Folate is a generic term for a family of structurally related compounds that have a common pteroylglutamic acid core consisting of pteridine and *p*-aminobenzoic acid (PABA) linked to one or more glutamate molecules. These compounds include naturally occurring food folates as well as synthetic folic acid, the fully oxidized monoglutamate form used to supplement and to fortify foods. Folates are synthesized by plants and most microorganisms via the condensation of PABA with dihydropterin pyrophosphate, but are essential nutrients for mammals (53). The structure of folates can vary by the addition of a series of one to ten glutamate molecules in a chain, by reduction of the pteridine moiety to form dihydrofolic acid and tetrahydrofolic acid (THF), and by substitution of one-carbon units at the nitrogen atoms at the 5 or 10 positions or both (**Figure 2.1**). Folates function as coenzymes in the acceptance, oxidation/reduction and transfer of one-carbon units, and are particularly important in amino acid metabolism and in the synthesis of nucleic acids (53). The coenzyme form is determined by substitution of one-carbon units, which include methyl ($-CH_3$), methylene ($-CH_2-$), methenyl (-CH=), formyl (-CH=O) or formimino (-CH=NH) groups, to the polyglutamate form of the tetrahydrofolic acid molecule.

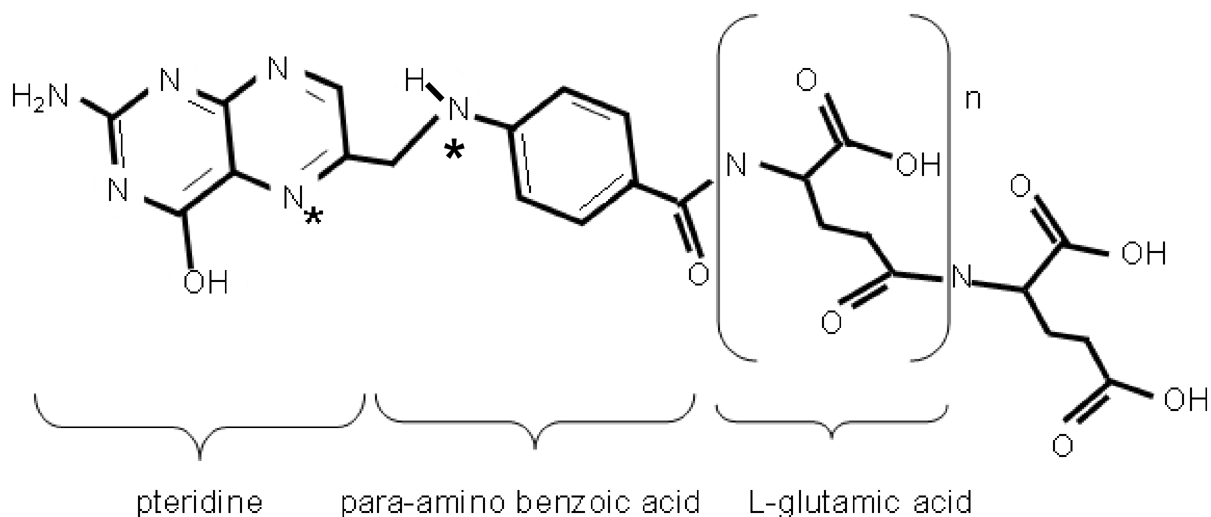

FIGURE 2.1 The structure of tetrahydrofolate. Carbon atoms are carried for transfer to other molecules at the positions shown by asterisks in the figure (positions 5 and 10).

2.2 HISTORY OF EARLY WORK ON FOLATE

Descriptions of human symptoms of folate deficiency may be found as early as 1824 in reports of macrocytic anemias that responded to treatment by dietary extracts of liver or yeast (54-57). Various extracts (Wills factor, vitamin M, vitamin Bc, Norite eluate factor), effective in reversing the effects of folate deficiency in animal models and of anemia in pregnancy, were eventually shown to contain folate as the active ingredient (58-59). The term "folic acid" was coined in 1941 after its extraction from spinach leaves (59). Pteroylglutamic acid was purified and crystallized in 1943, and synthesized in 1946 (59-63). Antifolates such as methotrexate were used in cancer treatment as early as 1947 (53). The production of folate in significant amounts by the intestinal flora of birds and mammals, including humans, was also recognized

in the 1940s and 1950s (64-71), as also was the availability of microbially-derived folate for absorption across the rodent small intestine, via the consumption of feces (coprophagy) (72).

2.3 DIETARY SOURCES OF FOLATE

2.3.1 Dietary sources of natural folate

Folate is found in most unprocessed natural foods in the form of reduced polyglutamate derivatives. It is particularly concentrated in yeast, and in liver and other organ meats (73). Among plant foods it is highest in green leafy vegetables, fresh oranges, asparagus, strawberries, peanuts and kidney and lima beans, however the levels in individual items are quite variable (74). The folate content of several foods is shown in Table 2.1. Most liver folate is in the form of pentaglutamyl conjugates (73), while among plant folates the heptaglutamyl form dominates (59, 73). These polyglutamate folates become bioavailable with their breakdown to pteroyl mono-glutamates due to the action of conjugases present in vegetable and mammalian tissues, and in the human intestine (75). Natural folates are vulnerable to oxidation, which is enhanced by heat, light and pressure in cooking or in other processing such as canning, leading to losses that may be over 55 % (76-77), while losses through pasteurization of dairy products are often less than10 % (78). Processing may also deconjugate vegetable folates leading to losses by diffusion into processing water (76). Orange juice is considered a major source of natural folate in the North American diet, but high losses of folate in frozen juices have been reported (77).

2.3.2 Dietary Sources of Synthetic Folic Acid

Many foods in Canada and the United States are fortified with folic acid. All cereal grain products labeled as enriched, and products containing these, are mandated in the United States to be fortified to contain 140 µg folic acid per 100g (79). As of November 1998, the addition of folic acid at 150 µg/100g to white flour, and 200 µg/100g to enriched pasta is

mandated in Canada (80-81). Due to industrial over-fortification as a measure against decomposition over the shelf-life of the products, even higher levels of folic acid, estimated by some to be twice the planned amounts, may be contained in foods made of enriched grain products (82). **Table 2.1** shows the folate content of some common unfortified foods. Breads, and fortified grain products such as rolls and crackers have been reported as the major sources of dietary folate in North America (83-85).

TABLE 2.1 The folate content of selected foods, before fortification (86-87)

Food Item	**Folate Concentration (µg per serving)**
Asparagus spears, canned	**104**
Boiled broccoli	**90-111**
Canned tuna	**1-2**
Cold breakfast cereal	**~100**
Egg noodles, enriched, cooked	**9.8**
Hamburger roll, enriched	**11.6**
Large hard boiled eggs	**22**
Raw oranges	**39.6**
Raw peaches	**3.3**
White granulated sugar	**0**
Whole fluid milk, 3.3% fat	**12.2**

2.4 QUANTITATIVE TECHNIQUES FOR FOLATE ANALYSIS AND THE STUDY OF FOLATE METABOLISM

2.4.1 Analytical techniques

Folates are vulnerable to destructive oxidation. This is best limited by precautions against exposure to light and heat, and by the addition of antioxidants such as ascorbate to samples. The vitamin also occurs in multiple chemical forms and the sensitivity of analytical techniques to different forms of folate must therefore be considered. Further, adsorption within protein matrices may require extraction of folates prior to analysis. Errors due to these factors tend to be in the direction of underestimation. Three main approaches have been used to measure folates (88-89).

(1) Microbiological assays measure total folate, often using *Lactobacillus rhamnosus* (ATCC 7469). Measurement by microbiological assay requires pretreatment of samples for deconjugation of polyglutamate forms and for extraction of folates from protein matrices and carbohydrates (90-95). Sample digestion with a series of enzymes is used to extract folates from sample matrices. Tri-enzyme digestion has been shown to increase yield but is its efficacy is variable (90-99).

(2) Protein-ligand binding methods have been used in clinical settings (100).

(3) A number of chromatographic approaches have been used (93), including Gas Chromatography (101), and more recently, high pressure liquid chromatography (HPLC) (92, 102-103) to allow separation of different folate forms. While earlier mass spectrometry studies used gas chromatography for preliminary separation of vitamers, HPLC coupled with mass spectrometry (LCMS), and tandem mass spectrometry (LC-MS/MS) allow shorter sample processing times with lower losses of folates to oxidative breakdown (104-105). As folate is vulnerable to degradation, electrospray ionization is more suitable than chemical ionization

methods (106). Folates may be separated and concentrated before instrumental analysis either by affinity purification or solid-phase extraction (106-108).

Round robin analyses have shown poor agreement both between different methods and different laboratories (108-110). For example, two- to nine-fold concentration differences were found in comparisons of serum and whole blood analyses (104-105, 110). The reliability of data cannot be assessed if the preparation techniques and analytical methods used are not specified.

2.4.2 Stable isotope studies of folate metabolism

The development of stable isotope methods has been particularly useful in combining the ability to detect different folate forms for tracing the metabolic fate of administered vitamer doses without jeopardizing human safety (108, 111-114). The use of double labels enables comparison of two routes of intake (for example oral intake *vs.* injection of the same vitamer with a different label) simultaneously (115-116). While LCMS is highly sensitive, it cannot distinguish between the same daughter fragments produced by different parent compounds. LC-MS/MS allows such separation as each parent-daughter transition may be monitored and the protocol more highly maximized for the problem at hand (106, 108, 117-119). LC-MS/MS is thus about 12-fold more sensitive to folate determination than LCMS, to <0.1 ng/mL on prepurified samples (106).

2.5 FOLATE METABOLISM

2.5.1 An overview of folate physiology

Dietary folates are absorbed chiefly across the proximal small intestine and enter the systemic circulation via the hepatic portal vein. Taken up by the liver they may be stored as polyglutamate folates, or monoglutamate folates may re-enter the systemic circulation via the

hepatic vein for transport to the periphery. A third destination for monoglutamate folates absorbed by the liver is secretion into the bile to enter the enterohepatic circulation for reabsorption via the small intestine and re-entry to the hepatic portal vein. A small amount of intact plasma folate is excreted in the urine though much is reabsorbed in passage through the kidneys. Folate is also oxidatively broken down in the tissues via a mechanism involving ferritin, and its breakdown products (*para*-acetamidobenzoylglutamate and its acetylated derivative acetamidobenzoylglutamate) are excreted in the urine (101, 120-121). Dietary folates are absorbed chiefly across the proximal small intestine and enter the systemic circulation via the hepatic portal vein. The majority of plasma folate is in the form of 5-methyltetrahydrofolic acid, with small amounts of 5- and 10-formyltetrahydrofolate (108). In higher intakes of folic acid (>200µg/d), such as in individuals taking supplements, unmetabolized folic acid may persist in plasma for several hours until metabolized by the liver to dihydro- and tetrahydrofolate (122-123). Taken up by the liver, reduced plasma folates may be stored as polyglutamate folates, or folates in the monoglutamate form may re-enter the systemic circulation via the hepatic vein for transport to the periphery. A third destination for monoglutamate folates taken up by the liver is secretion into the bile to enter the enterohepatic circulation for reabsorption via the small intestine and re-entry to the hepatic portal vein.

2.5.2 The role of folate in cellular metabolism

Folates cross cell membranes mainly in the form of monoglutamates (Figure 2.1) and elongation of the glutamate 'tail' of folate by the enzyme folylpolyglutamate synthetase is essential for retention of folate within cells (124). Cellular forms of folate have varying polyglutamate chain lengths, which may differ between cell types and cellular organelles (125).

A simplified outline of cellular folate metabolism is shown in **Figure 2.2**. Folate is metabolically active as fully reduced and polyglutamated tetrahydrofolic acid (3).

Tetrahydrofolate binds to one-carbon units of several oxidation states to form the folate vitamers 5-methyltetrahydrofolate, 5-formiminotetrahydrofolate, 5,10-methenyltetrahydrofolate, 5,10-methylenetetrahydrofolate, 10-formyltetrahydrofolate and 5-formyltetrahydrofolate. Some of these vitamers act as carbon donors with specific enzymes in cell metabolism. For example, 5,10-methylenetetrahydrofolate, 10-formyltetrahydrofolate are essential in DNA synthesis, 5,10-methylenetetrahydrofolate as donor for two carbons in the synthesis of purines, and 10-formyltetrahydrofolate in the methylation of uridylate to thymidylate (126). Vitamin B12 acts as a co-enzyme with methionine synthase reductase and methionine synthase to remethylate homocysteine to methionine (127-128). In this reaction 5-methyltetrahydrofolate serves as the methyl donor also necessary to regenerate tetrahydrofolate for the formation of the 5,10-methylenetetrahydrofolate needed for DNA synthesis.

Homocysteine is metabolized via the transulfuration pathway to form cystathionine which in the presence of vitamin B6 forms cysteine. Cysteine leads, via γ-glutamylcysteine, to the production glutathione, an important metabolite in defense against oxidative stress (129), or, via cysteine sulfinate and hypotaurine, to the formation of taurine, a non-protein amino acid and end-product of sulfur metabolism involved in diverse physiological activities including osmoregulation and cardiorespiratory function (130).

S-adenosylmethionine is the carbon donor in most cellular reactions requiring single carbon donation, including the formation of creatine and of neurotransmitters, and for the methylation of DNA (3). Over one hundred methyltransferases act with S-adenosylmethionine in the production of compounds acting in many cellular functions (3). Folate is also essential for the mitochondrial production of formyl-methionyl t-RNA which initiates protein synthesis (125). Its metabolism is compartmentalized: folate crosses the mitochondrial membranes as tetrahydrofolate (125, 131).

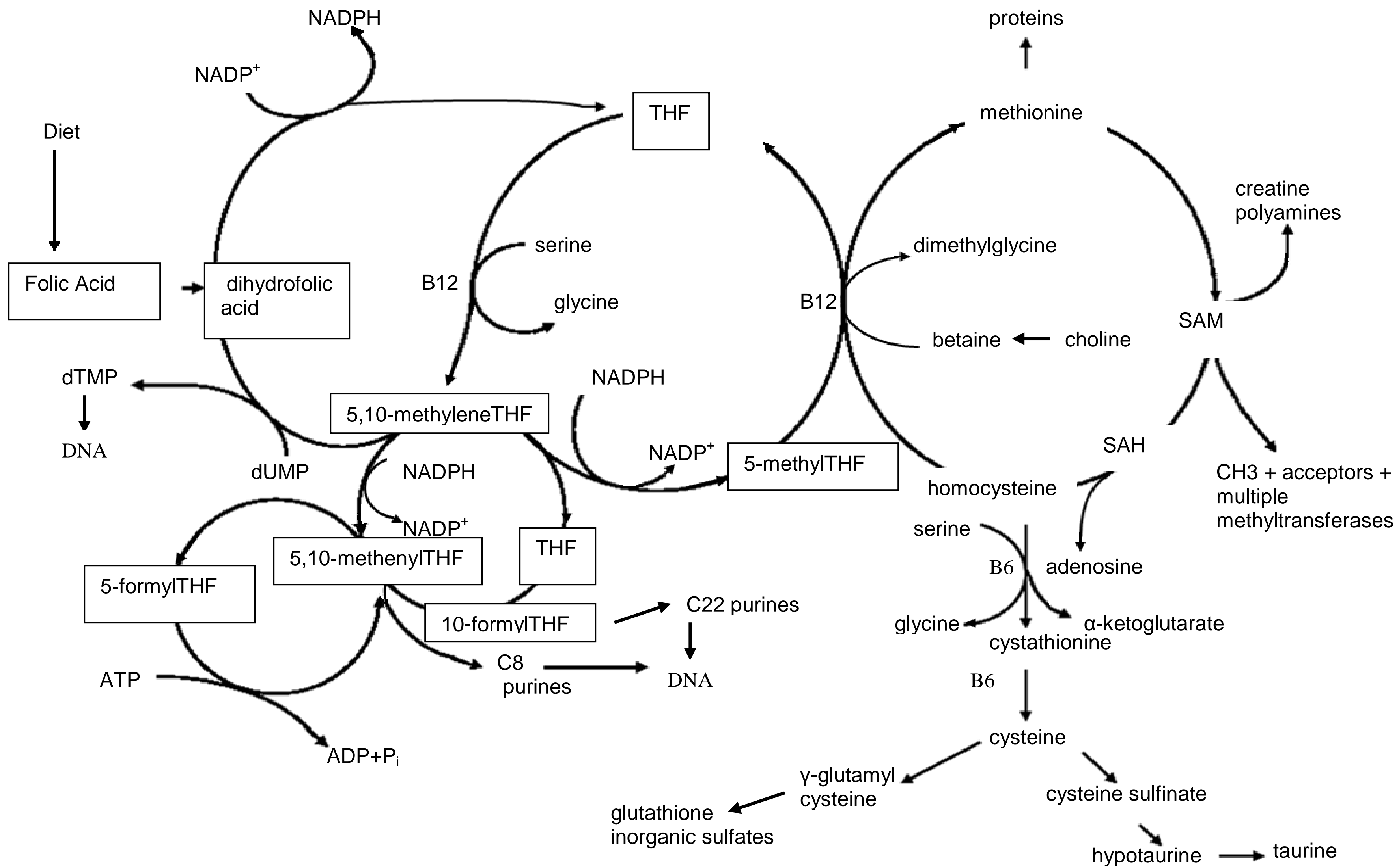

FIGURE 2.2 A simplified summary of cellular folate metabolism. (THF represents tetrahydrofolate).

2.6 FOLATE ABSORPTION

In mammals, folates are obtained from three main sources: from natural folates in foods, which are mainly in the form of poly-γ-glutamate derivatives; from supplements and folate added to fortified foods, mainly as folic acid (monoglutamate); and it is believed that microbially produced folate may be absorbed across the colon. Folate may be absorbed along the entire length of the small bowel (132) and has been shown to be absorbed across the colon of rats and of pigs (34-35, 133). In humans, *in vitro* studies have supported the presence of folate transport mechanisms in the colon, described below (36-38, 134). Most folate absorption of consumed food and supplements occurs in the proximal third of the small intestine, the jejunum (59).

2.6.1 Folate absorption in the small intestine

2.6.1.1 Intestinal deconjugation of food folates

Only short-chain folates are absorbed across the small intestine, in the form of mono- or diglutamates (135). The enzymatic hydrolysis of food folylpoly-γ-glutamates by specific hydrolytic enzymes (conjugases) is therefore a rate-limiting step in the absorption of food folate across the small intestine. Human intestinal γ-glutamylhydrolase (53) is the chief intestinal conjugase active in humans. Classified as glutamate carboxypeptidase II (EC 3.4.12.10) (GCPII) it is expressed in the jejunal brush border membrane and catalyzes the hydrolysis of folylpolyglutamates to the monoglutamate form (136-138). Pteroylhydrolase enzymes have been detected in many tissues in humans (139) and GCPII is found in the small intestine, prostate, brain, kidney and in tumor vasculature (140). GCPII is distinctive in its low pH optimum of 6.5 to 7.0 (141-142) and differs functionally and chromatographically from intracellular conjugases (59). It is not as abundant as intracellular folate hydrolase but is adequate for deconjugation of folates in the average diet (59), as the rate of folate

deconjugation is greater than the rate of absorption (139). GCPII is a zinc-activated exopeptidase, inhibited by certain drugs (salicylsulfapyridine, alcohol, diphenylhydantoin), and its activity is impaired at acid pH (2, 59, 136, 138, 143-146).

Deconjugases of the digestive system vary among animal species with respect to location and biochemistry (45, 147). In rodents and monkeys, significant conjugase activity is not found in the brush border membrane but rather in the soluble fraction of the lumen (44-45, 148). This activity is optimal at a pH of 4 to 5 (44, 148). Dogs show brush border conjugase activity (135, 149). Human and swine brush border conjugases are strikingly similar, and therefore the pig is considered the ideal model for human absorption of folate across the small intestine (44, 46, 142, 150-151). In pigs, and potentially in humans, pancreatic conjugase activity is also significant (152). It is stimulated by food consumption, shows an optimal pH of 4.5 and endo-random specificity: glutamates are hydrolyzed not from point of attachment to the pteroic acid moiety as with GCPII, but between glutamate residues (139). Pancreatic human conjugase has been suggested to potentially initiate deconjugation and to act in concert with brush border conjugase (139).

2.6.1.2 Intestinal transport mechanisms

2.6.1.2.1 Folate transport across the small intestine

Perhaps because it plays an essential role in basic cellular functions, there seems to be considerable redundancy in the mechanisms for folate uptake. There are three principal mechanisms for intestinal absorption. At high intraluminal concentrations (such as >10 μmol/L) a nonsaturable ion-mediated process of passive diffusion transports deconjugated folates through the enterocytes without modification, as a linear function of concentration (88, 139, 153). Reduced folate forms are transported more quickly than folic acid by this route (154). Passive diffusion may be the route of absorption of unreduced synthetic folic acid eaten

in excess of 266 μg as a bolus (123, 155-157). This mode of absorption is considered to play a minimal role in the intestine, because of the anionic, lipophobic nature of folates at physiological pH (158).

At lower luminal concentrations, but maximally between 10 and 20 μmol/L, monoglutamyl folates may be absorbed by active transport through the entire length of the small intestine (132). This process is saturable, pH- and energy dependent with an optimal pH between 5.0 and 6.0 (159). Carrier-mediated absorption occurs primarily in the proximal jejunum via a carrier protein that was long believed to be the reduced folate carrier (RFC). However the RFC functions at neutral pH while the luminal surface of the small intestine is an acidic environment. More recently mRNA for two genetically unrelated folate carrier proteins, the RFC and a proton-coupled folate transporter (PCFT), have both been shown to be expressed in the human intestine (160). Some of the characteristics of these two folate transporters are summarized in **Table 2.2**.

The PCFT, a high affinity folate transporter protein, is expressed along the length of the human intestine and in a number of other tissues, particularly in the small intestine, kidney, liver, placenta, retina and brain (161). Folate transport by PCFT is Na^+-independent and proton-coupled, has a low pH optimum and is electrogenic, adding to membrane potential as it co-transports >2 protons into the cell with each folate molecule (161-162). This transporter was previously thought to be a heme transporter and shows weak affinity for heme. Expression in cell culture is targeted to the apical membrane and therefore it is believed to act in luminal folate uptake (161). Along the intestine it is most highly expressed in the duodenum, less so in the jejunum, and at lower levels in the ileum, the cecum, segments of the colon and the rectum (162). The PCFT appears to be the main and perhaps the only active folate transporter in cultured human colon-derived Caco-2 cells (161-162). It shows high affinity for folates

including folic acid and the antifolate compound pemetrexed, and high stereospecificity for the 6S-natural folate steroisomers of 5-methyl- and 5-formyltetrahydrofolate, over their 6R-(synthetic) isomers (162-163). The PCFT functions optimally at low pH (5.5) but there is residual activity for 5-methyltetrahydrofolate transport as high as pH 7.4 (162).

In contrast to the PCFT, optimal activity for the RFC is around pH 7.4 with negligible activity below 6-6.5 (158). RFC transports reduced folates preferentially and displays higher affinity for 5-methyltetrahydrofolate, a very low affinity for folic acid, and shows a lack of sterospecificity (146, 153-154, 160, 164-171). Competitive inhibition for this carrier occurs between folic acid, 5-methyltetrahydrofolate, 5-formyltetrahydrofolate and several antifolates. The RFC, whose gene SLC19A1 is a member of the SLC 19 family of facilitative anion carriers, plays a fundamental role in tissue folate homeostasis: it is ubiquitously expressed in human tissues (160). Although it may function as an importer or an exporter of folate from cells, the RFC is expressed in the apical membrane along the length of the intestine, including the colon (163, 172). Expression is highest in the duodenum where the transporter has been localized on the upper half of the intestinal villus (160, 173). The RFC also transports thiamin phosphates and some other organic anions such as methotrexate, and is highly sensitive to the transmembrane gradient of phosphate anions which may be involved in the mechanism of folate transport (161).

Although both RFC and PCFT are expressed on the apical membrane of the enterocyte, though only the PCFT is thought to function at luminal pH. The transport of folates basolaterally is not well understood but it is thought to be mediated by a protein of the multidrugresistance-associated family of ATP-binding cassette exporters (158).

While the PCFT is believed to be the main mediator of folate absorption across the small intestine, some other folate binding proteins are also associated with intestinal expression

or transport. Members of the SLC21 family of carriers are expressed in the intestine and some transport folates (161). At least three other high affinity folate binding proteins, coded by three separate genes, are also expressed in humans. These are the folate receptors Fα and Fβ, proteins anchored on the cell surface by a glycosylphosphatidylinositol anchor and Fγ, a soluble receptor (174). These folate receptors (FRs) or folate binding proteins (FBPs) are not thought to be carriers in intestinal folate absorption but function rather at physiologic (nanomolar) concentrations in transferring folate across tissue cell membranes. FRs with a range of affiinities for reduced folates have been identified, specific to different mammalian tissues and fluids (165, 175-181). Soluble FBPs are thought to function to concentrate, store and protect folates from oxidation, while membrane-associated FBPs are involved in transport of folate between cells (146, 165, 177, 181). It has been suggested that FBPs modulate folate uptake in low folate extracellular folate milieus by concentrating folate due to their nanomolar affinities for the molecule, in the vicinity of folate transporters having micromolar affinities for folate (182). These proteins are not expressed in the intestine, but FBP in milk are thought to play a role in intestinal folate absorption by stabilizing folate during gastric passage (183-185). A number of folate binding proteins circulate folate (165, 186-189).

Other transporters of folate, such as the canalicular multispecific organic ion transporter, are also expressed in the small intestine of the rat and perhaps of other mammals, their role is unknown (190).

TABLE 2.2 A summary of some characteristics of two intestinal folate transport proteins, the reduced folate carrier (RFC) and the proton-coupled folate transporter (PCFT).

Criteria	RFC	PCFT
Intestinal Expression	Brush border membrane of jejunum, ileum, duodenum colon. More abundant in apical membranes of villus tip epithelium.	Duodenum, less so in jejunum, & at lower levels in the ileum, cecum, segments of the colon and rectum
Optimal pH	7.4	5.5
Substrate	Reduced folates, methotrexate. Poor affinity for folic acid. Also transports thiamine pyrophosphate, which competitively inhibits folate transport	Folates and methotrexate, no other organic anions. $THF>5CH_3THF>MTX>FA$. Inhibited by PABA
Gene locus	21q22.3	17q11.2
Other characteristics	No stereospecificity	Proton gradient across intestinal epithelium, protons co-transported with folate. NaCl not involved Stereospecificity for 6S- reduced folate isomers
K_m $5CH_3THF$	2-7 µM	1.67 µM

2.6.1.2.2 Folate absorption and transport across the colon

Absorption of colonic folate has been demonstrated clearly in two mammals: Rong *et al.* (1991) administered ^{3}H-labelled PABA to rats prevented from coprophagy, and showed that intestinally synthesized folates are bioavailable to the rat via the colon and are incorporated into liver and kidney (34). This was confirmed by Thoma *et al.* (2003) by the recovery of ^{3}H label in rat livers after cecal injection of rats with ^{3}H-labeled PABA (191).

Similarly, Asrar and O'Connor (2005) measured the absorption of folates across the colon of neonatal piglets (n=6), after cecal injection of ^{3}H-labelled folic acid, and ^{3}H-labelled PABA (35). Over 20% of the dose was found as folates in the liver, kidneys, feces and urine of the piglets within days. Tritiated folic acid was also shown to appear in the hepatic portal vein of 30 kg female pigs within minutes of infusion into the cecum with absorption of 10 % in 3.5 to 4 hours (133). However, *in vivo* data is lacking for human colonic folate absorption.

Mechanisms for absorption across the colon are not as well understood as for the small intestine but animal studies suggest it may be similar (5). Evidence for human colonic absorption is only available from *in vitro* studies. Studies of organ-cultured biopsy samples of human cecal and sigmoid colon mucosa found carrier-mediated uptake of folic acid (36). Methotrexate inhibits absorption in the cecal mucosa at pH 5.5 (36). A normal human colonic cell line (NCM460) was also shown to express a carrier-mediated absorption system, which was both pH dependent and regulated by a protein tyrosine kinase and cAMP-mediated pathway (37).

A carrier-mediated, pH dependent, anion exchange inhibitor-sensitive electroneutral process was investigated in apical membrane vesicles prepared from human colonic mucosal scrapings (134). The carrier showed relatively low affinity for folate ($K_m \sim 8$ μM) but a higher maximum velocity than in the small intestine. This was highest in the proximal colon

(V_{max} =19.8 pmol.mg^{-1}.s^{-1}) and slightly lower in the distal colon and implies the potential capacity for transport may be higher by 3 to 4 times than in the small intestine. Studies of basolateral membrane vesicles found that the RFC was expressed in these membranes as it also was in jejunal basolateral membrane. The highest level of expression was found in jejunal basolateral membrane, followed by colonic apical membranes, with lowest expression in colonic basolateral membrane (38). Colonic or jejunal apical membrane preparations also showed higher maximum velocity than did the colonic basolateral membrane (38).

As noted in the Section 2.6.1.2.1, more recent work on colon-derived cultured cells suggests the RFC is expressed only in the apical membrane of enterocytes (158). The PCFT, though active *in vitro* in colon-derived Caco-2 cells, is expressed in the colon to a lesser extent than in the small intestine, liver and kidneys (162). In mice, inactivation (+/-) of the RFC increases sensitivity to colon carcinogenesis (192). This may suggest that at least in the mouse, the RFC plays a role in folate supply to the colon. However, inactivation (+/-) of murine folate binding protein reduced colonic mucosal concentrations (192). RFC inactivation caused changes in folate homeostasis, particularly in the form of a reduction in plasma S-adenosylmethionine and S-adenosylhomocysteine (192). Thus inactivation of potential colonic absorption mechanisms also affects mouse body folate status. The determination of which transporter is active may be determined by local luminal pH, which varies greatly along the length of the colon (section 2.9) (158).

2.6.1.3 The regulation of intestinal folate absorption

In rats, dietary folate deficiency upregulates RFC expression at the level of mRNA, as shown by experiments with *in vitro* studies with jejunal everted sacs (193). Absorption via the RFC may be mediated via endocytosis (194). Production of this transporter is also regulated ontogenically in the rat intestine, and its expression follows the pattern of folate uptake (195-

196). In mice, four exons and promoters have been described and intestinal expression responds to changes in dietary folate intake (197). The human RFC is regulated posttranscriptionally as well as transcriptionally and transcripts are highly tissue specific (161, 198). Folate deficiency in rats was found to upregulate intestinal trans-epithelial folate transport, via an increase in V_{max} of the transport process, but with no change in the K_m, suggesting an increase in the number or activity of RFC molecules rather than in their affinity for folates (193). Steady-state increases in RFC mRNA indicate that the process is regulated at the transcription level. Increases in RFC mRNA were observed in both jejunum and ileum, as well as in the colon even though the RFC is not usually thought to be involved in folate uptake across the small intestine (193). Cell culture of human-derived intestinal Caco-2 cells in media over-supplemented with folate showed that long-term oversupplementation leads to downregulation of absorption (199).

2.6.1.4 Folate first pass metabolism

Once absorbed by the enterocyte, folates may be retained and function as coenzymes in mucosal metabolism, or are transported basolaterally to the circulation. Since the main circulating form of folate is 5-methyltetrahydrofolate, other reduced food- or potentially, microbially-derived folates are converted to this form in the enterocyte (200). In the rat, folates, including folic acid, once absorbed in the enterocyte are reduced to tetrahydrofolate and either methylated or formylated in the mucosa (164, 169, 201-203). In humans, reduction and metabolism to 5-methyltetrahydrofolate of folic acid is thought to occur in the liver (204-205). Reduction is easily saturated, and folic acid appears in human plasma and urine after oral folic acid ingestion of 200 to 260µg (123, 155, 157). About 10 to 20% of the folate joining the portal circulation from the mucosa either enters the liver on the first pass or is distributed to

other tissues (159). From the liver, folate is secreted in the bile primarily as 5-methyltetrahydrofolate and enters the hepatic circulation (206-208).

2.6.2 Bioavailability

2.6.2.1 Factors affecting bioavailability and its measurement

"Bioavailability" refers to the overall efficacy of utilization of a nutrient, be it supplied to the organism via absorption in the GI tract from food sources or through production by the microflora, or via clinical procedures. Both the amount and the form of a nutrient in food affect bioavailability. Efficacy of utilization depends on the efficacy of absorption as well as on all the other processes involved in its utilization such as transport, metabolism, and the kinetics of its catabolism, excretion and retention (88, 139). These processes may vary among individuals and may be complicated by such factors as dose-dependence, the effect of other dietary components on enterohepatic circulation, and by the activities of the intestinal microflora which may enhance the supply of a nutrient or hinder its availability for absorption, effects also potentially influenced by other dietary components (139). All these factors may combine to determine folate bioavailability.

Studies of folate bioavailability may focus on the proportion of folate absorbed from individual food items (short-term availability), or on the effects of an individual's dietary habits (longer term feeding studies). The primary indicator of long-term folate status is considered to be erythrocyte folate, which incorporates information on the availability of folate ingested during the average lifetime, 120 days, of the erythrocyte population in the bloodstream of an individual (1). Erythrocyte folate levels normally reflect tissue folate stores and are weakly correlated to liver folate (1, 209). In contrast, fasting plasma folate is responsive to recent folate intakes but repeated plasma sampling is also used to assess status. Subsequent to ingestion of a folate dose, changes in plasma folate (over several hours) or urinary excretion

levels (1-2 days) have chiefly been used to evaluate folate bioavailability from specific sources (210-213).

Single low-dose short term protocols address the kinetics of initial folate absorption and its metabolism and transport, rather than long-term changes in folate status (1, 88). A low folate dose more closely approximates the normal uptake of food folates. However, single low-dose determinations present a number of complications which must be considered and are described below. The response measured in terms of elevation of folate in plasma or urine is correspondingly also low, and therefore difficult to measure with accuracy and precision. Plasma response to low folate intakes is also highly variable (204).

Responses to high-dose protocols are greater and less variable, but at higher concentrations, absorption is likely to be mediated by passive diffusion rather than through active transport as with natural folates, and thus distribution and metabolism of higher doses may not reflect folate uptake at physiological concentrations. In order to enhance excretion of ingested doses, some study protocols monitoring urinary folates and folate decomposition products, include several weeks of folate supplementation for metabolic saturation of subjects, pre-dose (214-217). Results from such protocols do not represent normal physiological conditions and would not necessarily reflect normal physiological events. Moreover, *in vitro* studies of human intestinal and renal epithelial cells cultured at high levels of folate have shown that this down-regulates folate absorption (199). Expression of protein and mRNA levels of the RFC, as well as the PCFT and FR, decreases compared to cells cultured at maintenance levels of folate (199). It follows that bioavailability, and dose response, in terms of active uptake of folate, depend also on nutritional status.

Fluctuations in total plasma folates are sensitive to recent folate intakes, and a bolus dose of folate absorbed across the small intestine contributes to a rise of several hours duration

in the concentration of plasma folate. The rate of increase of plasma folate, the maximum concentration reached, the time to reach maximum, and the area-under the curve (AUC), have been used to describe this process and assess the amount of folate absorbed (204, 218). However, changes in total plasma folate after single physiological doses are small and highly variable (204). This may be partly due to displacement of tissue folates to the plasma upon absorption of the test dose (204, 219). These factors hampered the interpretation of results of single physiological folate dose studies difficult unless subjects were exposed to radioactive tracers, until the development of stable isotope labeling and Tandem mass spectrometry techniques.

To standardize the influence of diet, fasting is generally required of subjects before ingestion of the test dose. However, large amounts of folate are recirculated enterohepatically via the bile, which complicates measurement of fasting plasma folate. Fasting has been shown to interrupt enterohepatic circulation so that plasma folate levels rise as fasting continues (218, 220). Oral ingestion of a test dose reverses this effect, and once the absorbed folate dose reaches the systemic circulation, it raises plasma or serum folate proportionally.

In rats both folic acid and its reduced, carbon-substituted forms such as 5-formyl-tetrahydrofolate, are metabolized to 5-methyltetrahydrofolate in the intestinal mucosa (221). This was believed also to be so in humans and therefore folate bioavailability was thought convenient to express as relative to the bioavailability of a synthetic folic acid standard, consumed in fasting conditions. However some variability in human kinetic intestinal transport constants has been found for different monoglutamyl folates (166, 169, 204-205, 222-223). In humans delays in the appearance of 5-methyltetrahydrofolates after intestinal absorption of stable-isotope labeled synthetic folic acid were found to be longer than delays after ingestion of labeled 5-formyltetrahydrofolate (204-205, 223). This difference is now accepted to arise

because reduced folates are metabolized in the intestinal epithelium, once absorbed, but synthetic oxidized folic acid is reduced and metabolized in the liver (139, 224-225). Reduced absorbed folates such as 5-formyltetrahydrofolate are rearranged to 5-methyltetrahdyrofolate in the intestinal mucosa and subsequently transported to the systemic circulation (200). Metabolism of folic acid to 5-methyltetrahydrofolate takes place after transport via the hepatic portal vein to the liver (204-205).

2.6.2.2 The chemical forms of folate; inhibitors of conjugases or of absorption; folate destruction by processing

Only mono- and diglutamate forms of folate are absorbed across the small intestine, therefore polyglutamyl folate absorption depends on the efficiency of deconjugation. Incomplete deconjugation would limit the bioavailability of food folates, but this may not especially impact folate bioavailability on a mixed diet. Some early studies in human subjects found polyglutamyl folates were absorbed 50 to 80% as well as monoglutamyl folates (138, 211, 217, 226-231). However, differences may be due to differences in the metabolism of reduced natural folates and synthetic folic acid usually used as the monoglutamate form. Two studies did report equal utilization by humans of folic acid and polyglutamyl folate (210, 232). Factors that affect brush border conjugase necessarily affect polyglutamyl folate bioavailability. These include factors affecting the pH of the mucosal surface, which also then affect the rate of absorption. A slightly acidic pH allows highest bioavailability; highly acidic foods such as orange juice taken alone may lower folate bioavailability (233-234). Brush border conjugase may be inhibited *in vitro* by factors present in various foods or drugs that therefore could negatively affect the bioavailability of polyglutamyl folate (139, 235). Studies of legumes as sources of folates have shown the presence of a heat-activated inhibitor of several folate conjugases in the seed skin (236-237). Beans show an inhibitory effect specific

to absorption, shared by orange juice, pectin, blanched cabbage, wheat bran (139). Although yeast contains high amounts of folate, the concentrations found are greater than can be efficiently deconjugated and absorbed. Conjugase inhibitors are also present in yeast (139, 232, 238-239). Nevertheless, little impact is considered likely on a healthy mixed diet.

Reduction and carbon substitution of folates may affect the rate of folate metabolism and appearance in the systemic circulation (see section 2.1.8.1, above).

Naturally occurring, reduced folates are less stable than folic acid, and are likely to be cleaved into inactive fragments on oxidation (53). Plasma folate may be degraded *in vivo* by exposure to sunlight (240). Folates may be destroyed by thermal processing of foods industrially and in cooking (89), though the fraction remaining after such thermal losses has been found to be stable to heating (139, 241). Some folates are susceptible to destruction in passage through the extremes of gastric pH (88).

2.6.2.3 Interaction with food and intestinal contents

The bioavailability of endogenous folate in many foods is incomplete, for a number of possible reasons. The absorption of folate depends on its solubility. Endogenous folate may be bound in undissolved, insoluble or incompletely-digested food, preventing access to the mucosa (139, 242). Folate bioavailability in women was studied by Sauberlich *et al.*, 1987, who found the bioavailability of food folate of a mixed diet to be ≤50% of folic acid. The bioavailability of folic acid added to cereal-based foods was found by some to be similar to endogenous (216, 243). However folic acid taken with a light breakfast was about 85% as available as synthetic folic acid taken alone (1), perhaps because of adsorption to components of the food ingested with it hindering access to the intestinal wall for uptake.

Folate in the form of tablet supplements of folic acid is highly bioavailable but this may be limited by incomplete dissolution of the tablets leading to passage of undissolved tablets

with the feces (88). In considering the fortification of foods with folate, dietary reference intakes (DRIs) have been defined which take into consideration the effect of the chemical form of folate on efficacy of absorption (1). The DRIs are expressed as dietary folate equivalents (DFE), the mass (µg) of naturally occurring food folate plus 1.7 times the mass (µg) of synthetic folic acid. This definition is based on the ratio, 1.7, of the mean bioavailability of synthetic folate in food (85%) to the mean bioavailability of dietary folate (50%) (1, 88).

Most folate in milk is bound to soluble and particulate folate binding proteins (FBPs), found in milk of all species (165, 187-189). These proteins appear to function to concentrate and transport folate during milk secretion (244-245) , to shield folates from microbial uptake in the intestine (246), and to preserve them for absorption. They have been shown to enhance absorption by interaction with the mucosa *in vitro* both in isolated rat mucosal cells and in goat brush border membrane vesicles (247-248). Deconjugation is not impaired (152). FBP-bound folate is more gradually absorbed, increasing its availability to sucklings (249). In the rat, protein-bound folate is absorbed without dissociation, via a separate route than free folate (250). Human milk and milk-based infant formula have been shown to provide greater bioavailability of folate than other infant foods (251). Commercial milk pasteurization appears to partially denature the proteins and removes their ability to enhance absorption (252). Whereas folates in milk are shielded from microbial absorption, milk, particularly human milk, also appears to enhance the bioavailability of microbially-produced folates in the rat by enhancing the growth of folate-producing intestinal microbes (40, 253-254).

2.7 FOLATE AND HEALTH

2.7.1 Health and Folate Deficiency

Because of the central role of folate in cellular metabolism, an adequate supply of folate is crucial to the health of all mammals. This is critical in tissues with high rates of cell division

or metabolism, and at times of high anabolic activity, such as in pregnancy and fetal development, in lactation and childhood development, when folate needs are increased. The signs of inadequate intake appear gradually and are first seen in the blood, as decreases in the concentration of plasma and erythrocyte folate, and a rise in plasma total homocysteine. As sufficient folate is not available for DNA synthesis, megaloblastic changes appear in the bone marrow and other tissues with high rates of cell division, and within weeks, anemia develops which is characterized by weakness, irritability and difficulty concentrating (1).

Suboptimal folate intake is associated with a wide spectrum of health risks, including an elevated risk of neural tube defects, cleft lip and palate and other developmental disorders in offspring of women with low folate status, and preeclampsia and anemia in pregnancy, neuropsychiatric disorders increased risk of chronic degenerative disease, and stroke (2, 255-256), and a number of cancers, including neuroblastoma and leukemia and cancer of the breast, cervix, lung, pancreas, and in the GI tract, cancers of the esophagus and stomach and colorectal cancers (23, 257-259). Perturbance of folate metabolism and absorption by allelic variations in genes coding for folate dependent also influences the effects of lower folate intake (2).

2.7.2 Folate Overnutrition

Since folate is a water-soluble nutrient, it was long assumed that excess folates could be excreted in urine, and the focus of concern in high folate intake was the possibility of masking the symptoms of vitamin B12 deficiency, increasing the risk of neurological damage (1). Since fortification of the North American food supply with folic acid in 1998, blood folate levels have risen dramatically, especially in supplement takers, and while concern regarding inadequate intake of vitamin remains, further serious health risks associated with high folate intake have come to light (260). These risks include the possibility of reducing the effectiveness of antifolate drugs, prescribed for malaria, rheumatoid diseases, psoriasis and

cancer, of reducing immunity through reduction of natural killer cell cytotoxicity, accelerated cognitive decline among the older population, and in pregnant women, an increased incidence of obesity and insulin resistance in their offspring (11-15, 18-19). High folic acid intakes have been shown to facilitate the progression of preneoplastic cells to cancer in animals (17). These observations are supported by results from human intervention and epidemiologic studies of folate and cancers, particularly colon cancer (17, 261-263).

The potential mechanisms for cancer induction by folate depletion relate to the role of folate in synthesis of purines and thymidylate for DNA repair, and as donor in the methylation of DNA, but also include induced choline deficiency, impairment of natural immunity, faulty DNA synthesis and influences on viral mechanisms of carcinogenesis (257, 264-266). While the same routes may be functional in folate overnutrition, which may perturb folate metabolism by such possible mechanisms as saturation of receptor sites, efflux transporters and enzyme binding sites, the induction of relative deficiency of other essential micronutrients in folate metabolism (vitamins B12 and B6), and in potentially overwhelming regulatory mechanisms that evolved in a low folate environment, current evidence suggests that over supplementation of folate particularly folic acid, is associated with promoting the recurrence or progression of neoplasms (17-18, 257-258).

2.7.3 Folate and the colonic mucosa

Meenan *et al.* (1996) pointed out that colonocytes are continually exposed to a high folate luminal environment, and suggested colonic absorption as an explanation (267). They determined folate concentrations in colonocytes isolated from human biopsy samples and suspended in saline solution, and found folate concentrations to vary along the length of colon, with cells of cecal origin having lower folate concentrations than those of the sigmoid colon (267). However, they did not assess the association between the folate concentration in the

intestinal lumen with that of the colonocyte. It has been shown that colonic mucosal folate concentrations correlate well with blood folate indices in patients with adenomatous polyps (24) and both dietary folate intake and blood folate indices have been associated with an increased risk of colorectal cancer and adenoma (268-270). Kim *et al.* (2001) reported that blood folate concentrations, either as serum folate or erythrocyte folate, correlate well with folate concentrations in snap-frozen human colon biopsy samples, in subjects with physiological levels of folate intake (269). However, this relationship breaks down at the supraphysiological levels of intake achieved by taking supplements (269). These authors pointed out the special environment of the colonocyte in being exposed to high folate both luminally and systemically through the circulation, and the lack of knowledge of how this affects colonocyte folate levels and indeed what an appropriate concentration is for the colon. The finding that localized tissue folate deficiencies in buccal cells are associated with smoking, a destructive local environment, supports the potential importance of the luminal environment in colon health and folate absorption (20-21, 271). Overnutrition also affects colorectal cancer or its precursor, adenoma, a pattern supporting the dual model of folate in cancer: supplement takers show a higher rate of recurrence of adenomatous polyps than those taking no supplements (261). Little information is available for human colonic folate uptake. Early in vitro studies on absorption mechanisms of human derived cultures showed that at pH 7.5 folate absorption in the mucosa of the colon proceeds via a low-affinity carrier apparently by facilitated diffusion (36). However, mucosal accumulation of folate is higher at pH 5.5 than 7.5 with significant regional variation, accumulation being highest in cultures derived from the sigmoid colon (36).

2.7.4 High fibre diet and colorectal cancer

The relationship of dietary fibre with colorectal cancer has been studied extensively. Some epidemiological studies have suggested an inverse relationship between fibre intake and the risk of CRC (272-273). The association between consumption of fruits and vegetables and colorectal cancer in particular has been studied extensively with varied results. A recent prospective study examined this relationship in a European population and concluded that fruit and vegetable consumption was indeed inversely associated with an increased risk of colorectal cancer, especially colon cancer, but only in non-smokers, while the opposite was true in smoker (274). Given the dual relationship between folate and cancer, particularly colorectal cancer, it is notable that fruit and vegetables are prominent among folate-containing items in the North American diet, and that they are also natural sources of soluble fibre including some oligosaccharides considered to be prebiotics. It is enticing to suppose that this forms a link between fruit and vegetable consumption, microbial folate production and improved folate status and suggests that some of the benefit of fruit and vegetable consumption lies in the provision of another source of folate to the colon. Plant foods contain a broad diversity of components designated as fibre, with varied physical and chemical properties and biological effects, outlined below, which may contribute to the relationship between fruit and vegetable consumption and intestinal health.

2.7.5 Dietary fibre

Fibre is the name given to plant materials not digestible by human small intestinal enzymes, and in the past was therefore thought to be a non-nutrient component of foods. These plant materials may be divided into various classes on the basis of physical form and physiological effects and are composed mainly of the water-insoluble celluloses, hemicelluloses and lignins and the water-soluble pectins, gums and mucilage (275). **Table 2.3**

gives an example of such a classification of dietary fibre. Most plant foods contain both water soluble and insoluble fibre and while insoluble fibre contributes to fecal bulk, soluble fibres have important metabolic effects, determined by their viscosity, on rates of glucose absorption and on the enterohepatic circulation of bile acids (275). Fibre and other incompletely digested food components reach the colon where they may be fermented by the colonic microflora, whose metabolic products are available for absorption by the mucosa. The ingestion of dietary fibre has diverse long-term effects, on the induction of digestive enzymes, cholesterol absorption, on intestinal morphology, and on intestinal motility (275). Some effects are mediated by physical properties of the fibre, while others are related to microbial activity. The components of dietary fibre provide both fermentable substrate and the physical environment for the intestinal microflora and influence its composition (276-277).

Complex plant polysaccharides may be more slowly digested or undigestible by human digestive enzymes, and may therefore reach the colon substantially intact, with the potential to bind nutrients like folates, hindering their absorption at the level of the small intestine. Although endogenous folate of some plant-derived foods is only partially available, dietary fibre does not appear to substantially reduce the bioavailability of folates. Equivalent folate bioavailability was seen in comparing diets containing different dietary fibres in chicks (241), and in rats (278). *In vitro* equilibrium dialysis studies with several fibre types showed no evidence of folate binding (241). Brush border conjugase requires Zn^{2+}, and its activity may decrease *in vivo* due to zinc chelation by ionic polysaccharide (89), but several forms of fibre tested *in vitro* were reported not to inhibit conjugase activity (150, 152).

TABLE 2.3 A classification of some dietary fibres (adapted from Southgate (279)).

Fibre	Solubility	Class of Polysaccharide
α-Cellulose	Water , alkali soluble	Cellulose (glucan)
Hemicelluloses	Water insoluble	Arabinoxylans. Galactomannans
	Soluble in alkali	Xyloglucans
Lignins	Insoluble in 12M H_2SO_4	Polyphenolpropane Non-carbohydrate
Pectic substances	Water soluble	Galactouronans Arabinogalactans β-glucans Arabinoxylans
Gums	Water soluble or dispersible	Galactomannans Arabinogalactans
Mucillages	Water soluble or dispersible	Many branched and substituted galactans

Some negative effects on folate bioavailability have been reported. Some forms of fibre inhibit the absorption of polyglutamyl and monoglutamyl folates in rats, and some insoluble digesta of food have been shown to bind folates slightly (280). Wheat bran has been shown in several studies to affect folate bioavailability. Wheat bran and pectin were shown in rats on hydrated diets to slow the uptake of polyglutamyl and monoglutamyl folates (120). Wheat bran slowed absorption in rats, of added heptaglutamyl folate compared to folic acid absorption (281-282), and of polyglutamyl folates in humans, although the rate of absorption of folic acid

was unaffected (210). These effects may be due to the fibre slowing diffusion of folate and impeding its access to the mucosa, and to brush border conjugase (139, 242). The relationship of fibre to the availability of microbially-produced folate is discussed below.

2.8 THE COLONIC ENVIRONMENT

2.8.1 The human colon

The human colon is about 1.5 m long with a surface area of $0.3 m^2$ (283). It contains the undigested residues of ingested foods and supplements, unabsorbed bile, mucin and sloughed intestinal cellular debris, and the microorganisms that digest and metabolize them. It is a site of absorption of water, electrolytes and of the products of bacterial metabolism. About 4 L of water are absorbed daily chiefly in the proximal and transverse colon though this can be delayed in the presence of slowly fermenting water-holding fibre which may increase water content of the distal colon (283). While the area available for absorption is smaller than in the small intestine (0.3 vs. 120 m^2) the residence time of the colon contents is much longer, two to three days (283). The consecutive sections of the colon, the cecum, the ascending, transverse and descending colon, rectum and anal canal differ in their anatomy, neurology, blood supply and absorption characteristics (283). The cecum and proximal colon are fermentation vessels with a highly active bacterial metabolism and a higher absorptive capacity than the distal colon (283). While some absorption takes place in the distal colon, the luminal contents are semisolid and access to the mucosa may determine whether a metabolite is absorbed. The mucosal surface of the colon changes with age. At birth, it resembles the small intestine. Loss of villi soon leave this surface flat with deep crypts, with a decreasing number of non-goblet crypts and more fecal water with aging (283).

The mean luminal pH of the ileum of normal subjects is 7.5 but the pH drops sharply in the cecum. Lewis and Heaton measured the pH of the proximal colon at 5.21. The pH rises gradually along the length of the colon, to 6.6-6.8 mid colon, and 7.04-7.14 in the distal colon (284-285). As in the small intestine, the microclimate at the mucosal surface is more acidic than the pH of the bulk of colon contents. A pH of 5.8-6 in a zone ~ 840μ thick is maintained by the secretion of protons due to the activity of membrane carbonic anhydrase (286-289). This buffers luminal pH, for example, when the luminal pH is 7.51 and 5.96, the surface microclimate is lowered to 6.26 and 6.8, respectively (286). The acidity of this zone is also promoted by the products of bacterial metabolism: short chain fatty acids stimulate the mucosal secretion of HCO_3^- (290). This effect of short chain fatty acids on the surface pH of the colon seems likely to facilitate the transport of microbial folates via the PCFT.

The residence time of luminal contents in the colon is controlled by motility patterns in the contractions of the colonic wall, and is increased by such factors as awakening from sleep, the size of a meal, diet and stress (283). The pattern of transit times through the sections of the colon varies with individuals and by area: full passage can take 3-5 days, but monitoring a small liquid bolus injected into the colon showed the proximal colon emptied within 90 minutes, while in the transverse colon transit was extremely slow (283, 291). Low residue diets are associated with slower transit through the GI tract while a high fibre diet increases stool output, and if fermented, shortens transit time (283).

The relationship between the pH of intestinal contents and diet has also been studied: intake of some types of high fibre diets are associated with lower fecal and intestinal pH (292). Lewis and Heaton (1997) have shown that a high fibre diet and the consequently accelerated whole gut transit time is associated with decreased pH in the distal colon, and suggested that unabsorbed butyrate produced by the microflora contributed to decreasing pH (285).

2.8.2 The colonic microflora

The normal colonic microflora has been compared to the liver in its metabolic activity and diversity. In humans, this bacterial population is made up of about 400 species of which about 40 are found commonly (293). Sixty to seventy percent of the feces, or 15-20% dry weight, are made up of bacteria (294). About twenty bacterial species predominate (295), maintaining a relatively stable composition over time, but variable between individuals (295). The great majority of the bacteria are obligate anaerobes, the most prevalent among these being the bacteroidaceae (296). After obligate anaerobes, the next most prevalent groups are eubacteria and anaerobic lactobacilli, followed by peptococcaceae, and after these, bifidobacteria. Bifidobacteria are present with an average number of 10^{10} per gram of wet feces (296).

This microflora is able to ferment carbohydrates and protein, and metabolizes many other substances including bile acids, steroid hormones, fats and drugs (297-298). The chief substrates for the endogenous flora are fibre, unabsorbed sugars and oligosaccharides, and starch that has escaped digestion in the small intestine (299). Accordingly, the flora may be altered by diet, as has been shown, for example, in humans, rats and pigs (276, 278, 300-305). Both the bacterial load and the profile of intestinal bacterial species may be altered by diet (276, 304).

The distribution of the microflora in the human gut is determined by stomach acidity and the propulsive motor activity of the small intestine, which keep the upper parts of the GI tract almost sterile (298). The flora of the upper part of the GI tract is determined largely by microbial food contaminants and tends to be transient. In the distal small intestine the microflora increase reaching 10^{11} microorganisms per gram wet weight of contents in the colon, with some increase in weight towards the distal colon (295). In disease, and in

developing countries, greater numbers of bacteria are found in the human small intestine than among healthy humans of industrialized nations, perhaps due to greater incidence of malnutrition and diarrhea (298) or a greater microbial contamination of food and water (293).

At least two ecosystems exist in the lower GI tract: the luminal flora and the mucosal flora (306). The composition and activity of the luminal flora is mainly determined by available nutrients and by antimicrobial substances entering the gut. That of the mucosal flora is influenced by the expression of specific adhesion sites on the enterocyte membrane, which promote adhesion and survival of specific organisms. The local chemical environment also influences the survival of specific microorganisms. This environment is determined by the rate of mucus production and the production of secretory immune globulin, by cellular material from the enterocyte membrane, present in the mucus which may provide nutrients for this microflora, as well as by lumen contents (306).

2.8.3 Development of the human colonic microflora

Diet has a strong influence on the initial colonization of the human gut. Coliforms, staphylococci, and streptococci appear within days of birth (307). Their growth reduces the redox potential (Eh) leading to colonization by anaerobes such as bifidobacteria, bacteroides and eubacteria (307). As many as 400 different bacterial are found in human intestinal contents, with at least 20 different genera represented in the human colon (293). Viable bacterial cell concentrations in human intestinal contents range in concentration from 10^{10}-10^{12}/g, the vast majority being anaerobes (308). The most dominant genus is Bacteroides, followed by Bifidobacteria (308).

Bifidobacteria are non motile, gram-positive bacteria, natural inhabitants of the gut of warm blooded animals and man (309). They play a significant role in controlling the pH of the colon through the production of lactic and acetic acid, and are found in colonic contents at

concentrations of 10^8 - 10^{11} colony-forming units/g (309). This restricts the growth of many pathogens and putrefactive bacteria. Bifidobacteria appear to have anticarcinogenic and anticholesterolemic properties (310). While Mitsuoka, 1982, observed low total counts in the small intestine of humans, increasing from 10^4 to 10^6 towards distal end of the small intestine, bifidobacteria inhabit principally the colon (310).

Bifidobacterium bifidum, *B. infantis*, *B. breve*, *B. longum*, *B. angulatum*, *B. catenulatum* and *B. pseudocatenulatum* are found in humans (311). *B. longum* is the most prevalent species in adults (312). High numbers of bifidobacteria are found in both the adult and infant colon (311) but different species predominate (307, 313). Bifidobacteria distribution is highest in the cecum, from birth (310). In infants, bifidobacteria are the largest group, outnumbering others 100-fold, but shift to the third largest in adults (313). Their dominance in the infant gut is thought to be due to factors in breast milk (307, 314): n-acetyl glucosamine, glucose, galactose and fucose and oligosaccharides (40, 315). The low protein content and reduced buffering capacity and the presence of lactoferrins in human milk are believed to facilitate increased bacterial growth (316-319). As infants mature, *B. infantis* and *B. breve* are replaced by *B. adolescens* and *B. longum* (313). The colonic flora is relatively stable in adults, though the bifidobacterial count may be reduced among the elderly (307, 320).

Bifidobacteria and some other intestinal flora can metabolize carbohydrates such as oligosfructose and fructo- and galactooligosaccharides (28-29, 313, 321-324). These oligosaccharides appear to be effective bifidogenic substances (311). Bovine casein digest and yeast extract are also reported to consistently promote the growth of bifidobacteria but the oligosaccharides are more selectively used by them (311, 325). In theory, bifidobacteria produce acetic and lactic acid in molar ratios of 3:2, but deviations from this ratio are found and acetate, formic acid and ethanol may be produced (313). Bifidobacteria are among known

folate producers, some producing folate at up to 100 ng/mL (326). Folate production by the microflora is reviewed in Section 2.10.3, below.

2.8.4 The intestinal microflora of the pig

The intestinal microflora differ between different species (310). In humans, monkeys and guinea pigs, bifidobacteria outnumber lactobacilli. In pigs and some other species however, lactobacilli outnumber bifidobacteria, though bifidobacteria are present in almost all animals (310). Enumeration studies of bifidobacteria in nursing piglets demonstrated that their populations were variable and constituted less than 1% of the total bacterial population. However, this population is quite diverse. *B. boum, B. choerinum, B. globosum, B. minimum, B. pseudolongum, B. suis, and B. thermophylum* are among bifidobacteria species reported in pigs (327-329). The porcine cecum and its epithelial layer contain fifteen distinct strains of bifidobacteria (329).

The most numerous bacterial species in the normal pig cecum is *Bacteroides ruminicola*; Selenomonas, Butyrivibrio, Lactobacilli, Peptostreptococcus and Eubacterium are also present. The predominant Bifidobacterium species is *B. pseudolongum* (Type A) (309). Bacteria are found along the entire length of the GI tract of pigs (the stomach, small intestine, cecum and colon). A stratified squamous epithelium lines the *pars oesophagea* of the stomach (293). To this surface, lactobacilli and certain streptococci can adhere forming a bacterial layer (293). Epithelium-associated lactic acid bacteria are not found in humans as only the distal anal canal has a stratified squamous epithelium (293). In pigs, the ileum is also colonized by bacteria to a greater extent than in humans (up to 10^9 colony forming units (CFU/g intestinal contents) in pigs compared to 10^6 in humans) (330). In the pig colon bacterial cell concentrations are $>10^{10}$/g of contents (331). Oligosaccharide fermentation in pigs occurs in the small intestine as well as the cecum and colon and has been shown to affect villus height and

crypt depth of the distal small intestine (332). Reports in the literature conflict: some consider that inulin may be 20-30% fermented in the upper GI tract of pigs, versus 10% in humans (330, 333), however Yasuda *et al*. (2007) examined digesta from the upper and lower jejunum, the cecum and the proximal, mid and distal colon of weanling pigs fed inulin and concluded that the cecum was the main site of degradation, sufficiently so to support the pig as a model for human studies of inulin digestion (334).

While bacterial colonization affects the whole gastrointestinal tract, transit time varies greatly, affecting the degree of fermentation in each section. Both intestinal fluids and small particles move quickly through the pig stomach though larger particles can be retained there for long periods and appear to be digested microbially (335). Digesta move relatively rapidly through the small intestine and cecum but colon transit times are prolonged and production of volatile fatty acids suggests the highest rates of fermentation occur in the pig colon (335). Transit times for pellets in the colon of suckling piglets have been measured at 9.5 to 30.5 hours, but this time increases once the piglets are no longer on a liquid diet (336).

In pigs, weaning affects the profile of microflora in the jejunum, ileum and cecum (337). Enumeration of piglet jejuna, ileal and cecal contents for *E. coli*, lactobacilli, bifidobacteria and total anaerobes showed that weaned piglets had lower lactobacilli populations than their nursing littermates, and that *E.coli* and lactobacilli populations decline with weaning in the small intestine and cecum while fecal bifidobacteria populations increased (337). Bifidobacteria were significantly more numerous, and volatile fatty acid concentrations higher, in the cecum than in the ileum or jejunum of piglets (337).

Piglets have been used to model areas of human nutrition and gastrointestinal physiology including colonic fermentation (50, 338-341). The piglet colon has a larger fractional volume, due mainly to length rather than circumference, compared to that of humans

(342). This is especially evident in the size of the piglet cecum, which has a length ~3.6 times, but a volume about 1.5-2.2 times that of a human cecum (343). While diet is not greatly influenced in suckling piglets, in growing pigs, this requires a greater proportion of dietary fibre than in humans, which should have the effect of increased short chain fatty acid production (342). In spite of these differences, they are considered the premier model in this area, over rats, because of rat coprophagy which is targeted in rats on cecally-fermented feces, affecting the digestion of resistant starch in rats and greatly affecting the production of short chain fatty acids (342).

2.8.5 The Intestinal biosynthesis of folate

Many microbial species synthesize folate *de novo* via the condensation of *p*-aminobenzoic acid (PABA) with dihydropterin pyrophosphate, a pathway requiring at least three, and up to nine genes unique to microbial cells (64, 344-346). Sulfonamide antibacterial drugs block microbial folate synthesis by preventing this condensation reaction (345). A number of bacterial species in the intestinal tracts of birds and mammals synthesize folate (65-67, 69-71, 347). These include some Lactobacillus species, *E. coli* and some Clostridium, Streptococcus and Enterococcus species (348), and prominently, as mentioned above, some Bifidobacterium species (30, 349). Folate is also consumed by bacteria, for example lactobacilli in yoghurt cultures have been shown to deplete folate in skim milk (348).

Bacterially produced folates may be released by bacteria, or accumulated intracellularly, both among folate producers and consumers. Bacteria may also lyse during passage through the intestinal tract and thus release folates and folate conjugases, aiding absorption of folates by the host or other bacteria (326). Investigation of bacterial folate production for commercial application has shown that folates released by viable bacteria in culture carry short polyglutamate tails of 1 to 3 units, and would therefore be potentially bioavailable (350).

Cultures of *L. casei* were found to release mono and diglutamated 10-formyltetrahydrofolates, the monoglutamated form being more rapidly released to the medium (351). Cultures of *Streptococcus thermophilus* were found to extensively excrete folates, chiefly 5-formyltetrahydrofolates and 5,10-methenyltetrahydrofolates (326). Predominant forms found in foods including dairy products included tetrahydrofolate, 5-methyltetrahydrofolate and 5-formyltetrahydrofolate (326). Among lactic acid bacteria examined by Sybesma *et al*. (2003), all folate producers excreted some folate but *Streptococcus thermophilus* was the most productive (350). Folate production was highest at pH 5.5 but decreased at higher pH, and more folate was retained in bacteria at higher growth rates (350). The ability to produce folate is specific to strains of bacteria (348, 352). Some strains of many bacterial species produce folates, including species of bifidobacteria, lactobacilli, lactococcus, propionibacteria and streptococci (353) As not all folate producers are able to synthesize all precursors, in some strains of folate producers, the concentration of the precursor *p*-aminobenzoic acid (PABA) influences the rate of folate production (352). Most of this data was based on food bacteria although many species listed could also be found in the colon. Kim *et al.* (2004) analyzed human fecal folates and found infants excrete 93.2 ± 92.8 nmol/d in feces, of which 66% was in a short chain, and thus bioavailable form, and 52.5% was 5-methyltetrahydrofolate (5).

Very early work in pigs, rats and dogs suggested that dietary intake of folate is not essential to these animals, as bacterially-synthesized folates are sufficient for normal growth and hematology (277). Rats were fed diets with milk solids from human, cow and goat milk. Human milk solids are bifidogenic, *i.e.* they increase the number of bifidobacteria, and rats fed human milk solids show increased numbers of bifidobacteria in the cecum that correlate with enhanced folate status (40). Studies with rats fed diets containing bifidogenic human milk solids contain higher cecal folate concentrations than rats fed similar diets with cow's milk

(253). Keagy and Oace (1989) showed that rats fed diets containing the partially soluble hemicellulose fibre, xylan, had higher liver folate concentrations than rats fed wheat bran (354). However, the effects of colonic folate absorption on folate status are not always broadly apparent even among rats. Thoma *et al.* (2003) showed increased plasma, erythrocyte and colonic tissue folate levels but no changes in liver folate, in rats fed diets with citrus pectin compared with those fed cellulose-containing diets (191). Sepehr *et al.* (2003) also found unchanged liver folate levels in rats on various fibre-supplemented diets (wheat bran, oat bran, ground corn, wheat germ) (41).

In humans, fecal folate is higher than dietary folate intake would predict, and has been reported as high as 300 to 500 μg/day, likely the result of colonic microbial production (355). The microbiota of the human small intestine produce folate that is reported to be absorbed but in humans, microbial production in the small intestine is considered to be only a minor source of the total folate absorbed (356-357). The impact of microbial folate production on human nutrition, and the fate of this large metabolic pool of folate, is essentially unknown. However, secondary analyses of data collected from an observational and an intervention human study support a significant influence of colonic folate on blood folate status (358). Houghton *et al.* reported that the consumption of dietary fibre was positively associated with serum folate concentrations in women (n= 224) even after controlling for folate intake pre-fortification of the food supply in Canada ($P<0.001$) (358). In fact, serum folate increased by 1.8% with each gram of dietary fibre ingested. Likewise, in a randomized controlled trial of type 2 diabetics, investigators showed serum folate was significantly higher in subjects treated with miglitol versus metformin (359). Miglitol, an α-glucosidase inhibitor, improves glycemic control by competitive inhibition of carbohydrate digestion. In contrast, metformin promotes glycemic control by affecting insulin sensitivity and hepatic glucose output. In both studies, increased

colonic bacterial growth secondary to increased availability of fermentable substrate was proposed as the mechanism for the observed increase in serum folate content among high fibre consumers and miglitol users.

2.8.6 Probiotics, prebiotics and the microflora

The long standing concept of modification of the intestinal microflora by probiotics, products containing bacterial cultures for consumption, in order to obtain health benefits, was first published in 1907 by Metchnikoff (360). The benefits of probiotics are believed to be based on the protective effect of the intestinal flora against infection, in forming a barrier to pathogenic and opportunistic organisms (361). Generally probiotics function through the production of substances inhibitory to other bacteria, blocking access of pathogenic micro-organisms to adhesion sites, competition with pathogens for nutrients, degradation of toxin receptor, and through the stimulation of immunity in the host (361). Probiotics have also been called live microbial food supplements (362), since large amounts of the products of microbial metabolism are absorbed in the colon of the host. Substrate flow between intestinal microflora and the human host is potentially very high: breast fed infants fed ^{15}N-labeled *bifidobacteria* absorbed ~90% of the label, about 70% of which was retained in the infants' protein (363).

Prebiotics are products not digestible by the host, which are used to modulate the growth and metabolism of the intestinal microflora, in order to selectively stimulate the growth and/or activities of beneficial intestinal bacteria (364). The term 'synbiotic' was coined by Gibson and Roberfroid (1995) for the combination of prebiotics with probiotics (365-367). Prebiotics used to date have been polysaccharides, ranging from small sugar alcohols and disaccharides to oligosaccharides and large polysaccharides (361, 368-369).

Non-digestible carbohydrates are beneficial to health. Potential effects include an increase in fecal bulk, in intestinal microbial mass, in short chain fatty acid (SCFA) production,

in mineral absorption (370). Systemically, the ingestion of fibre is associated with decreases in cholesterol, serum triglycerides, ammonia, urea, and increases in immune function (371). Specific prebiotic products have been associated with specific health benefits. For example, high amylose starch fed with *Bifidobacterium longum* to pigs was shown to result in higher fecal numbers of this bacterium than pigs fed *B. longum* with a low amylose cornstarch (372). Inulin and its derived fructooligosaccharides are found in many plant species as storage compounds; foods containing inulin include wheat, onion, bananas, garlic, chicory (373). Inulin is a polydisperse ß-(2-1) fructan, that is, a mixture of fructooligosaccharides, of mixed chain length, from 2 to 60 sugar units, polydispersed on average about 10 units (373). The term fructooligosaccharide, or fructan, refers to mixtures of short chains, of fructose bound by ß-(2-1) linkages, attached to a terminal glucose unit linked by an (α1- ß2) bond (**Figure 2.2**). Fructooligosaccharides are the short chain polymers of linked fructose units produced by hydrolysis of inulin, but may also be synthesized enzymatically from sucrose or lactose (374).

Oligofructose is defined by the IUB-IUPAC Joint Commission and AOAC as a fructose oligosaccharide with 2 to 10 monosaccharide residues (375). Synthesized oligofructose contains only chains with terminal glucose units, and oligofructose derived from chicory contains both chains of only fructose and chains of fructose with terminal glucose units (373).

Human milk contains about 1% oligosaccharides, the third largest component, comprising > 130 different structures of great complexity (376). These oligosaccharides are composed of multiple subunits of glucose, galactose, N-acetylglucosamine, fucose and sialic acid (377). They are not commercially available but a mixture of fructooligosaccharides and galactooligosaccharides with a similar molecular weight profile to human milk oligosaccharides has been used in several studies successfully, to mimic the bifidogenic properties of human milk (28-29, 378-379).

Galactooligosaccharides are chains of galactosyl sugar residues linked by β-1,6 bonds attached terminally to a glucose unit via an α-1,4-bond, and may be produced by bacteria from lactose via the enzyme β-galactosidase (380). Galactooligosaccharides are structurally similar to the core structure of human milk oligosaccharides (378). Both fructo- and galactooligosaccharides have been shown to be bifidogenic (323, 368, 381-387).

2.8.6.1 Human response to oligosaccharide supplementation

Chicory inulin and its hydrolysis products showed a marked bifidogenic effect in human volunteer studies, with increases in the numbers of bifidobacteria found to the same extent with oligofructose feeding as with inulin (26). A fall in the pH of fecal slurries was suggested to be indicative of fermentation *by bifidobacteria* (26). Chicory-derived FOS appear to selectively stimulate the growth of bifidobacteria in humans, a "prebiotic effect" (388). The addition of neosugar, synthetic FOS, to the human diet also resulted in increases in bifidobacteria concentrations in the large bowel. Studies of the bifidobacterial flow in humans fed 8g/day showed increases of 10 to 79-fold (381, 389).

Studies of oligosaccharide supplementation to the diet of infants have examined effects on the microflora (29, 52). Addition of a 90%:10% GOS:FOS mixture to preterm formula over a 28 day feeding period led to dose-dependent, highly significant increases in the upper range of bifidobacterial counts and decreases in fecal pH (28, 32). Similarly, addition of 8 g/L of a 9:1 GOS:FOS mixture to formula of 7-8 week old infants showed a significant increase in faecal bifidobacteria (52). The stool characteristics in the supplemented formula-fed infants were more similar to infants fed human milk.

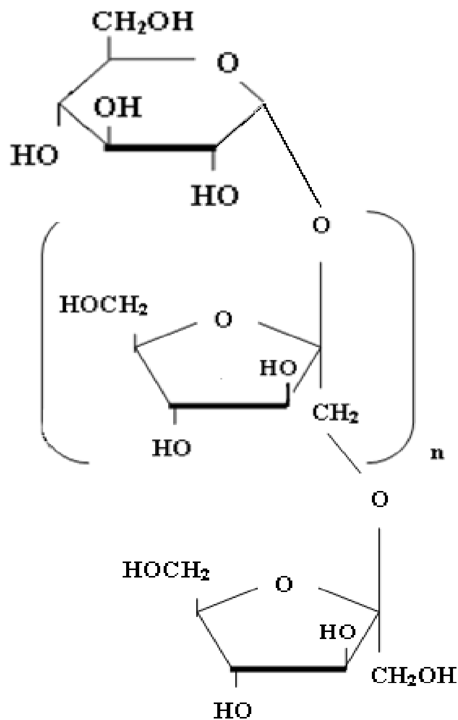

FIGURE 2.3. The structure of fructooligosaccharides (adapted from Yun, with permission) (390).

2.8.6.2 Animal Studies of Prebiotics and Probiotics

2.8.6.2.1 Rat studies

Inulin and inulin-derived fructooligosaccharides are the subject of numerous studies on the effects of non-digestible carbohydrates on intestinal bacteria, many in rodents (391-394). Fructooligosaccharides are degraded in the colon by the microflora (395). They have been shown to increase the endogenous growth of intestinal *lactobacilli* and *bifidobacteria* in both animals and humans an effect considered beneficial to health (31, 396-398). Studies of rats, *in vivo* (398) and rat-derived microfloral cultures *in vitro* (382) have shown that FOS fermentation decreases pH, produces SCFA and lactate, and increases the proportion of butyrate in the SCFA

produced. Butyrate especially is the main source of energy for colonocytes, stimulates water and sodium absorption, and modulates intestinal motility (374, 399).

Dietary inulin has been shown to suppress the formation of aberrant crypt foci in rats and to be associated with a significant increase in the proportion of apoptotic cells in colonic tumors (the apoptotic index) (391, 394, 400). It has been suggested that the production of butyric acid is at the root of this effect, though fecal bulking by fiber has also been shown to be protective (31, 365, 391, 398, 401). Many rodent studies examined both short- and long-term effects of FOS feeding (374). Three to twelve weeks are necessary to stabilize the cecal bacterial mass and metabolic activity (374, 402). A long-term rat study of FOS effects found the cecal concentration of total SCFA strongly increased mainly in butyrate over 27 weeks, though cecum weight did not differ from controls (374).

A butyrogenic effect appears to be common to both *bifidobacteria* and FOS feeding in rats. Fermentation produces lactic acids and short chain fatty acids. In rats, there is a decrease in cecal pH, and an increase in the cecal pool of SCFA. The molar ratio of butyrate is also increased compared with rats on a starch-rich diet without added oligosaccharides (398, 403-407). Roberfroid has related this to the effect of butyrate on intestinal tissue causing hyperplasia of the mucosa, increased wall thickness in the small intestine and cecum (404, 407).

2.8.6.2.2 Studies of prebiotics and probiotics in pigs

In pigs, prebiotic and probiotic use has been supported by the claim that probiotics influence the indigenous microflora to promote growth and improve feed conversion, and control health. Just after weaning there is a critical point in development of the colonic microflora in piglets, when there is a large decrease in *lactobacilli* and an increase in *E. coli* and with age, the percentage of hemolytic coliforms increases, associated with the weaning

diarrhea of young piglets (408). The use of certain *lactobacilli* as probiotics has been shown to have beneficial effects against such pathogens (408).

Inulin in weaned piglets is digested mainly *via* microbial activity in the hindgut. It modifies the pool of fermentation metabolites towards an increase in *n*-valerate and propionate at the expense of acetate and ammonia (409). In the small intestine, inulin can block adhesion of F4+ *E.coli* to small intestinal villi. Studies using fermentable carbohydrates (raw potato starch) and digestible carbohydrates (gelatinized maize starch) in growing pigs found that fermentation mainly increased the colonic empty weight although fermentable non-starch carbohydrates increased the empty weights of both stomach and colon (410).

Perhaps because there is a more substantial microflora in the small intestine of pigs than in humans, there is some evidence for pre-cecal action of prebiotic oligosaccharides, including inulin (337, 411-412). When young adult male castrated pigs, were fed a diet with 2% oligosaccharides, either FOS or α–galactooligosaccharides (GOS) *ad libitum*, the oligosaccharides were degraded 40-50% in the small intestine. They also contributed to fermentation in the distal gut, and were not detected in the feces (413). Studies of the digesta of inulin–fed young pigs found some evidence of prebiotic activity by inulin in the ileum but the major site of inulin digestions is the cecum. When digesta from the ileum, cecum and proximal colon of young pigs fed for 6 weeks on an inulin–supplemented diet were compared in their ability to digest inulin, highest activity was found in the cecum, followed by digesta from proximal colon, and ileum (337).

Oligosaccharides may act as effective prebiotics in the piglet colon. In piglets, in the absence of antibiotics, stress and physiological changes during weaning are associated with low growth and increased diarrhea and mortality, and prebiotics are a means to support piglet health during weanling (414). A number of studies document weanling piglet response to prebiotic

treatments, although suckling piglets and weaned piglets have shown differences in microflora and potential differences in response to prebiotics (337, 415-416).

Among suckling piglets, formula-fed neonatal piglets consuming 0 or 3g/L fructooligosaccharides for 6d showed a trend to greater numbers of fecal bifidobacteria (P=0.08), a result deemed to suggest enhancement of bifidobacteria populations by fructooligosaccharides (302). A human milk oligosaccharide, fed to 3 day old piglets at 0.2 g/d for 30 days was found to increase both bifidobacteria and lactobacilli in the piglets (417). When creep feed was supplemented with oligofructose at 2g/kg, in combination with a mixed probiotic, and fed to seven-day old suckling pigs, they showed increases in the numbers of colonic bifidobacteria ($P<0.05$) (418).

Weanling pigs whose diet was supplemented with oligofructose at a dose of 3g/d also showed increases in numbers of colonic bifidobacteria, as did weanlings supplemented at 3g/d concurrently administered probiotic lactobacilli for 20d (419-420). Weanling piglets fed fructooligosaccharides (0.5g/kg) for 4 weeks also showed significant increases in bifidobacteria numbers ($P<0.05$) (421). Male weaned 28d old piglets fed 0, 16 or 40g/kg GOS or 16g/kg inulin showed significant increases in colonic bifidobacteria only when fed 40g/kg GOS (422). A study supplementing the diet of 28d old weanlings with inulin at 3g/kg of dry feed, found that inulin increased the number of piglets with colonic bifidobacteria (423). Three week old piglets fed acid-resistant oligosaccharides at 5g/kg showed increases in numbers of bifidobacteria, as did six week old piglets fed high amounts (138 and 221 g/kg, respectively) of chicory roots and sweet lupine and fourteen week old pigs fed fructooligosaccharides with a high-amylose starch diets concurrent with a probiotic (424-426).

Other studies found a mixed or negative relationship between oligosaccharide intake and changes in bifidobacteria and lactobacillus content of the colon of piglets. For

example, 17 day old sucklings fed fructooligosaccharides and arabinogalactan 4d prior to weaning showed no effects after weaning, on total anaerobes or bifidobacteria (427). In neonatal pigs, dietary supplementation with FOS for fifteen days did not change cell counts of *bifidobacteria* or total anaerobes, cecal pH or the concentrations of SCFA, but cecal mucosal cell density and cell number increased with FOS, as did proximal colonic mucosal crypt height, leading edge, and cell density, and proliferation zone (302). Distal colonic cells showed a similar response. When neonatal pigs were fed either 0 or 3 g FOS/L of formula for 6 days, and feces were collected every two days, greater numbers of *bifidobacteria* were found in feces of FOS-fed piglets only on day 6 (p=0.08) (302). This data was considered to support that FOS promote *bifidobacteria* growth in piglets and lead to changes in the microbial environment, but that detection of such an increase may depend on timing.

In weaned piglets, dietary inulin supplemented at 30g/kg dry matter for 3 and 6 weeks was not followed by change in the numbers of bifidobacteria or lactobacilli, and similarly, after 15g/kg Jerusalem artichoke flour- or 15g/kg oligofructose-supplementation of 28 day old piglet diets, for 4 weeks, there was no change in numbers of bifidobacteria, anaerobes or coliforms (423, 428-429).   Others have found increases of lactobacilli or decreases in bifidobacteria in the cecum, colon or feces of pigs fed yeast mannan oligosaccharides at a dose of 0.1 g/kg, or fructooligosaccharides at a dose of 3g/d (430).

3 RATIONALE, HYPOTHESIS AND OBJECTIVES

3.1 RATIONALE AND HYPOTHESES

There is a large depot of folate, much of bacterial origin, in the colon of mammals, including humans (5, 273). However, while the pool of human colonic folate is greater than recommended dietary intakes, little is known about its bioavailability (273). Animal studies have demonstrated that folate crosses the colon of the rat and the pig in substantial amounts (34-35, 133). For example, in piglets injected with [^{3}H]-folic acid, the liver, urine and kidneys accounted for 12.3% 3.9% and 1.7% of recovered [^{3}H]-folic acid, respectively (35). *In vitro* studies on human colon-derived biopsy scrapings and cell lines have suggested that considerable amounts of colonic folate could be absorbed (36-37), by mechanisms similar to that of the small intestine (36, 38-39). Nevertheless, direct evidence that folate is absorbed across the intact human colon is lacking. Providing such evidence is one of the objectives of the work described here.

Little is known about luminal colonic folate and the factors affecting bacterial folate synthesis and influencing colonic folate absorption. Our long term goal in this research is to explore the relationship between dietary fibre, the modulation of folate production by the microbial community, and its effects on folate status and colonic health. To date, the effect of dietary modulation on colonic folate production has been tested only in rats by dietary supplementation of various types of fibre (40-41, 191). However, in rats, the difficulty of preventing coprophagy leads to uncertainty whether absorption of bacterially-synthesized folate occurs across the small intestine. The piglet is an established model for studies of gastrointestinal physiology, sugar and dietary fibre digestion and infant development (48, 50-51, 431-432). It is the premier animal model for human folate absorption, in that absorption across the porcine small intestine is similar to that in humans (44-47).

To provide a basis for investigating the relationship between diet, human colonic folate production and health, we conducted two studies. For the first study, recognizing a need to verify that folate is absorbed across the intact human colon, we designed a protocol to test such absorption using a dose of labeled folate introduced into the colon by colonoscopy. We recruited healthy subjects from patients awaiting colonoscopy screening at St Michael's Hospital in Toronto. Using colonoscopy, we verified that the colon was intact and then administered a physiologic dose of labeled folate directly into the cecum. The rate of absorption was monitored by appearance in plasma of ^{13}C-methylfolate.

For the second study, since among the bacteria groups found in the human microflora, bifidobacteria are known to include potent producers of folate, we reasoned that feeding bifidogenic dietary fibre would increase folate production and allow increased absorption of folate (30, 33). Five day old piglets were fed for 28 days on one of two experimental milk-based formula diets: one supplemented with prebiotic bifidogenic oligosaccharides in the form of 5g/L inulin and 5g/L galactooligosaccharides, the other with a control carbohydrate, maltodextrin. For this study, we examined the effects of dietary manipulation on the colonic microflora by enumeration of the colonic aerobic and anaerobic bacterial load and of bifidobacteria and lactobacilli, and by determination of folate in the colonic contents. We monitored the response of the piglets by determining folate indices in blood and analyzing folate levels in their liver and kidneys, by measuring their growth in body weight and the weights of the colon, liver and kidneys.

Hypotheses for Study I:

1. Folate is absorbed across the colon of humans.

2. A physiological bolus dose of [6S]-^{13}C-glutamyl-5-formyltetrahydrofolic acid infused directly into the cecum of healthy adults will be detected in their plasma within four hours.

Hypotheses for Study II:

1. Feeding of 5g/L inulin and 5g/L galactooligosaccharides with a milk formula diet to young piglets will stimulate the growth of colonic bifidobacteria, known folate producers, compared to the growth of bacteria in piglets on a control diet

2. A relative increase in colonic bifidobacteria will increase the amount of folate in the piglet colon and more folate will be available for absorption and will also be absorbed across the piglet colon

3. Differences will be seen in indices of piglet folate status and in growth between the two groups

3.2 OBJECTIVES

Objectives for Study I:

1. To determine whether folate is absorbed across the intact human colon by infusing ^{13}C-labeled 5-formyltetrahydrofolic acid directly into the cecum of humans during colonoscopy

2. To estimate the bioavailability of a naturally occurring form of folate (^{13}C-labeled 5-formyltetrahydrfolic acid) by comparing the area under the curve (AUC) of the appearance in plasma of $^{13}C_5$-5-methyltetrahydrofolic acid following infusion to the cecum during colonoscopy, versus the AUC of the appearance in plasma of $^{13}C_5$-5-methyltetrahydrofolic acid, following IV injection of $^{13}C_5$-5-methyltetrahydrofolic acid.

Objectives for Study II:

1. In piglets fed milk-based diets containing 5g of inulin and 5 g of galactooligosaccharides per liter of diet, to ascertain whether the total bacterial load and the concentration of bifidobacteria, known synthesizers of folate, increase, compared to piglets fed a diet containing 5g/L maltodextrin.

2. To set up a piglet model to study the effect of manipulation of the colonic microflora on net folate microbial biosynthesis.

3. To determine the effect of feeding milk-based diets containing 5g of inulin and 5 g of galactooligosaccharides per liter of diet on the folate status of piglets, by measuring indices of folate status in piglet blood, liver and kidneys.

4 STUDY I: FOLATE IS ABSORBED ACROSS THE COLON OF ADULTS: EVIDENCE FROM CECAL INFUSION OF ^{13}C-LABELED [6S]-5-FORMYLTETRAHYDROFOLIC ACID

4.1 ABSTRACT

Folate deficiency increases the risk of several human diseases. Likewise, high intakes of folate, particularly synthetic folic acid intake, may be associated with adverse health outcomes in humans. A more comprehensive understanding of the “input side” of folate nutrition may help to set dietary recommendations that strike the right balance between health benefits and risks. It is well known that the microflora in the colon produce large quantities of folate that approach or exceed recommended dietary intakes; however there is no direct evidence of the bioavailability of this pool in humans. Our objective was to determine whether, and to what extent, the natural folate vitamer 5-formyltetrahydrofolic acid is absorbed across the intact colon of humans.

During screening colonoscopy, 684 nmol (320 µg) $^{13}C_5$-glutamyl-5-formyltetrahydrofolic acid was infused directly into the cecum of six healthy adults. Three or more weeks later, each subject received an intravenous injection of the same compound (172 nmol). Blood samples were collected before and after each treatment. The ratio of labeled to unlabeled folates was determined in plasma by tandem mass spectrometry. The apparent rate of folate absorption across the colon of a bolus dose of $^{13}C_5$-5-formyltetrahydrofolic acid infused into the cecum was 0.6 ± 0.2 nmol/h, as determined by the appearance of $^{13}C_5$-5-methyltetrahydrofolic acid in plasma. In comparison, the rate of appearance of $^{13}C_5$-5-methyltetrahydrofolic acid after an intravenous injection of $^{13}C_5$-5-formyltetrahydrofolate was 7 ± 1.2 nmol/h. In conclusion, physiological doses of natural folate are absorbed across the intact colon in humans.

4.2 INTRODUCTION

Folate deficiency has been implicated in numerous negative health outcomes including neural tube defects (NTDs) and other congenital defects, vascular disease, neuropsychiatric disorders, and cancer (1). The case for folate in the risk reduction of NTDs led to mandatory folic acid fortification of the North American food supply in 1998. A subsequent decrease in the incidence of NTDs was observed (6-7). Recently, there have been calls to increase the level of folic acid fortification to prevent a further 25% of NTDs suspected to be folate related (433-434). However, concern remains that high folic acid intakes could delay diagnosis of vitamin B12 deficiency leading to the onset and progression of potentially irreversible neurologic damage (1, 16, 18). In addition, a number of other health risks associated with folic acid fortification and supplementation have been recently proposed (18). For example, it was suggested that high folic acid intakes are associated with decreased natural killer cell toxicity, a reduction in the effectiveness of antifolate drugs used against malaria, rheumatoid arthritis, psoriasis and cancer, and in pregnant women an increased incidence of obesity and insulin resistance in their offspring (11-15, 18-19). High folic acid intakes have also been shown to facilitate the progression of preneoplastic cells to cancer in animals (17). These observations are supported by results from human intervention and epidemiologic studies of folate and colorectal cancer (CRC) (261, 263).

To date, calculation of folate intake in the examination of the relationship between exogenous folate supply and health outcomes has been based solely on oral intake (1). Another potential source of folate is the depot synthesized by microflora residing in the colon. It known that the intestinal microflora produce large quantities of folate that approach or exceed dietary intakes of the vitamin (1, 355, 435-436). Recently we showed that the forms of folate synthesized are available for absorption, at least they would be if present in the small intestine

(436). If a significant proportion of folate from the colon can be absorbed, it should be considered when examining the relationship between folate supply and health, particularly given its proximity to the colonocytes and our understanding that both low and high levels of the exogenous folate may influence the development and progression of CRC (18, 261, 263). Data from animal studies suggest a relationship between intestinal folate biosynthesis and folate status (34-35, 40, 278, 437). However in many studies, coprophagy could have facilitated absorption of microbially synthesized folates across the small intestine, despite investigator efforts to prevent.

While *in vitro* data are suggestive, there is no direct evidence to support that folate is absorbed across the intact human colon (36, 38, 134). The objectives of this study, then, were to determine whether, and to what extent, folate is absorbed across the colon of humans. A physiological dose of ^{13}C-labeled 5-formyltetrahydrofolic acid was infused into the cecum of humans undergoing colonoscopy. Here we report results of plasma analyses of $^{13}C_5$-labeled and unlabeled folates.

4.3 SUBJECTS AND METHODS

4.3.1 Study population and recruitment

Healthy adult male and nonpregnant female subjects with normal blood chemistry and folate status were recruited between February and May 2007 from patients waiting to undergo screening colonoscopies at St Michael's Hospital, Toronto. Folate status was assessed by determining red blood cell (RBC) folate concentration using microbial assay (97). Blood chemistry included complete blood counts and measurement of serum electrolytes, glucose, renal function, and liver enzymes. Individuals who consumed > 1 alcoholic drink/d, had a known sensitivity to leucovorin (5-formyltetrahydrofolic acid), or had documented or suspected

gastrointestinal disease that could interfere with folate absorption and metabolism (e.g., celiac disease, inflammatory bowel disease) were excluded. Additional exclusion criteria were pregnancy, use of oral contraceptives, and medications known to affect folate metabolism (*e.g.* dilantin, phenytoin, primidone, metformin, sulfasalazine, triamterene or methotrexate). All subjects were screened for the 5,10-methylenetetrahydrofolate reductase (MTHFR) C677→T polymorphism by the Taqman SNP Genotyping Assay (Applied Biosystems, Foster City, CA) using DNA extracted from whole blood as described previously (438). Individuals homozygous for the T allele were excluded from the study to minimize potential inter-subject differences in how folate was metabolized.

Subjects were asked to refrain from using vitamin and/or mineral supplements for at least two weeks prior to study initiation. Bowel preparation for the colonoscopy included clear fluids and consumption of the standard polyethylenglycol solutions followed by fasting the day before the scheduled procedure. All subjects gave written informed consent. The study protocol was approved by the human research ethics boards at St Michael's Hospital and The Hospital for Sick Children.

4.3.2 Test Compound

$^{13}C_5$-[6S]-Glutamyl-5-formyltetrahydrofolate was synthesized by Merck Eprova AG (Schaffhausen, Switzerland) in the form of a calcium salt and purchased in powdered form, with a molecular formula of $C_{15}{}^{13}C_5H_{21}CaN_70_7$. The isomeric structure and labeling pattern of the test compound is shown in **Figure 4.1**. The form of folate administered, 6S-5-formyltetrahydrofolic acid, is the naturally occurring folate vitamer of greatest chemical stability and is among the folate vitamers synthesized by lactic acid bacteria in culture (326, 350). The 5 carbons of the glutamic acid portion of the molecule were occupied by ^{13}C atoms. A contaminant comprising ~9% of the labeled compound proved on analysis by LC-MS/MS to

be enriched by only four ^{13}C carbon atoms. The stereochemistry of two chiral carbon atoms, at position 6 of the pteridinyl ring and at the α-carbon of the glutamic acid moiety were each confirmed at >98% purity (Figure 4.1) (Merck Eprova AG, Schaffhausen, Switzerland). The concentration (97.4%) and stability (for one month) of [6S]-5-formyltetrahydrofolic acid in the product was confirmed by infrared spectroscopy and HPLC (Merck Eprova AG, Schaffhausen, Switzerland). Liquid solutions of the test compound were prepared under aseptic conditions by dissolution of the purchased powder in sterile physiological saline (pH 7.0) (193.6μmol folate/L). To ensure that absorption was tested at a physiological concentration of folate, a dose lower than human adult fecal folate concentrations reported in the literature was chosen for infusion was (355, 439). Test solutions were stored at 4ºC shielded from light and used within one month of preparation. The folate concentration of each compounded lot was confirmed by microbial assay to be within ±10% of the target concentration. Sterility and safety of each compounded lot was confirmed by analysis for pyrogens and heavy metals before administration to subjects (Nucrotechnics Inc, Scarborough, Canada). For intravenous (IV) injection, 1 mL aliquots of test solution were used; for cecal infusion, four mL aliquots of test solution were diluted to 100 mL with sterile physiological saline (pH 7.0) immediately before use.

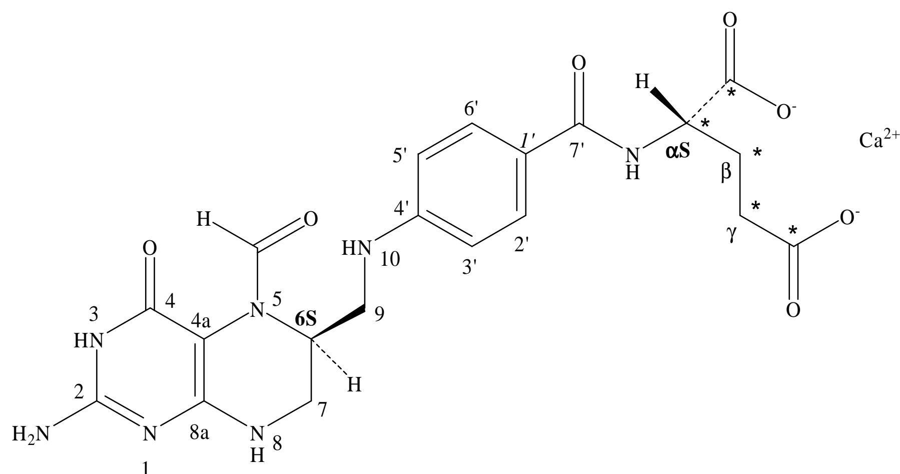

Figure 4.1 The chemical and isomeric structure and labeling pattern of the test compound, $^{13}C_5$-(6S)-5-formyltetrahydrofolate-Ca. The stereochemistry of carbon atoms at position 6 of the pteridinyl ring and at the α carbon of the glutamate moiety are shown in boldface. The positions occupied by ^{13}C are shown by asterisks.

4.3.3 Study protocol

An indwelling catheter was inserted in one arm of each subject to collect a baseline fasting blood sample (5 mL) shortly before colonoscopy, and for blood sampling after cecal infusion. After confirming via colonoscopy that the colon was free of any lesions including polyps proximal to the rectum (polyps in the rectum were allowed), 684 nmol (320 µg) of $^{13}C_5$-labeled 5-formyltetrahydrofolic acid in 100 mL physiological saline was delivered to the cecum of each subject via an irrigation catheter in the biopsy channel of the colonoscope (CF 160L, Olympus America Corporation). The ileocecal valve of each subject was intubated prior to

cecal infusion of the labeled folate in order to rule out subclinical Crohn's disease or other mucosal diseases involving the terminal ileum. After confirming that the ileocecal valve was normal and was competent, we infused the labeled folate in the cecum via spray catheter at the opposite side of the ileocecal valve and kept the ileocecal valve in the superior position (i.e., 11 - 12 o'clock position) to prevent reflux of the test dose into the terminal ileum. The translocation of the infused labeled folate in the cecum was observed for at least 5 minutes and no obvious reflux of the cecal content into the terminal ileum was noted. Cecal infusion was chosen in order to expose as much of the colon as possible to the test dose, as the dose would move distally towards the rectum. After cecal infusion, blood (5 mL) was taken at 30 minute intervals for 4 hours.

Three or more weeks after colonoscopy, in the Clinical Investigation Unit at The Hospital for Sick Children, fasting blood samples were collected from each subject and an intravenous injection of 172 nmol (80 µg) of $^{13}C_5$-5-formyltetrahydrofolic acid, dissolved in 1 mL of sterile saline was administered. Post-injection, blood samples (5 mL) were collected from each subject at 30 minute intervals for 4 hours, via an indwelling catheter inserted in the arm not used for injection of the test dose. For 4 hours after both the cecal infusion and IV injection, subjects were provided with beverages and snacks that we confirmed were low in folate content by direct analyses in our laboratory.

Blood samples throughout the study were collected in EDTA-treated tubes, and processed within two hours of collection. For measurement of RBC folate (baseline only), 100 µL aliquots of whole blood were diluted tenfold with ascorbic acid and deionized water (1% wt:vol) and incubated at 37 ºC for 30 minutes to convert folates into their microbiologically assayable form. Plasma at all collection times was separated from whole blood by centrifugation (1500 x g for 20 minutes at 4 ºC) and portioned for future folate analyses with

added sodium ascorbate (1 % wt:vol) to prevent the oxidation of folate. All samples were frozen on dry ice immediately after processing and stored at -80 ºC.

4.3.4 Biochemical and Mass Spectrometry Analyses

The total folate content of plasma samples was determined by the standard microbial assay according to the method of Molloy and Scott using the test organism *Lactobacillus rhamnosus* (ATCC7649; American Type Tissue Culture Collection, Manassus, VA) (97). The folate content of foods consumed during the 4-hour post cecal infusion and IV injection period were determined by microbiological assay after tri-enzyme treatment of samples as described by Hyun and Tamura (95). The accuracy and reproducibility of these assays were assessed using a whole-blood control 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 yielded a folate content of 31.7 ± 1.0 nmol/L, with an interassay CV of 4.6 %. Red blood cell folate was calculated from whole-blood folate by subtraction of plasma total folate using a correction for red blood cell volume.

Plasma enrichment of the infused $^{13}C_5$-5-formyltetrahydrofolic acid and its $^{13}C_5$-labeled metabolite, 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 as previously described . Prior to LC-MS/MS analysis, folates were extracted from plasma (275 µL) that was diluted with formate buffer (825 µL, pH 3.2) and ascorbic acid (1g/L), using phenyl solid phase extraction (SPE) cartridges. Extracts (20 µL) were then loaded onto a Luna C-8 analytical column for chromatographic separation using an isocratic mobile phase. Mass to charge ratios of transitions of interest [(M+0) and (M+5)] were

monitored in positive ion mode via turbo ion electrospray on a Sciex API 4000 triple quadrupole MS system (Applied Biosystems Group, Foster City, CA).

To verify that area ratios were reproducible and matched theoretical molar ratios, we prepared standard curves at three folate concentrations (20, 60, and 100 nmol/L) with varying proportions of $^{13}C_5$-labeled/^{13}C-unlabeled 5-formyl- and 5-methyltetrahydrofolic acid (0-50% label; standard compounds obtained from Merck Eprova AG). The standard mixtures were prepared over multiple days and analyzed either directly by LC-MS/MS, or after carrying them through SPE cartridges. Because the $^{13}C_5$-5-formyltetrahydrofolic acid used in this study contained a small portion of the (M+4) label (9 % of the [M+5] label), we also monitored this transition to ensure that we're capturing the entire signal produced by this compound. As expected, measured area ratios (expressed as the sum of [M+4] and [M+5] over [M+0]) were very reproducible from day-to-day (2-4% CV), were not affected by the SPE cartridges, and matched theoretical molar ratios within ± 5%.

After these initial experiments, we only used areas from the (M+5) channel for 5-formyl- and 5-methyltetrahydrofolic acid to calculate area ratios ([M+5]/[M+0]) because there is no metabolic conversion from (M+5) to (M+4), and (M+4) areas were typically small and might therefore increase imprecision. No correction for natural abundance of isotopes was necessary since the contribution at (M+5) due to the presence of ^{13}C, ^{15}N, and ^{17}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 >30% enrichment for $^{13}C_5$-5-formyltetrahydrofolic acid. Samples collected after cecal infusions were all below 30% enrichment.

4.3.5 Quantification of the Plasma Folate Response

In addition to reporting our data as molar ratios of 5-formyl- and 5-methyltetrahydrofolic acid, we chose to quantify the plasma folate response and pharmacokinetic data to produce the lexicon most familiar to readers without a background in stable isotopes—*i.e.* sum of peak areas, and nmol folate/person. This was done with a number of important caveats. Most importantly, to calculate the sum of all peak areas, we added the peak areas for labeled (M+5) and unlabeled (M+0) 5-formyl- and 5-methyltetrahydrofolic acid (**Figure 4.2**). This required adjustment of the peak areas for 5-formyltetrahydrofolic acid (divided by 2.3) to account for the differences in the LC-MS/MS signal between 5-formyltetrahydrofolic acid and 5-methyltetrahydrofolic acid. Second, to quantify the total amount (nmol/L) of labeled (M+5) 5-formyltetrahydrofolic acid or 5-methyltetrahydrofolic acid, we took the peak area for each labeled metabolite and converted it to nmol folate per litre plasma using the total plasma folate concentration determined by microbial assay for each subject (*i.e*. either cecal infusion or IV injection) as shown in the following equation:

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

Where X = the concentration (nmol/L) of labeled 5-formyltetrahydrofolic acid or 5-methyltetrahydrofolic acid.

The lowest plasma folate concentration determined by microbial assay for each subject and treatment was used, rather than simply the baseline concentration, to avoid the distortion in blood folate content introduced by interruption of bile flow during fasting (218). Finally, in order to express our data on a whole body basis (*i.e.* convert from nmol/L to nmol/person) as shown in Figure 4.4, we determined the total plasma volume of each subject. Blood volumes were estimated using the values 75 mL/kg for males and 66.5 mL/kg for females of normal

weight (440). Plasma volumes were then calculated from the estimated blood volume by correcting for RBC volume (hematocrit). To account for the different hydration of lean versus fat mass, we adjusted the blood volume for each individuals using the relationship of blood volume and deviation from ideal weight described by Feldshue and Enson (441).

4.3.6 Statistical analysis

SAS for WINDOWS (Version 9.1; SAS Institute Inc, Cary, NC) was used to generate descriptive statistics (*i.e.* mean, SEM). Changes in the total plasma folate concentration determined by microbial assay or molar ratios of either 5-formyltetrahydrofolic acid or 5-methyltetrahydrofolic acid were determined by repeated-measures ANOVA (PROC MIXED) using sample as the main effect and quadratic sample or cubic sample as necessary. These analyses included baseline RBC folate concentration in the statistical model. The apparent plasma half-life (one-phase exponential decrease over time) of ^{13}C-labeled 5-formyltetrahydrofolic acid after IV injection for each subject, was determined using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California USA) using the slope of the descending portion of each plasma response curve. The rate of appearance of ^{13}C-labeled 5-methyltetrahydrofolic acid in plasma over time, after cecal infusion, was determined from the linear slope of the ascending portion of each plasma response curve.

4.4 RESULTS

4.4.1 Subject characteristics

Subject characteristics are summarized in **Table 4.1**. Ten subjects were recruited from colonoscopy screening patients at St Michael's Hospital. Two were excluded because they were homozygous for the MTHFR C677→T variant, and two were excluded due to the detection of polyps proximal to the rectum (both cecal polyps) at the time of colonoscopy. All

six remaining participants were Caucasian and 50 to 63 years of age. Erythrocyte folate concentrations of the subjects varied widely but were well above a cut-off of 360 nmol/L associated with tissue folate depletion. Three of the 6 participants were heterozygous for the MTHFR C677→T polymorphism. One subject had suboptimal B12 status (127 pmol/L: normal ≥150 pmol/L) (442). Three were obese as defined by a BMI of >30. Two participants regularly consumed folic acid-containing vitamin supplements prior to enrollment; the first and second discontinued supplementation at least 5 and 2 weeks, respectively, before the study intervention. Rectal polyps in three subjects were excised in a standard fashion (one microadenoma, one adenoma, and one hyperplastic polyp) and, as allowed for in the inclusion criteria, these individuals were included in the study. Mean (±SD) dietary intake of folate during the 4-h blood collection period post IV injection and cecal infusion was 14.1± 3.7 and 14.8 ± 4.0 µg, respectively. All participants completed both clinic visits with complete blood sample collection.

TABLE 4.1 Subject characteristics

Subject characteristics	Mean±SD (n=6)	Range
Sex (M/F)	4/2	
Age (y)	56±2	50 – 63
Weight (kg)	96±9.8	60.5 – 128.4
BMI[1] (kg/m^2)	33. ±3.1	23.1 – 43.7
Plasma volume[2] (L)		2.37 – 4.29
Blood screening Data:		
MTHFR[3] C677→T genotype (CC/CT)	3/3	
Red blood cell folate (nmol/L)	1163 ± 196	638 – 1931
Total plasma folate (nmol/L)	48.1 ± 10.1	21.2 – 50.8
Vitamin B12 (pmol/L)	258 ± 45	127 – 403
Dietary intake of folate during blood collection:		
Post intravenous injection (µg)	14.1 ± 3.7	3.6 – 27.2
Post colonoscopy (µg)	14.8 ± 4.0	0.72 – 23.8

[1]BMI = body mass index. [2]Whole blood volume was calculated using an estimate of 75 mL/kg for males and 66.5 mL/kg for females of normal weight (440). Plasma volume was estimated from whole blood volume using hematocrit determinations and corrected for the deviation of each subject's actual weight from their ideal body weight as described by Feldshue and Enson (441) [3]MTHFR = methylene tetrahydrofolate reductase.

4.4.2 Plasma folate response

The plasma folate responses to IV injection and cecal infusion of $^{13}C_5$-5-formyltetrahydrofolic acid, as determined by microbial assay and LC-MS/MS, are shown in Figure 4.2**.** The change in the plasma folate concentration over time, as determined by microbial assay following IV injection of the test compound, was statistically significant (P=0.0005). The pattern for total folate (sum of peak areas) calculated by adding both labeled (M+5) and unlabeled (M+0) peak areas for 5-formyl- and 5-methyltetrahydrofolic acid generally followed the same trend as shown for the folate concentration determined by microbial assay following IV injection but the change did not reach statistical significance (P=0.1551). As illustrated, plasma folate rose briefly after IV injection of $^{13}C_5$-5-formyltetrahydrofolic acid and returned to baseline within the 4 hour observational period. After cecal infusion, neither the change in plasma folate concentration, as determined by microbial assay (P=0.7318) or LC-MS/MS (P=0.1490) was statistically significant.

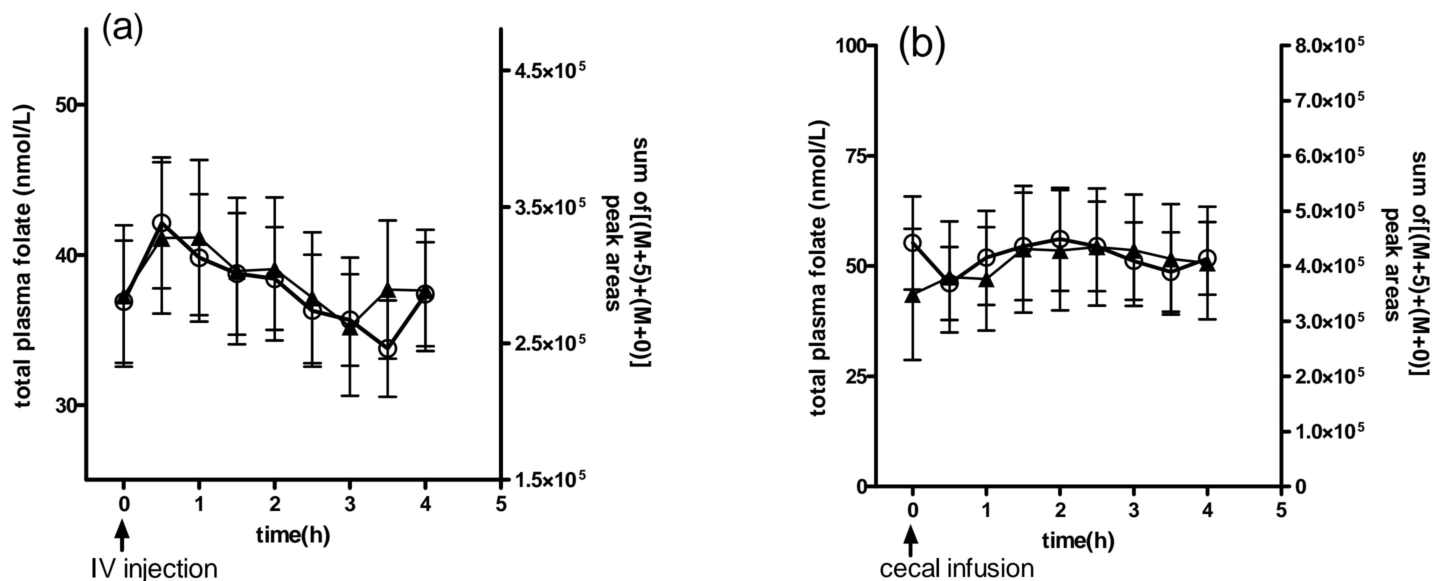

FIGURE 4.2 Mean plasma folate response (n=6, mean ± SEM) following administration of $^{13}C_5$-labeled 5-formyltetrahydrofolic acid by **(a)** IV injection, and **(b)** cecal infusion. Data are presented as plasma folate concentration (nmol/L) from results obtained by microbial assay (○), or sum of the peak areas as measured by LC-MS/MS, (▲). Note that y axes differ. The change in total plasma folate after IV injection, determined by microbial assay, was statistically significant (*P*=0.0005, repeated measures ANOVA). No statistically significant change in total plasma folate was observed after cecal infusion. The change in summed peak areas measured by LC-MS/MS did not reach statistical significance after either IV injection (=0.1551) or after cecal infusion (*P*=0.1490)

The plasma molar ratios (M+5/M+0) for 5-formyltetrahydrofolic acid and 5-methyltetrahydrofolic acid following administration of $^{13}C_5$-[6S]-formyltetrahydrofolic acid are found in **Figure 4.3**. There was a statistically significant change after IV injection in the molar ratios for 5-formyltetrahydrofolic acid (*P*=0.0037). The change in the molar ratios for 5-methyltetrahydrofolic acid following IV injection did not reach statistically significance (*P*=0.0974).

Following cecal infusion of the test dose, there was no statistically significant change in the molar ratios for 5-formyltetrahydrofolic acid but there was for 5-methyltetrahydrofolic acid (*P*<0.0001). We did not see detectable levels of $^{13}C_5$-5-formyltetrahydrofolic acid or $^{13}C_5$-5-methyltetrahydrofolic acid following cecal infusion of the test compound for 3 and 1 of the six subjects, respectively.

In **Figure 4.4**, we have converted the LC-MS/MS data for $^{13}C_5$-5-formyltetrahydrofolic acid (M+5), unlabeled 5-formyltetrahydrofolic acid (M+0) and $^{13}C_5$-5-methyltetrahydrofolic acid (M+5) to nmol/person. The amount of labeled 5-formyltetrahydrofolic acid rose after IV injection of the test dose, to a maximum of 12 ± 1.2 nmol followed by a rapid decline to baseline four hours post-injection (*P*<0.0001). $^{13}C_5$-5-Methyltetrahydrofolic acid content also showed a rapid rise in plasma after IV injection of the test dose, followed by a slight decline then maintenance at a mean incremental increase of about 6 nmol per person at the end of the 4 hour study period (*P*<0.0001). After cecal infusion of the test dose, the mean $^{13}C_5$-5-formyltetrahydrofolic acid content showed a small, insignificant increase, while the mean $^{13}C_5$-5-methyltetrahydrofolic acid content showed an approximately linear increase with no decrease over the 4 h blood sampling period (*P*<0.0001). The mean plasma unlabelled 5-formyltetrahydrofolic acid content remained constant for 1.5 h after cecal infusion, and rose linearly thereafter with no decrease over the 4 h blood sampling period (*P*=0.0009).

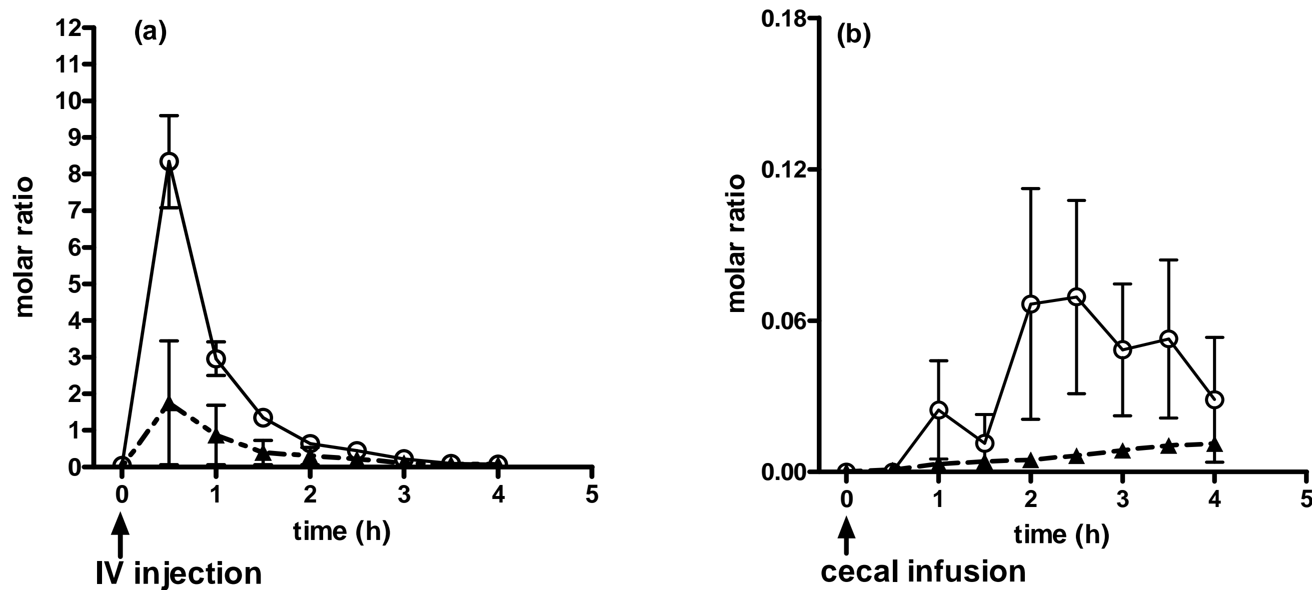

FIGURE 4.3 The mean ± SEM (n=6) molar ratios of (M+5)/(M+0), for 5-formyltetrahydrofolic acid (○), and 5-methyltetrahydrofolic acid (▲), after **(a)** IV injection and **(b)** cecal infusion of ^{13}C- 5-formyltetrahydrofolic acid. Note y axes differ between Figure 2a and b. The change in the molar ratio of 5-formyltetrahydrofolic acid (*P*=0.0037, repeated measures ANOVA) but not 5-methyltetrahydrofolic acid (*P*=0.0974) was statistically significant after IV injection of $^{13}C_5$-5-formyltetrahydrofolic acid. The change in the molar ratio of 5-formyltetrahydrofolic acid was not significant after cecal infusion but was for 5-methyltetrahydrofolic acid (*P*<0.0001).

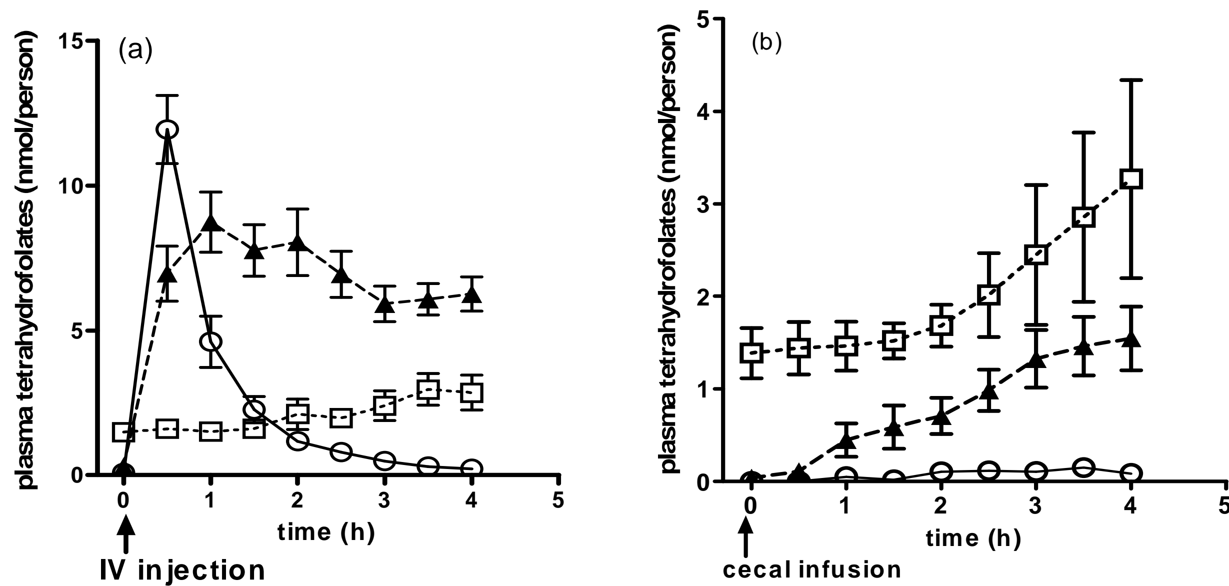

FIGURE 4.4 The mean ± SEM (n=6) plasma folate content (nmol/person) of $^{13}C_5$-5-formyltetrahydrofolic acid (○, M+5), its metabolite $^{13}C_5$-5-methyltetrahydrofolic acid (▲, M+5), and unlabeled 5-formyltetrahydrofolate (□, M+0), following administration of ^{13}C-5-formyl tetrahydrofolic acid to 6 subjects (**a)** by IV injection, and **(b)** by cecal infusion. Note that y axes differ between Figure 3a and b. The change in $^{13}C_5$-5-formyltetrahydrofolic acid was highly significant after IV injection but was not significant after cecal infusion (*P*<0.0001 and *P*=0.1669, respectively). The change in $^{13}C_5$-5-methyltetrahydrofolic acid was highly significant after IV injection and after cecal infusion (*P*<0.0001). The change in unlabeled 5-formyltetrahydrofolate was not significant after IV injection but was so cecal infusion (*P*= 0.2660, *P*= 0.0009, respectively).

4.4.3 Pharmacokinetics

In **Table 4.2** we have summarized the pharmacokinetic data for the study. The mean (± SEM) maximal content (C_{max}) of $^{13}C_5$-5-formyltetrahydrofolic acid after IV injection was 12 ± 1.2 nmol. Labeled 5-formyltetrahydrofolic acid content in plasma decreased from its maximum after IV injection with a half-life of 0.3 ± 0.04 h. The mean C_{max} for $^{13}C_5$-5-methyltetrahydrofolic acid was 9 ± 0.8 nmol per person. After cecal infusion, $^{13}C_5$-5-methyltetrahydrofolic acid increased after a mean delay of 0.8 ± 0.3 h, at a mean rate of 0.6 ± 0.2 nmol/h reaching a mean C_{max} of 1.7 ± 0.3 nmol per person 4 h post-cecal infusion. A table summarizing the characteristics of plasma response of the unlabeled folates determined, after cecal infusion of $^{13}C_5$-5-formyltetrahydrofolic acid can be found in the supplementary data provided in the Appendices (**Table 8.1).**

TABLE 4.2 Pharmacokinetic data after IV injection and after cecal infusion of $^{13}C_5$-5-formyltetrahydrofolic acid[1]

Subject	Intravenous Injection				Cecal Infusion			
	$^{13}C_5$-5-formyltetrahydrofolic acid		**$^{13}C_5$-5-methyltetrahydrofolic acid**		**$^{13}C_5$-5-methyl tetrahydrofolic acid**			
	C_{max}	$t_{1/2}$	C_{max}	Rate of Appearance[2]	Plasma Volume	t_{delay}	C_{max}	Rate of Appearance[2]
	(nmol)	(h)	(nmol)	(nmol/h)	(L)	(h)	(nmol)	(nmol/h)
a	13.5	0.315	12.3	3 ± 1	4.29	25	2.22	0.98 ± 0.2
b	9.75	0.174	5.35	10.87	3.82	1	1.53	1.5 ± 0.5
c	11.5	0.342	8.11	3 ± 1	3.08	0.5	2.13	0.58 ± 0.05
d	15.9	0.358	8.06	7 ± 4	3.63	0.5	1.98	0.44 ± 0.03
e	13.1	0.285	10.24	10 ± 4	2.37	0.	1.76	0.41 ± 0.04
f	7.83	0.467	9.30	9 ± 1	2.98	ND	ND	ND
mean±SEM	12 ± 1	0.3 ± 0.04	9 ± 1	7 ± 1	3.44 ± 0.3	0.8 ± 0.3[3]	1.7 ± 0.3[3]	0.6 ± 0.2[3]

[1]C_{max}= maximal content; $t_{1/2}$= apparent plasma half-life; t_{delay}= time lag before detection of the labeled vitamer. [2] Determined by the ascending slope of the appearance of $^{13}C_5$-5-methyltetrahydrofolic acid in plasma. [3]A zero value was used for subject “f” as $^{13}C_5$-5-methyltetrahydrofolic acid was undetected following cecal infusion of the test dose.

4.5 DISCUSSION

Results from this study provide the first direct *in vivo* evidence that folate can be absorbed across the colon in humans. We predict the rate of folate absorption across the colon to be 0.6 ± 0.2 nmol/h based on the appearance of $^{13}C_5$-5-methyltetrahydrofolic acid in plasma after cecal infusion of 684 nmol $^{13}C_5$-5-formyltetrahydrofolic acid. In comparison, the rise in $^{13}C_5$-5-methyltetrahydrofolic acid following IV injection of 172 nmol of $^{13}C_5$-5-formyltetrahydrofolic acid was 7 ± 1.2 nmol/h. The apparent rate of folate absorption across the colon reported, herein, is considerably lower than that reported in the literature for the small intestine (204-205). Extrapolating data from that published by Wright *et al*. in which the appearance of $^{13}C_5$-5-methyltetrahydrofolic acid in plasma was monitored after an oral dose of 431-569 nmol $^{13}C_5$-5-formyltetrahydrofolic acid, we estimate the rate of folate absorption across the small intestine to be 34 nmol/h (204-205). It is important to acknowledge, however, that while 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 hour) is considerably shorter than that in the colon (24-72 hours) allowing for a greater opportunity for absorption to occur in the colon (443-446). Further, while folate absorption across the small intestine relies on oral intake and hence is intermittent, the availability of folate for absorption across the colon is continuous, since microbial synthesis of folate occurs 24 hours a day. Earlier reports confirm that the colons of adults, with an intact microbiota, contain folate well in excess of the test dose administered herein (355, 435).

Our observations that folate absorption occurs across the colon are consistent with our earlier work in which we injected [^{3}H]-*para*-aminobenzoic acid, a precursor of bacterially synthesized folate, into the cecum of 11 day old piglets and were able extract titrated folates from the liver and kidneys and urine over the subsequent 3 days . In this latter study we

predicted that about 18% of the dietary requirement for the piglet could be met by folate absorption across the colon. Similarly Rong *et al.* injected [^{3}H]-*para*-aminobenzoic acid into the cecum of rats and observed that bacterially synthesized tritiated folate was incorporated into the liver of rats despite the prevention of coprophagy (34).

Secondary analyses of data collected from an observational and an intervention human study are similarly supportive of the findings in the present study (358-359). We reported that the consumption of dietary fiber was positively associated with serum folate concentrations in women (n= 224) even after controlling for folate intake pre-fortification of the food supply in Canada ($P<0.001$) (358). In fact, serum folate increased by 1.8% with each gram of dietary fiber ingested. Likewise in a randomized controlled trial of type 2 diabetics, investigators showed serum folate was significantly higher in subjects treated with miglitol versus metformin (359). Miglitol is an α-glucosidase inhibitor that improves glycemic control by competitive inhibition of carbohydrate digestion. In contrast, metformin promotes glycemic control by affecting insulin sensitivity and hepatic glucose output. In both studies increased colonic bacterial growth secondary to increased availability of fermentable substrate was proposed as the mechanism for the observed increase in serum folate content among high fiber consumers and miglitol users.

We did not assess what percentage of the test dose was metabolized within the colonocyte, however it is known that a significant fraction of other nutrients are absorbed at the level of the small intestine. For example, select amino acids and folate are metabolized within the enterocyte and the liver, by so-called first-pass splanchnic metabolism (51, 205, 447-448). Natural dietary folates, including 5-formyltetrahydrofolate, absorbed across the human small intestine are metabolized to 5-methyltetrahydrofolic acid within the enterocyte before export to the liver via the portal vein, while synthetic folic acid passes through the enterocyte and is later

reduced and metabolized in the liver (68, 200, 204-205). From the liver, the metabolite, 5-methyltetrahydrofolate, may be released to the systemic circulation for distribution to the periphery. A substantial proportion of reduced folate absorbed by the small intestine may be retained by the splanchnic organs (204). **Figure 4.5** shows the first pass metabolism of 5-formyltetrahydrofolic acid in the enterocyte. Our results show that the greater part of the $^{13}C_5$-5-formyltetrahydrofolate absorbed across the colon into the plasma was similarly converted to $^{13}C_5$-5-methyltetrahydrofolate: after colonic infusion small amounts of $^{13}C_5$-5-formyltetrahydrofolate were detected in the plasma of only 3/6 subjects. Given the proximity of colonocytes to the depot of folate in the colon, it is enticing to contemplate what role this pool of folate may have on colonic health and CRC specifically. Folate has been linked to CRC at the low and high ends of intake (1, 17, 449). To date, the complex relationship between folate and CRC has been examined in relation to oral folate intake alone. However, if the colonocyte can use bacterially synthesized folate, and the size of this pool can be influenced by diet (*i.e.* fiber), this could explain why the association between folate intake and CRC risk is stronger for dietary folate (relative risk for high vs. low intake = 0.75; 95% CI = 0.64-0.89) than for total folate (folate from foods and supplements: relative risk for high vs. low intake = 0.95; 95% CI = 0.81-1.11) (450). Natural sources of folate in the diet are typically excellent sources of fiber.

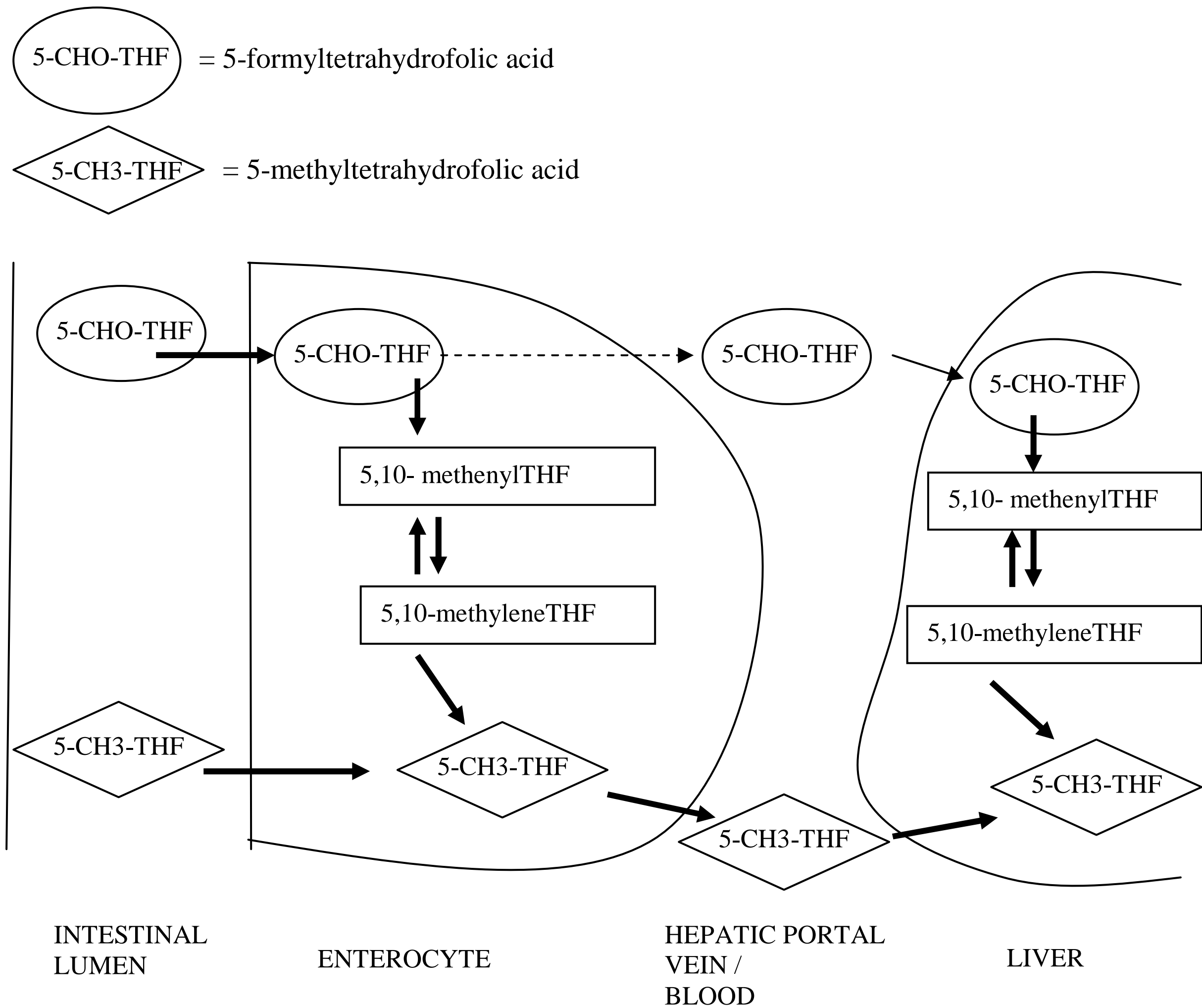

FIGURE 4.5 A sketch of the first pass intestinal and liver metabolism of 5-formyltetrahydrofolic acid to 5-methyltetrahydrofolic acid. THF represents tetrahydrofolic acid. While both 5-formyl- and 5-methyltetrahydrofolic acid are absorbed by the enterocyte, most 5-formyltetrahydrofolic acid is metabolized to 5-methyltetrahydrofolic acid before transport via the hepatic portal vein to the liver (200, 204-205, 451).

As illustrated in Figure 4.4, there was a significant rise in unlabeled mean 5-formyltetrahydrofolic acid after cecal infusion of $^{13}C_5$-5-formyltetrahydrofolic acid (*P*=0.0009), suggesting displacement of endogenous folate from the colonocyte and/or liver. Wright *et al.* (2003) made the same observation at the level of the small intestine following oral ingestion of ^{13}C-5-formyltetrahydrofolic acid (204). These data serve as a caution in interpreting folate absorption studies using "unlabelled" folates, as the portion of the plasma response due to the test dose cannot be distinguished from that due to displacement of endogenous folate, and hence would lead to over-estimation of folate bioavailability.

The proposed potential mechanisms of how folate is absorbed across the colon include passive diffusion, and active transport by two solute carriers—reduced folate carrier and proton-coupled folate transporter (162, 452). Reduced folate carrier is widely expressed in the body including the small intestine and colon and has a neutral pH optimum (452). The proton-coupled folate transporter is expressed in the colon at concentrations lower than in the small intestine with a pH optimum of 5.5 (162). The pH of the colon varies along its length across the pH optimum of these two carriers, from about 5.2 in the proximal colon, rising to 6.4-6.9 mid-colon, to over 7 in the distal colon (285).

We acknowledge several limitations of this work. First, we originally planned to compare the two areas under the curve produced by measuring $^{13}C_5$-5-methyltetrahydrofolic acid in plasma following cecal infusion versus IV infusion of the test dose. This would allow us to determine the % bioavailability of our test dose. However at the end of the blood collection phase following cecal infusion, the $^{13}C_5$-5-methyltetrahydrofolic acid concentration in plasma had not returned to baseline, in fact, it continued to rise (Figure 4.4b). Unlike folate absorption across the small intestine, the calculation of % bioavailability across the colon using an area under the curve approach would require blood sampling for much longer than 4 h. The

second limitation of this study is that we measured folate absorption after bowel cleansing in preparation for colonoscopy (453). It is possible that both of these factors impacted on our study results. Third, though we took every precaution to avoid reflux of our test compound from the cecum back into the small intestine and we believe unlikely, we can't absolutely rule this out. Lastly, we appreciate the generalizability of our findings from 6 subjects to the larger population is limited. All of our subjects were $\geq$ 50 years of age, three were obese, one was on folic acid supplements 16 days prior to the study, one had a low vitamin B12 value and, as a group, our subjects had high blood folate concentrations, at least compared to pre-fortification of the food supply. While the C677→T polymorphism in the gene for MTHFR is known to decrease plasma folate concentration, individuals with the MTHFR *CT* genotype were not excluded by our protocol, and labeled folate absorption in these subjects was similar to that of the homozygous (*CC*) subjects (454). For the most part, it does not appear that aging in the absence of disease affects folate absorption (1, 455) ; however, there is evidence in the literature that adiposity may impact folate metabolism (456-457) and that high levels of folate exposure may down-regulate folate absorption (199). To account for this some investigators allow for a 5 to 6 week washout of supplements (458-459). Finally, severe vitamin B12 deficiency is known to block the uptake of folate into tissues (460) though the appearance and disappearance of $^{13}C_5$-5-methyltetrahydrofolic acid in plasma of our subject with moderate vitamin B12 deficiency was remarkably similar to that of others.

In conclusion, results from this study provide the first direct *in vivo* evidence that physiological concentrations of folate can be absorbed across the colon in humans. The impact of the large depot of folate found in the colon on whole body folate status, and perhaps more importantly colonic health, is worthy of future investigation.

5 STUDY II: THE AMOUNT OF BACTERIALLY-SYNTHESIZED FOLATE IN THE COLON OF PIGLETS CAN BE MODIFIED BY ADDING OLIGOSACCHARIDES TO THEIR DIET

5.1 ABSTRACT

A large quantity of folate, often exceeding dietary intake, is present in the colon of humans. Recently we demonstrated that this pool of folate can be absorbed. The purpose of the present study was to investigate whether the net amount of folate synthesized by bacteria in the colon of piglets could be modified by incorporating prebiotics, known to selectively alter the composition of the microflora, into their diets. Five day old male piglets (n=12) were randomly assigned to be fed a milk-based formula diet with either 5g/L inulin + 5g/L galactooligosaccharides (IN-GOS), or 5 g/L maltodextrin (control) for 28 days. No difference in body weight was found between groups during the feeding intervention. After 28 days, the mean weight of colon tissue (37.9 %) and colon contents (178 %) was higher in the IN-GOS group than in the control group (P=0.0044, P=0.0003, respectively). Likewise, the total bacterial load in the colon of piglets fed IN-GOS was 531% greater, and the total amount of folate found in the colon contents 53.1% greater than that of the controls (P=0.0022, P=0.0218, respectively). Despite these observations, indices of blood folate status (plasma and erythrocyte folate and total plasma homocysteine) and folate concentrations in liver and kidneys did not differ between groups. Both groups of piglets showed evidence of folate deficiency, exhibiting low RBC folate (56±23 nmol/L) and elevated homocysteine (24±7 µmol/L), respectively. In conclusion, dietary supplementation with 5g of inulin + 5g of galactooligosaccharides increased colon tissue mass, bacterial load and total folate content in the colon of piglets; however these changes were insufficient to modify indices of whole body folate status. Future studies investigating the impact of feeding prebiotics on localized folate status at the level of the colonocyte are warranted.

5.2 INTRODUCTION

Folate, as a donor and acceptor of 1C units, plays an important role in amino acid metabolism, DNA synthesis and repair and methylation. Suboptimal intakes of folate have been associated with anemia in pregnancy and neural tube defects, low infant birth weight, stroke, neuropsychiatric disorders and cancers, notably colorectal cancer (23, 461-468). After public policy makers in North America mandated fortification of the food supply in 1998, blood folate concentrations rose and the number of neural tube defects decreased by ~50% in Canada (7, 469). Despite the tremendous success of the folic acid fortification strategy in reducing neural tube defects, there is concern that high dietary intakes of folate, and particularly supplemental intakes of folic acid, may not be beneficial to everyone. It is known that high levels of supplemental folic acid intake could delay diagnosis of vitamin B12 deficiency (16, 470-471). Other serious health risks have been proposed to be associated with higher folate intakes including breast, prostate and colorectal cancer (15, 18, 43, 258, 261, 263, 472-479).

It has long been known that the microflora in the colon of mammals produce large quantities of folate which approach or exceed levels of dietary intake (111, 355, 435, 439). We recently demonstrated that folate can be absorbed across the intact colon of humans (480) confirming earlier observations in animal models (34-35). Thus given its size and availability, a more comprehensive understanding of the contribution of microbially synthesized folate to the "input side" of folate nutrition may help to set dietary recommendations that strike the right balance between health benefits and risks.

We know that folate status in rats can be modulated by altering microbial production of folate in the colon through incorporation of additional fibre or prebiotics in the diet (40, 254, 278, 437). Inclusion of a fermentable source of fibre in the diet presumably increases folate production in the colon by increasing total bacterial load and hence the number of bacteria

synthesizing folate. In contrast, prebiotics selectively stimulate the growth and/or activity of one or a limited number of bacteria. Prebiotics such as the neutral oligosaccharides (galacto- and fructooligosaccharides) found in human milk, result in a net increase in bifidobacteria, potent synthesizers of folate (30, 71, 311, 326, 352, 481). An obvious limitation in extrapolating data collected from rat studies to humans is that these animals are coprophagic and hence, despite attempts by investigators to prevent its occurrence, in rats folate synthesized by microorganisms in the colon and excreted in feces could be absorbed across the small intestine.

The aim of the present study was to systematically investigate whether microbial biosynthesis of folate could be altered by diet, specifically by prebiotics, using the piglet as an animal model. Due to their similarities to the human in gastrointestinal physiology and development, piglets have been successfully used as an animal model in many areas of human nutrition, including colonic microbial fermentation and the digestion of fibre (48-50, 338-339, 482-483). Piglets offer the advantage of smaller size and the relative ease of manipulating their microflora compared to adult animals. Unlike rats, pigs do not practice coprophagy and have a similar mechanism of folate absorption to humans (44-47).

5.3 METHODS AND MATERIALS

5.3.1 Animal care

Twelve 5-day old male Yorkshire piglets were randomly assigned to one of two dietary treatments. As per usual animal husbandry practice, the piglets received an injection of iron (200 mg Fe as iron dextran) (Gleptosil, Champion Alstoe Animal Health Inc, Whitby ON) at birth. Piglets were housed in groups of 3 with a 12-h light: dark cycle at a controlled temperature of 23-24°C. Tap water was supplied *ad libitum*. Pens contained feeding troughs, a

polypropylene toy, and in one corner a cotton sheet and heat lamp for comfort. For the first two weeks pens contained wood shavings for bedding.

Piglets were fed milk-based formula diets every six hours, freshly prepared with warm water. The diets (Research Diets Inc., New Brunswick, NJ) were free of antibiotics and met the nutrient requirements for piglets as described by the National Research Council (1998). The compositions of diets are shown in **Table 5.1**. A common basal diet was supplemented with either 5 g/L maltodextrin (Control diet) or a 1:1 mixture of 5 g/L each of two oligosaccharide products: inulin (Raftiline HP Gel, Orafti Active Food Ingredients, Malvern PA), and galactooligosaccharides (Vivinal GOS, Borculo Domo Ingredients, the Netherlands).

Maltodextrin (Lodex 10, Cargill, Minneapolis, MN) is a mixture of polysaccharides and oligosaccharides with a DE of 10. It is frequently used as a carbohydrate source in infant formula, has been similarly used as a control carbohydrate in human infant studies, is well absorbed at the level of the small intestine and supplied about 20 kcal/L in the reconstituted formula (484).

The combination of inulin (source of long chain fructo-oligosaccharides) and short chain galactooligosaccharides was chosen to mimic the prebiotic, and specifically the bifidogenic effect of human milk (322). Human milk contains a complex matrix of oligosaccharides that selectively stimulate bifidobacteria and lactobacillus. Among the former are potent synthesizers of folate and among the latter, a greater proportion of species and strains with an obligate requirement for preformed folate (348).

Raftiline HP® is a chicory-derived, long chain inulin made up of fructose units linked by β-(2$\rightarrow$1) glycosidic bonds, with a terminal glucose unit, and an average chain length of $\geq$23.

Vivinal GOS is a mixture of 57% w:w transgalactooligosaccharides, 23% lactose, 22% glucose and 0.8% galactose (485). Together the insulin and galactooligosaccharide products are estimated to provide 20 kcal/L in the reconstituted piglet formula (32, 322, 378).

The amount of formula consumed was determined four times daily by measuring the volume of milk remaining in feeding troughs versus that provided. As a precaution against osmotic diarrhea, for the first two days of the study piglets were supplied with formula mixed in a 1:1 ratio with warm water. Formulas were gradually brought to full concentration over days three and four. Piglet body weight was determined at the same time daily (± 10g), using a MBS 2010 Digital Baby Scale (My Weigh, Vancouver, BC). The experimental protocol was reviewed and approved by the Animal Care Committee at The Hospital for Sick Children, Toronto.

TABLE 5.1
The composition of piglet formulas, reconstituted with warm water four times daily.

Ingredient	**Control Diet** *g/L*	**Inulin and Galactooligosaccharide-supplemented (IN-GOS) Diet** *g/L*
Skim milk powder	110	110
Whey powder	35	35
Maltodextrin 10	5	0
Inulin[1]	0	5
Galactooligosaccharides[2]	0	5
Soybean oil	16.5	16.5
Hydrogenated coconut oil	14	14
Safflower oil	29.25	29.25
Flax seed oil	0.25	0.25
Vitamin Mix[3]	0.5	0.5
Mineral Mix[4]	11	11
Total	221.5	226.5

[1]Raftiline HP Gel, Orafti Active Food Ingredients, Malvern PA,USA.
[2]Vivinal GOS, Borculo Domo Ingredients, the Netherlands.
[3]Vitamin.mix, per L prepared diet: vitamin A palmitate 2.2mg; vitamin D3, 2.2 mg; vitamin E acetate, 32 mg; menadione sodium bisulfite, 0.5 mg; biotin , 5 mg; cyanocobalamin, 17.5 µg; folic acid, 0.05 mg; nicotinic acid, 15 mg; calcium pantothenate, 10 mg; pyridoxine-HCl, 1.5 mg; riboflavin, 3.5 mg; thiamin HCl, 1 mg; choline chloride, 250 mg; sucrose 177 mg.
[4]Mineral mix, per L prepared diet: calcium carbonate, 1.0 g; calcium phosphate, 7.46 g; magnesium oxide, 332 mg; sodium chloride, 1.5 g; chromium potassium sulfate, 13 mg; cupric carbonate, 10.5 mg; potassium iodate, 0.25 mg; ferric citrate, 235 mg; manganous carbonate, 8.4mg; sodium selenite, 0.5 mg; zinc carbonate, 96 mg; sucrose, .344.35 mg.

Table 5.2 The calculated energy and protein content of piglet formulas, reconstituted with warm water four times daily

Criteria	Control Diet	Inulin and Galactooligosaccharide-supplemented (IN-GOS) Diet
Kcal/L	539	536
g protein/L	67.8	67.8

5.3.2 Sample collection

After 28 days of feeding, the piglets were serially anesthetized with Acepromazine (1mg/kg) (CDMV, St Hyacinthe, QC, Canada). Arterial blood samples were collected from the ear or the femoral artery in 4 mL tubes containing K_3 EDTA (Vacutainer, Becton-Dickinson, Franklin Lakes, NJ). Anesthesia was maintained using halothane, oxygen and nitrous oxide (2L/min oxygen, 2L/min nitrous oxide and halothane 1.5%) for six piglets (3 piglets per treatment), while for the remaining six (3 per treatment) halothane, and oxygen (2L/min oxygen, and halothane 1.5%) were used. Piglets were not fasted prior to surgery to allow for collection of intestinal contents.

The small intestines of piglets were removed, cleaned and weighed (Mettler, Toledo Inc, Mississauga, ON). To prevent mixing of intestinal contents, sections of the colon (1) cecum, (2) proximal coil, (3) distal coil and (4) terminal colon distal to the coil) were isolated prior to excision, using adjacent ligatures of umbilical thread (**Figure 5.1**). The proximal and distal coil in the piglet roughly equates to the ascending, transverse and a portion of the descending colon in the human (335, 483).

Immediately after weighing, the colon (with contents intact) was transferred to an anaerobic bag under an atmosphere of 80 % N_2, 10 % CO_2 and 10 % H_2 (AtmosBagTM, Sigma

Aldrich Co). Samples (average sample weight 0.9±0.6 g) of intestinal contents were collected directly from the cecum and from the mid points of each of the proximal and distal sections of the coil, into preweighed vials containing 12-mL aliquots of phosphate-buffered peptone water (Difco, Becton Dickinson and Company, MD), 0.03% cysteine hydrochloride, and 1 mL glycerol (299, 394). Additional samples of the intestinal contents were collected from the same locations in sterile vials for determination of folate concentration and moisture content, and frozen on dry ice until stored at -80°C. Thereafter the colon contents were removed, each section was rinsed with physiological saline and the tissue was weighed. The moisture content of intestinal contents was determined by weighing samples before and after drying to a constant weight in a drying oven (Blue M Electric Company, Blue Island IL). Piglets were euthanized after removal of the intestinal tract (sodium pentobarbital, 240 mg/mL). The liver and kidneys were perfused *in situ* with physiological saline and then removed. The liver was cleaned of fatty tissue and the organs weighed and cooled on ice until stored at –80°C.

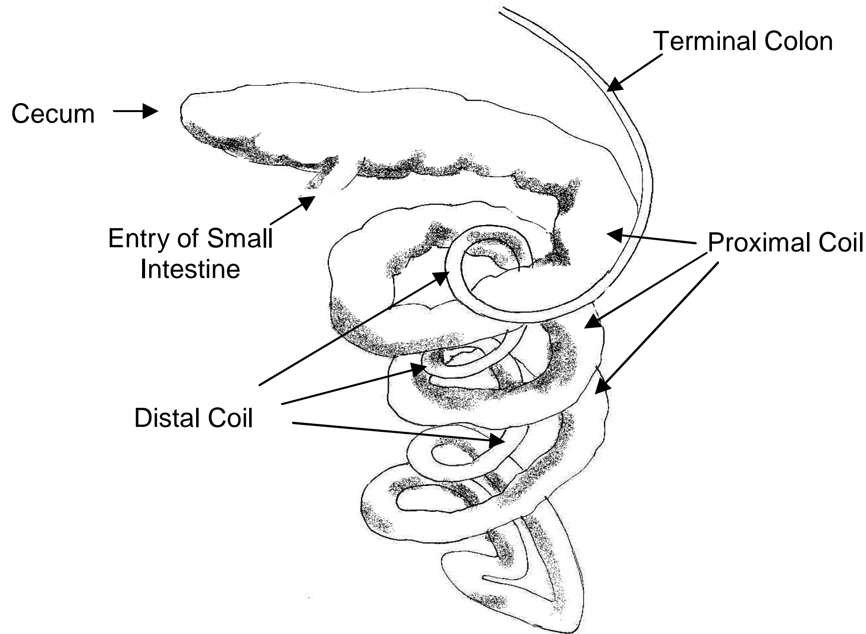

FIGURE 5.1 Sketch showing the structure of the piglet colon. Tissue and intestinal content weights were determined at 4 anatomical locations: (1) cecum, (2) proximal coil (3) distal coil and (4) the terminal colon. The proximal and distal coil roughly approximates the ascending, transverse and a portion of the descending human colon (335, 483).

5.3.3 Histology

Small tissue samples were excised at the time the piglets were killed throughout the colon of 6 piglets (3 per treatment) and fixed in 4% paraformaldehyde at room temperature overnight. The next day, the samples were serially dehydrated in ethanol, and paraffin embedded. Seven micron sections were made for each of three regions of the colon: (1) at the

beginning of the proximal coil, (2) at the beginning of the distal coil and (3) at the end of the distal coil. The sections were subjected to routine hematoxylin and eosin (H&E), and periodic acid-Schiff (PAS) histochemical staining, the latter to highlight goblet cell morphology. Colonic crypt depths were measured for each region of the colon. Ten fields were measured for each slide and the data averaged (Openlab 5.5, Improvision Inc, Waltham, MA).

5.3.4 Microbiology

Samples of anaerobically collected intestinal contents were thawed and weighed. Sample weight was calculated by difference. Vials were vortexed before removal of 1 mL aliquots for serial dilution in buffered peptone water. Aliquots of 0.1 mL of the diluted samples were spread-plated on Schaedler agar (Becton, Dickinson and Company, MD) for enumeration of total anaerobic load (TAN), and on Schaedler agar with 5% defibrinated horse blood (Cedarlake Laboratories, Hornsby, ON) for enumeration of total aerobic load (TAE). Rogosa agar (Merck KGaA, Darmstadt, Germany) was used for enumeration of lactobacilli, and MRS agar (Becton, Dickinson and Company, MD) for the enumeration of bifidobacteria. Media and incubation parameters are shown in **Table 5.3**. Anaerobic conditions were maintained in Gas Pak jars using a BBL CampyPak Plus Microaerophilic System (Becton, Dickinson and Company, Maryland). After incubation, plates containing 30-300 colony forming units were enumerated on a darkfield Quebec colony counter (American Optical Co., Buffalo, NY). Bacterial counts are expressed as the logarithm of colony forming units per gram of intestinal contents.

TABLE 5.3 Media and incubation parameters for enumeration of microorganisms

Microorganism	Media and incubation parameters
Total aerobes	Schaedler agar[1], 37 ^{o}C, aerobic, 72 h
Total anaerobes	Schaedler agar, 37 ^{o}C, anaerobic, 72 h
Lactobacilli	Rogosa agar[1], 37 ^{o}C, anaerobic, 72 h
Bifidobacteria	MRS agar[1] plus 0.03% cysteine-HCl agar, 37 ^{o}C, anaerobic, 72 h

[1]BDH Laboratories, Toronto, ON

5.3.5 Blood, tissue and intestinal contents and diet formula sample preparation and analysis

Blood samples were processed for analysis within two hours of collection. Hematocrit was determined in duplicate by centrifugation of freshly collected blood. A portion of whole blood was diluted 1:10 with a 1% solution of ascorbic acid and incubated for 30 minutes at 37 ^{o}C to convert folates to their microbiological assayable forms (short-chain folylpolyglutamates) then immediately stored at –80 ^{o}C until analysis. Plasma was separated from whole blood by centrifugation (1500 x g for 20 minutes at 4° C) and stored at –80 ^{o}C with and without added sodium ascorbate (1%). Folate was extracted from milk-based diet, organs and intestinal contents by homogenization of one gram of sample in 10 volumes of HEPES/CHES buffer (50 mmol/LHEPES, 50 mmol/L CHES, pH 7.85) containing 20g/L sodium ascorbate and 10 mmol/L 2-mercaptoethanol (91, 486). Homogenates were treated with α amylase (EC 3.2.1.1), protease (EC 3.4.24.31) (Sigma Chemical Co., St Louis, MO) and rat serum conjugase (Harlan Bioproducts for Science, Indianapolis, IN), as described by Lim *et al.* (1998), in order to free folates from cellular matrices, and to convert folates to their microbiologically assayable form (95-96). All enzyme solutions were treated with activated charcoal to remove residual folates.

In order to inactivate plasma folate binding proteins, piglet plasma and whole blood samples were diluted in 0.5% sodium ascorbate and heated (100 °C, 5 minutes), cooled on ice and vortexed immediately before bioassay (487-488).

Whole blood and plasma folates, and folate in enzyme-treated liver, kidneys and intestinal contents were determined by microbiological assay as described by Molloy and Scott with minor modifications, using *Lactobacillus rhamnosus* (ATCC #7469, American Type Tissue Culture Collection) as the test organism (489). Erythrocyte folate content was determined by difference, using the analyzed whole blood folate concentration minus the plasma folate content corrected for hematocrit.

The accuracy and reproducibility of folate extraction and enzyme treatment of diets, organs and intestinal contents was assessed by repeated measurement of a standard reference material of pig's liver with a certified value (13.3±1.3 mg/kg dry matter, CRM 487 Pig's Liver, European Commission Joint Research Centre Institute for Reference Materials and Measurements, Geel, Belgium). Analysis of the pig liver standard in our laboratory yielded a measured value of 14.7±0.9 mg/kg with an interassay CV of 6.1 %. The accuracy and reproducibility of blood folate analyses was assessed by repeated measurements of a whole blood standard reference material with a certified value (29.5 nmol/L)(Whole Blood 95/528; National Institute of Biological Standards and Control, Hertfordshire, United Kingdom). Analysis of this standard in our laboratory yielded a measured value of 32.2±1.8 nmol/L. with an interassay CV of 5.6 %.

5.3.6 Analysis for homocysteine by HPLC

Plasma total homocysteine was determined according to the method of Cole *et al*. (1998) by HPLC. The HPLC system consisted of a P580 pump with an ASI100 autosampler (Dionex Canada, Oakville, ON), a 4x50 mm OmniPac PCX-500 precolumn, a 4x250 OmniPac

PCX-500 analytical column and an EDJO electrochemical detector with a gold working electrode. System management and data processing were performed using Chromeleon Version 6.20 software. Reproducibility of homocysteine analysis was assessed by repeated measurement of pooled blood standard and yielded a CV of 1.6%.

5.3.7 Statistical analyses

Statistical analyses were performed using the Statistical Analysis System for Windows, version 9.01 (2002-2003). All data were tested for normality of distribution (PROC UNIVARIATE procedure) and where possible normalized by log transformation. Differences between treatment groups for normally distributed data were determined by ANOVA (PROC GLM procedure). Differences between treatment groups for data we were unable to transform to produce a normal distribution were analyzed using the Wilcoxon rank-sum test. Differences in daily body weights of piglets and in the volume of formula ingested at each meal were determined by repeated-measures ANOVA (PROC MIXED). A value of $P \leq 0.05$ was used to determine significance. Values are presented as means ± SD.

5.4 RESULTS

5.4.1 Piglet formula intake and body, tissue and intestinal content weights

The volume of formula consumed by piglets in the IN-GOS group (351±63 mL/kg/d) did not differ from that of the control group (356±73 mL/kg/d) during the 28 day feeding period. Likewise, the body weight of piglets in the two feeding groups did not differ (**Figure 5.2**). The energy intake and protein intake of the piglets, are shown in **Table 5.4**. Estimated daily dietary folate, vitamin B12 and choline intake exceeded NRC recommendations (**Table 5.5**). Samples of intestinal contents were obtained from the ceca, proximal coils and distal coils of all piglets. Samples were collected in the terminal colon of only four of twelve piglets, in the form of soft pellets, and statistical data for analytical data of terminal colon contents are

therefore not presented. The weights of intestinal contents, normalized to piglet body weight, are shown in **Table 5.6**.

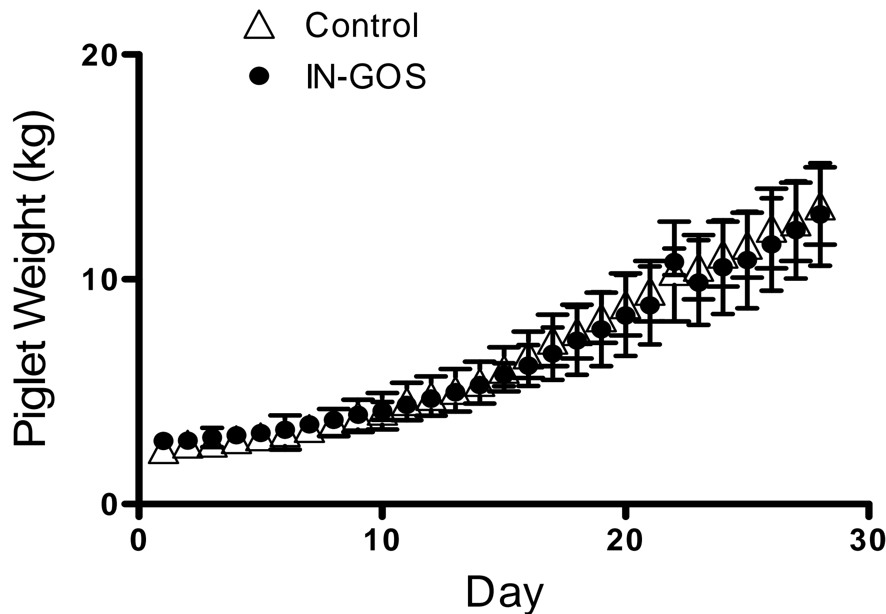

FIGURE 5.2 The body weights of piglets fed for 28 days on milk-based formula diets supplemented either with maltodextrin (Control diet) or with inulin and galactooligosaccharides (IN-GOS diet). Data are expressed as means ± SD. There was no statistically significant difference between the two feeding groups.

Table 5.4 The estimated energy and protein intake of piglets fed for 28 days on milk-based formula diets supplemented either with maltodextrin (Control diet) or with inulin and galactooligosaccharides (IN-GOS diet).

Criteria	Control diet	IN-GOS diet
energy intake	189 kcal/kg/d	188 kcal/kg/d
protein intake	23.7 g/kg/d	23.7 g/kg/d

Table 5.5 The estimated intake of folate, vitamin B12 and choline, for piglets who consumed milk-based formula diets at a rate of 0.351L/kg/d compared to the NRC recommendations for piglets (490).

Piglet weight *(kg)*	Nutrient	Estimated intake	NRC recommendation
3	Folate *(mg/d)*	0.19	0.08
3	Vitamin B12 *(µg/d)*	18	5
3	Choline *(mg/d)*	260	150
5	Folate *(mg/d)*	0.30	0.08-0.15
5	Vitamin B12 *(µg/d)*	31	5.0-8.8
5	Choline *(mg/d)*	440	150-250
10	Folate *(mg/d)*	0.60	0.15-0.30
10	Vitamin B12 *(µg/d)*	61	8.75-15
10	Choline *(mg/d)*	880	250-400

Both the wet and dry weights of the contents of the entire colon of IN-GOS fed piglets were greater than that of the control animals ($P = 0.003$ and $P<0.0001$, respectively). Similarly, the wet and dry weights of cecal contents, of proximal coil contents and the wet weight of distal coil contents of the IN-GOS group were significantly greater than those of the control group. Differences in the dry weights of distal coil contents approached significance. These differences were not related to differences in hydration of the contents as there was no difference in the moisture of intestinal contents of the two groups of piglets (**Figure 5.3**). Piglet groups did not differ in the mean weight of the liver or kidneys or in the mean weights of the

small intestine or distal coil (**Table 5.7**). However, the mean weights of the cecum, proximal coil and terminal colon, excluding intestinal contents, of the IN-GOS group were greater than those of the control group (*P*=0.0441, *P*=0.0083 and *P*=0.0042, respectively) (Table 5.7). The mean weight of the colon (including the cecum) of the IN-GOS group was greater by 39% that of the control group (*P*=0.0044) (Table 5.7).

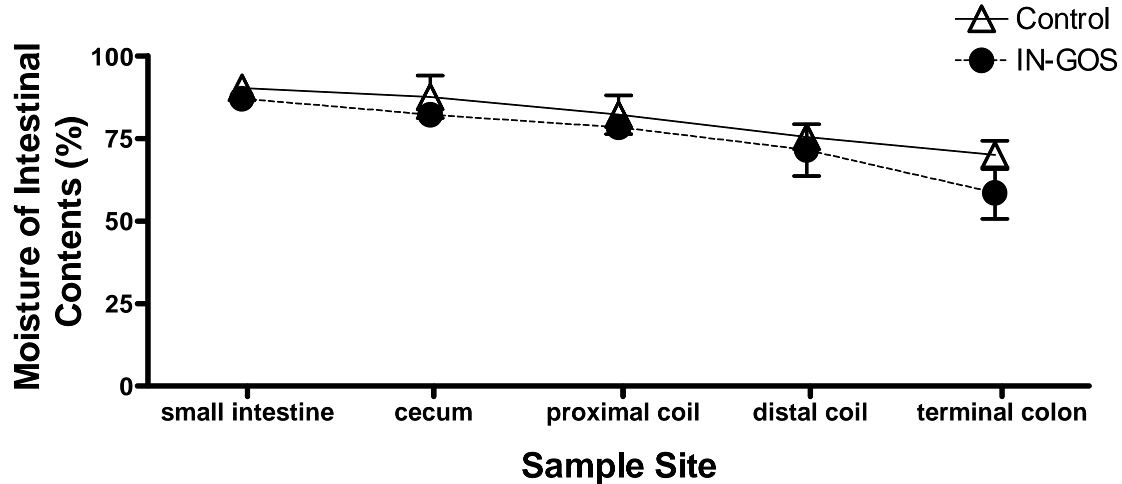

FIGURE 5.3. The percent moisture of intestinal contents of two groups of piglets, fed for 28 days on milk-based formula diets supplemented either with maltodextrin (Control diet) or with inulin and galactooligosaccharides (IN-GOS diet), for 28 days. Data are expressed as means ± SD. There was no difference between the two groups of piglets.

TABLE 5.6 The weights of the intestinal contents of piglets (n=12) fed milk-based formulas supplemented with either maltodextrin (Control) or inulin and galactooligosaccharides (IN-GOS) for 28 days[1].

Intestinal section	Control Diet *(g/kg)*	IN-GOS Diet *(g/kg)*	*P*
Entire Colon : wet weight	3.7±1.5	10.3±2.6	0.0003
Entire Colon : dry weight	0.8±0.3	2.7±0.7	<0.0001
Cecum: wet weight	1.1±0.4	2.0±0.8	0.0232
Cecum: dry weight	0.1±0.1	0.4±0.2	<0.0001
Proximal coil: wet weight	1.5±0.8	4.4±1.3	0.0010
Proximal coil: dry weight	0.2±0.1	0.4±0.2	0.0153
Distal coil: wet weight	0.8±0.5	1.9±0.7	0.0110
Distal coil: dry weight	0.2±0.1	0.5±0.3	0.0512
Terminal colon: wet weight	0.3±0.4	1.9±0.9	0.0085
Terminal colon: dry weight	0.10±0.1	0.8±0.4	0.0052

[1]Data are presented as means ± SD

TABLE 5.7 The body, and tissue weights of piglets (n=12), fed milk-based formulas supplemented with either maltodextrin (Control) or inulin and galactooligosaccharides (IN-GOS) for 28 days.[1,2]

Criteria	Control Diet (g/kg)	IN-GOS Diet (g/kg)	*P*
Body weight, age 5 days *(kg)*	2.3±0.2	2.5±0.4	0.3033
Body weight, age 33 days *(kg)*	13.5±1.7	12.9±2.0	0.5849
Liver *(g/kg)*	30.5±2.6	31.7± 3.7	0.5260
Kidneys *(g/kg)*	7.4±0.8	7.6± 1.3	0.8056
Small intestine *(g/kg)*	37.1±9.9	45.8±12.9	0.2179
Entire Colon[2] *(g/kg)*	9.5±1.8	13.1±1.7	0.0044
Cecum *(g/kg)*	1.3±0.3	1.6±0.2	0.0441
Proximal coil *(g/kg)*	3.7±1.0	5. 9±1.3	0.0083
Distal coil *(g/kg)*	1.9± 0.4	2.5±0.6	0.1015
Terminal colon *(g/kg)*	2.6±0.3	3.1±0.3	0.0042

[1]Data are presented as means ± SD

[2]Weights of colon tissues do not include the weight of intestinal contents

5.4.2 Histology

No appreciable histological changes were found between the two groups of piglets as seen under H&E staining (not shown). PAS staining which delineates goblet cells also did not show any differences in morphology in the three regions of the colon examined (**Figure 5.4**).

Mean colonic crypt depths were equivalent between control and experimental piglets regardless of the region of colon sampled (**Figure 5.5**).

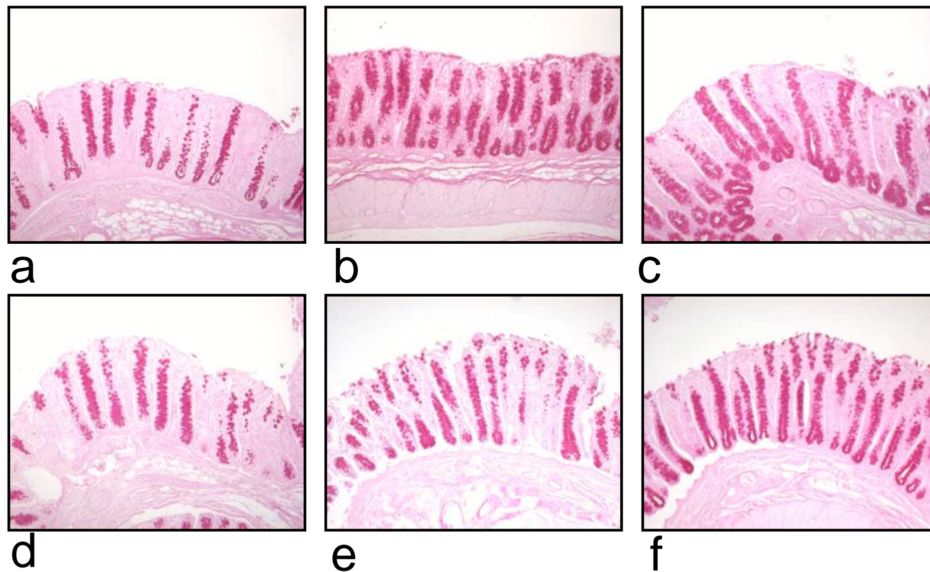

FIGURE 5.4 Periodic acid-Schiff (PAS) -stained sections from the colon of piglets (n=6) fed milk-based formulas supplemented with either maltodextrin (a, b, c) or inulin and galactooligosaccharides (d, e, f), for 28 days. PAS staining highlights goblet cell morphology. Sections were taken from the following locations of the colon: (a) and (d) the beginning of the proximal coil; (b) and (e) beginning of the distal coil; (c) and (f) beginning of the terminal colon. No difference in the morphology of sections from experimental and control piglets was found.

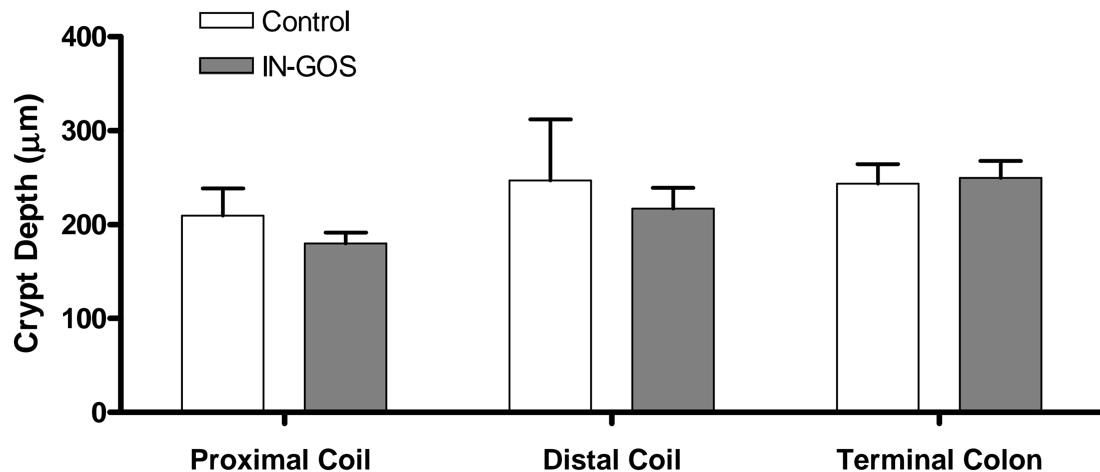

FIGURE 5.5 The colonic crypt depths measured in PAS-stained sections from the colon of piglets (n=6) fed milk-based formulas supplemented with either maltodextrin (Control) or inulin and galactooligosaccharides (IN-GOS), for 28 days. PAS staining highlights goblet cell morphology. There were no differences in crypt depth. Data are shown as means ± SD.

5.4.3 Microbiology

The results of bacterial enumeration of the colon contents are shown in **Tables 5.8** and **5.9**. The increase in total bacterial load and the anaerobic load expressed on a concentration basis (per gram wet weight) approached and reached statistical significance, respectively, for piglets fed the IN-GOS formula compared to that of the control group (P=0.0523 and P=0.0319). However, the concentrations of bifidobacteria and lactobacilli did not differ between the two feeding groups of piglets. Likewise, the total bacterial count in the colon (concentration x content wet weight) was significantly higher in the IN-GOS group than in controls (P=0.0022). No statistically significant difference was found in the cecum.

TABLE 5.8 Bacterial enumeration expressed on a per g wet weight basis, of the colonic contents of piglets (n=12) fed for 28 days on milk-based formulas supplemented either with maltodextrin (Control) or inulin and galactooligosaccharides (IN-GOS)[1]

Criteria	**Location**	**Control Diet** $log_{10}CFU^2/g$	**IN-GOS Diet** $log_{10}CFU/g$	***P***
Total bacterial load	Entire Colon	10.83±10.51	11.27±11.10	0.0523
	Cecum	10.40±10.36	10.70±10.75	0.3483
	Proximal coil	10.39±10.25	10.91±11.22	0.4267
	Distal coil	10.59±10.26	11.13±11.17	0.1501
Total anaerobic load	Entire Colon	10.69±10.30	11.25±11.10	0.0319
	Cecum	10.21±10.2	10.67±10.73	0.2066
	Proximal Coil	10.32±10.27	10.90±11.22	0.6991
	Distal Coil	10.08±10.11	10.72±10.68	0.0706
Total aerobic load	Entire Colon	10.27±10.37	9.77±9.61	0.2165
	Cecum	9.97±10.10	9.45±9.44	0.2527
	Proximal Coil	9.53±9.48	9.23±9.30	0.3095
	Distal Coil	9.78±10.11	9.15±9.29	0.4078
Bifidobacteria	Entire Colon	9.63±9.81	9.94±10.29	0.6069
	Cecum	9.40±9.62	8.39±8.43	0.5887
	Proximal Coil	8.64±8.56	9.9±10.28	0.5887
	Distal Coil	9.12±9.34	8.59±8.62	0.6991
Lactobacilli	Entire Colon	9.86±9.86	9.61±9.42	0.3573
	Cecum	9.60±9.67	9.44±9.42	0.9372
	Proximal Coil	9.39±9.51	8.84±9.02	0.2319
	Distal Coil	8.86±8.67	8.84±8.84	0.9293

[1] Data are expressed as means ± SD. [2]CFU = Colony Forming Units

TABLE 5.9 Bacterial enumeration expressed on a per section basis, of the colonic contents of piglets (n=12)fed for 28 days on milk-based formulas supplemented wither with maltodextrin (Control) or inulin and galactooligosaccharides (IN-GOS) [1]

Bacteria	Location	Control Diet *(log_{10} CFU^2)*	IN-GOS Diet *(log_{10} CFU^2)*	***P***
Total bacterial load	Entire Colon	12.08±11.89	12.88±12.89	0.0022
	Cecum	11.62±11.59	12.12±12.15	0.1645
	Proximal Coil	11.70±11.49	12.70±12.96	0.4848
	Distal Coil	11.44±11.59	12.10±12.07	0.0813
Total anaerobic load	Entire Colon	11.84±11.52	12.83±12.90	0.0022
	Cecum	11.230±11.19	12.07±12.011	0.1320
	Proximal Coil	11.55±11.36	12.64±12.95	0.3939
	Distal Coil	11.11±11.14	12.08±12.06	0.0488
Total aerobic load	Entire Colon	11.52±11.61	11.27±11.05	0.9919
	Cecum	11.13±11.31	10.85±10.83	0.4897
	Proximal Coil	10.92±10.91	10.96±11.03	0.9798
	Distal Coil	11.05±11.41	10.41±10.46	0.9372
Bifidobacteria	Entire Colon	10.77±10.89	11.74±12.11	0.5190
	Cecum	10.43±10.53	9.78±9.82	0.1675
	Proximal Coil	9.98±9.90	11.73±12.11	0.5710
	Distal Coil	10.35±10.62	9.93±9.99	0.9284
Lactobacilli	Entre Colon	11.07±11.13	11.07±10.82	0.5887
	Cecum	10.77±10.87	10.82±10.80	0.5887
	Proximal Coil	10.70±10.80	10.58±10.76	1.000
	Distal Coil	9.96±9.97	10.15±10.07	0.4278

[1] Data are expressed as means ± SD. [2]CFU = Colony Forming Units

5.4.4 The folate content of formula diets, intestinal contents, blood and organs

Analysis of formula diets consumed by the piglets in this experiment confirmed that the diets conformed to NRC recommendations for piglets: the folate content of the milk formula diets were determined to be 0.15 ± 0.01 mg/L and 0.14 ± 0.01 mg/L for the control formula and IN-GOS formula, respectively (490). Folate levels determined in intestinal contents are shown in **Table 5.10**. There was no difference in folate concentration per gram of colon contents. Considering the total colon contents, piglets on the IN-GOS diet showed higher folate than piglets on the control diet (P=0.0218). There were higher amounts of folate in the intestinal coil (P=0.0023), both in the proximal coil and in the distal coil of the piglet colon (P=0.0016, P= 0.0117, respectively). The two groups of piglets did not differ in the mean concentrations of plasma folate, erythrocyte folate or plasma total homocysteine (**Table 5.11**). Mean liver and kidney folate of the two groups also did not differ (Table 5.11).

TABLE 5.10 Folate content in the colons of piglets (n=12) fed milk-based formulas supplemented with either maltodextrin (Control Diet) or inulin and galactooligosaccharides (IN-GOS Diet) for 28 days.[1]

Criteria	**Folate** *(nmol/g wet weight of contents)*		
	Control Diet	**IN-GOS Diet**	***P***
Cecum	11±7	11±3	0.5689
Proximal Coil	15±10	18±7	0.9571
Distal Coil	15±9	17±6	0.7774
Terminal Colon	0.1	0.05±0.01	-
	Total folate *(nmol/intestinal section/kg piglet)*		
Entire Colon	597±266	914±362	0.0218
Cecum	175±135	132±61	0.5018
Proximal Coil	243±139	628±169	0.0016
Distal Coil	129±73	263±78	0.0117
Terminal Colon	0.03±0.04	0.09±0.04	-

[1]Data are expressed as means ± SD.

TABLE 5.11 Folate concentration in the blood, liver and kidneys, and plasma homocysteine concentrations of piglets (n=12) fed milk-based formulas supplemented with either maltodextrin (Control) or inulin and galactooligosaccharides (IN-GOS) for 28 days.[1]

Criteria	**Control Diet**	**IN-GOS Diet**	***P***
Plasma folate (nmol/L)	43.7 ± 5.7	36.3 ± 14.9	0.2826
Erythrocyte folate (nmol/L)	57 ± 32	55 ± 15	0.9045
Total plasma homocysteine (µmol/L)	23.6 ± 7.2	23.7 ± 6.2	0.9883
Liver folate (nmol/g)	3.19 ± 1.11	3.14 ± 0.41	0.9179
Kidney folate (nmol/g)	1.41 ± 0.49	0.98 ±0.65	0.2070

[1]Data are expressed as means ± SD.

5.5 DISCUSSION

Results from this study suggest that feeding 5g/L of inulin in combination with 5g of GOS in milk-based formulas for 28 days increased both the weight of the colon of piglets (37.9%, P=0.0044), as well as the total bacterial load of their colonic contents (317%, P=0.0022). These increases were not accompanied by increases in the volume of formulas consumed, in body weight, or organ weight nor did they affect colonic crypt morphology after 28 days of feeding. Our observations herein indicating an increase in the weight of the colon with the IN-GOS diets are consistent with previously published studies of rats and pigs fed diets containing various sources of fibre or resistant starch including raw potato starch and

human milk solids (rich in galacto- and fructooligosaccharides) (40, 302, 398, 404, 407, 491-492). Not only was the total dose of maltodextrin in the control formula lower than the dose of oligosaccharides added to the IN-GOS formula, but because of its digestibility, the added maltodextrin should have been completely absorbed in the small intestine. The mechanisms by which soluble fibre and resistant starch increase intestinal proliferation are not completely understood but *in vivo* studies of rat and pigs, and *in vitro* studies using fecal and microbial cultures from humans and pigs suggest increased microbial synthesis of butyrate plays an important role (33, 40, 332, 374, 422, 493). In piglets, increased epithelial proliferation along the length of the colon, particularly in the proximal colonic epithelium has been linked to microbial butyrate production (50, 494-495). In suckling piglets, neither colonic butyrate or inulin affects serum GLP-2 thus the effect of butyrate may to supply a source of energy to the colon (493). It has been shown *in vitro* that microbial butyrate synthesis associated with lactic acid bacteria may be a result of cross feeding among the swine microflora, specifically that in swine cecal digesta, lactate and acetate produced by lactic acid bacteria provides substrate to butyrate-producing bacteria (496). In rats short-term intake of fructooligosaccharides is associated with increases in lactic acid bacteria, while longer feeding of these prebiotics elevates cecal butyrate concentration (374). In piglets, fructooligosaccharide intake has also been found to increase colonic butyrate (420, 497-498).

The oligosaccharides used in this study, inulin and GOS are preferentially fermented by bifidobacteria and lactobacilli in humans (31, 313, 321, 397, 499). Unlike most reported studies of human infants fed these prebiotics, we did not observe an increase in concentration of either bifidobacteria and/or lactobacilli in the colon contents (29). The dose of oligosaccharides (5g/L inulin and 5g/L galactooligosaccharides) totaling approximately 3.5 g/d wet weight (4.4% of dry matter in feed), fed in the current study was well within the range of

that provided in human infant formula feeding trials where changes in bifidobacteria and/or lactobacilli counts in stools were observed (29, 32). Also, the concentration of inulin and GOS in the piglet formula were well within the range of oligosaccharides found in human milk (29). Likewise the forms of specific oligosaccharides fed, inulin and GOS, have both been shown to be bifidogenic in human infants (32, 52, 386, 499-503).

Whether, or not, oligosaccharides have a bifidogenic effect on piglets, and at what dose, is less clear than in the human infant literature. In piglets, in the absence of antibiotics, stress and physiological changes during weaning are associated with slower growth, increased diarrhea and mortality, and prebiotics are a means to support piglet health during weaning (414). As a result, more information is available on the weanling piglet's response to prebiotic treatments, although suckling piglets as weaned piglets have shown differences in microflora and potential differences in response to prebiotics (337, 415-416).

Among suckling piglets, formula-fed neonatal piglets consuming 0 or 3g/L fructooligosaccharides for 6d showed a trend to greater numbers of fecal bifidobacteria ($P=0.08$), a result deemed to suggest enhancement of bifidobacteria populations by fructooligosaccharides (302). A human milk oligosaccharide, fed to 3 day old piglets at 0.2 g/d for 30 days was found to increase both bifidobacteria and lactobacilli in the piglets (417). Seven-day old suckling pigs fed creep feed supplemented with oligofructose at 2g/kg, in combination with a mixed prebiotic, showed increased numbers of bifidobacteria in the colon ($P<0.05$) (418).

Weanling pigs whose diet was supplemented with oligofructose at a dose of 3g/d showed increases in numbers of colonic bifidobacteria, as did weanlings supplemented at 3g/d concurrently administered probiotic lactobacilli for 20d (419-420). Weanling piglets fed fructooligosaccharides (0.5g/kg) for 4 weeks also showed significant increases in bifidobacteria

numbers ($P<0.05$) (421). Male weaned 28d old piglets fed 0, 16 or 40g/kg GOS or 16g/kg inulin showed significant increases in colonic bifidobacteria only on 40g/kg GOS (422). One study supplementing the diet of 28d old weanlings with inulin at 3g/kg of dry feed, found that inulin increased the number of piglets with colonic bifidobacteria (423). Three week old piglets fed acid-resistant oligosaccharides at 5g/kg showed increased bifidobacteria numbers, as did fourteen week old pigs fed fructooligosaccharides with a high-amylose starch diets concurrent with a probiotic, and six week old piglets fed high amounts (138 and 221 g/kg, respectively) of chicory roots and sweet lupine (424-426).

We determined substantial numbers of bifidobacteria and lactobacilli in the colon contents of all the piglets, but there was no clear bifidogenic effect. Both groups of piglets had similar bifidobacterial concentrations and high standard deviations were measured, and comparatively high bifidobacteria concentrations were found. Bifidobacteria were enumerated in all piglets. A possible explanation for these results may be that the basic diet formula supported the growth of bifidobacteria to a degree sufficient to confound demonstration of a dose-response relationship. In humans, demonstration of a dose response to inulin and oligofructose depends on low initial bifidobacterial growth in the subjects (504).

However, others have found a mixed or negative relationship between oligosaccharide intake and changes in bifidobacteria and lactobacillus content of the colon of piglets. For example, 17 day old sucklings fed fructooligosaccharides and arabinogalactan 4d prior to weaning showed no effects after weaning, on total anaerobes or bifidobacteria (427). The numbers of colonic bifidobacteria were found to increase after neonatal piglets were fed diets supplemented with oligofructose at 3g/L after 6 days but not after 15 days (302).

In weaned piglets fed inulin at 30g/kg dry matter for 3 and 6 weeks, no change was found in the numbers of bifidobacteria or lactobacilli, and similarly, after 15g/kg Jerusalem

artichoke flour or 15g/kg oligofructose supplementation of 28 day old piglet diets for 4 weeks, there was no change in numbers of bifidobacteria, anaerobes or coliforms (423, 428-429). Others have found increases of lactobacilli or decreases in bifidobacteria in the cecum, colon or feces of pigs fed yeast mannan oligosaccharides at a dose of 0.1 g/kg, or fructooligosaccharides at a dose of 3g/d (430). Despite variable results, Flickinger (2003) commented on the increasing use of oligosaccharides for health promotion during the weaning period by maintenance of bifidobacteria, in Japan and Europe (505).

A possible influence on our results may that both lactobacilli and bifidobacteria may ferment oligosaccharides, however while strains of bifidobacteria are reported to synthesize folates, lactobacilli are more often consumers than producers of folate (348). One author remarked that in pigs, fructooligosaccharides stimulate lactobacilli but not bifidobacteria (296). However, we did not observe increases in the concentrations or the total numbers of either lactobacilli or bifidobacteria.

As the concentration of neither bifidobacteria and/or lactobacillus changed in the colons of our piglets, it is not surprising that the concentration of folate in the intestinal contents likewise did not differ between the two feeding groups. Nonetheless, the total bacterial load was greater in piglets fed formulas containing IN-GOS compared to maltodextrin, and the total amount of folate produced was similarly greater.

Piglets in our study showed no difference in the folate concentrations of (wet) intestinal contents. Individual piglets carried between 0.6 and 3.9 µmol folate in the lumen of the colon, with a range of folate concentrations of 7.2-19.0 µmol/L in the cecum, rising to from 7.2 to 32.7µmol/L in the intestinal coil. While these concentrations may be dependent on homeostatic interrelationships among the microflora of each intestinal region, they may also be affected by folate absorption across the colon, and by pH, which in turn is influenced by bacterial

metabolism. The colons of piglets of the IN-GOS group have a larger luminal surface area, and the higher amounts of folate produced by the oligosaccharide-treated microflora may therefore be continually depleted by intestinal absorption. By comparison, Kim *et al.* (2004), found that 10d old piglets, younger and thus with a less developed microflora than in our study, fed a similar milk-based formula diet but without addition of prebiotics, excreted about 0.3 µmol/d of folate (5). This fecal folate represents folate not absorbed during intestinal transit.

Despite a 53 % increase in the total amount of folate present in the colon of piglets fed IN-GOS versus maltodextrin, we did not see differences in blood, liver or kidney folate concentrations between the two feeding groups. There may be a number of reasons for this observation. First, folate may not be absorbed across the colon, or inadequate folate may be absorbed, to affect folate status. However, it has been shown previously that microbial folate is absorbed across the piglet colon (35).

The piglets do not show abnormal indices of folate status: blood folate concentrations and liver and kidney folates in our piglets are similar to literature values for piglets of their age (47, 506-508). However, plasma homocysteine levels of piglets at four to five weeks of age are relatively high, and are believed to arise from their very rapid growth rate, which creates a high demand for S-adenosylmethionine as a carbon donor, especially for creatine synthesis (509-510). This high growth rate may lead to a period of effective deficiency with respect to micronutrients such as folate. Folate promotes the formation of methionine from homocysteine, and methionine is needed to form S-adenosylmethionine (509, 511). The piglet GI tract is known to have a high methionine requirement, and metabolism of this amino acid generates a significant contribution of homocysteine to the systemic circulation, in the absence of adequate folate to regenerate methionine from homocysteine (51, 512-513). At the relatively low dietary folate intake levels of these rapidly growing piglets, plasma homocysteine would

therefore be expected to rise in the treatment group relative to controls, associated with their higher colon tissue weight and consequent a higher need for folate. Nevertheless, differences in plasma homocysteine between the two feeding groups were not observed. Folate has been shown to effectively lower plasma homocysteine in pigs (514). We suggest that higher amounts of colonic folate absorbed across the greater colonic surface area available, compensated for higher homocysteine production in the enlarged colons of the IN-GOS piglets. Additional folate absorbed across the colon of the IN-GOS piglets may have been insufficient to noticeably raise plasma or liver folate, in the conditions of high nutrient demand of rapidly growing piglets, but analysis of tissues with rapidly dividing cells, including the colonic mucosa, may have revealed increased folates.

In conclusion, supplementation of the piglet diet with a combination of inulin and GOS increases the weight of the colon, the total bacterial load in the colon, and the amount of folate in colon contents, but does not result in increased indices of folate status. These oligosaccharides do not promote the growth of bifidobacteria in piglets but appear to exert their effect by increasing the supply of energy to the microflora in the form of fermentable substrate. Increases in colonic cell proliferation in the treatment group, unaccompanied by increased total plasma homocysteine, suggest that folate absorbed across the colon supplied the incremental folate used in cell division. Further investigation may be warranted to examine the role of colonic folate in gastrointestinal development.

6 CONCLUSIONS AND FUTURE DIRECTIONS

6.1 CONCLUSIONS

The focus of this thesis is the absorption of microbially synthesized folate across the colon. There is a large amount of folate in the colonic lumen of mammals, a high proportion of it in a form that would be bioavailable if in the small intestine (5). Animal studies, chiefly of rats, suggest that microbially produced folate impacts folate status (40, 191, 254, 278). The absorption of microbial folate across the colon of both rats and pigs has been confirmed by tracer studies, although in rats coprophagy may enable the absorption of microbially-produced folate via the small intestine (34-35). In humans, *in vitro* work suggests folate may be absorbed across the colon (36-38, 134). Secondary analyses from an observational and an intervention study have shown that an increased supply of carbohydrate to the colonic microflora is associated with increased folate status in humans (358-359). These studies suggest that an increase in the supply of fermentable substrate, such as dietary fibre, to the microflora, increases the amount of folate available for absorption across the colon. However, there is no direct evidence for folate absorption across the intact human colon, and little is known about the impact of dietary fibre on microbial folate synthesis or on host folate status.

In the first study described in this thesis, *in vivo* evidence is presented for the first time that folate is absorbed across the intact colon of humans. During colonoscopy, a physiological dose of ^{13}C-5-formyltetrahydrofolic acid was delivered in the form of an infusion to the cecum of six adults, enabling estimation of a rate of folate absorption by measurement of the appearance of ^{13}C-5-methyltetrahydrofolic acid in plasma. We found that folate was absorbed at an estimated rate of 0.6 ± 0.2 nmol/h. In comparison, the rate of increase of ^{13}C-5-methyltetrahydrofolic acid in plasma was 7 ± 1.2 nmol/h after an IV injection of one quarter the dose of ^{13}C-5-methyltetrahydrofolic acid delivered to the colon. The rate determined for colonic absorption of folate is much lower than the rate estimated for absorption of a similar

dose of the same form of folate across the small intestine, 34 nmol/h. We monitored the absorption of folate across the colon for four hours after infusion and during this time interval the amount absorbed increased linearly, with no clear maximum. We hypothesize that this pattern may be characteristic of absorption across the colon, given that the residence time for colon contents is of the order of 1-3 days (283). While folate absorption across the small intestine occurs at intervals comprising 2-3 hours after ingestion of food, it is likely that folate uptake across the colon continues between meals and during periods of fasting, and may provide a source of folate for distribution to tissues, including colonocytes, with high rates of cell division or metabolic activity requiring a steady folate supply. This supply would likely be replenished by microbial activity, concurrent with luminal absorption.

Differences among subjects in the rates of metabolism of the absorbed folate were revealed by stable isotope labeling of the administered folate dose and tandem mass spectrometry. 5-Formyltetrahydrofolic acid is thought to be metabolized in the mucosal epithelium to 5-methyltetrahydrofolic acid before transport to the systemic circulation (200, 205). However, unmetabolized $^{13}C_5$-5-formyltetrahydrofolate was determined in the plasma of some subjects, but only its metabolite, $^{13}C_5$-5-methyltetrahydrofolate, was detected in others. This variation in first pass metabolism may reflect differences in the expression of folate enzymes in colonocyte. It may be significant and warrant further investigation, that all the subjects with unmetabolized $^{13}C_5$-5-formyltetrahydrofolate were obese, and that all were taking a common proton-pump inhibitor.

Limitations of this study include the restrictions imposed by the subject population of screening colonoscopy patients. Bowel cleansing removes the influence of the bulk of the normally resident microflora from the colonic environment (160, 162). The presence of an undisturbed microflora may reduce folate absorption by competition for available folate;

alternatively, it may increase folate absorption by the effect of the microflora on the luminal environment, such as by influencing the pH of the mucosal surface to be closer to optimal conditions for activity of folate transporters. Colonic absorption of folate in the presence of an intact microflora remains to be investigated.

Further, the generalizability of results from 6 subjects may be limited. All the subjects were $\geq$ 50 years of age, three were heterozygous for the C677→T polymorphism in the gene for MTHFR, three were obese, one had a low vitamin B12 value and one was taking folic acid supplements 16 days prior to the study. Aging in the absence of disease does not appear to impact folate absorption, but both the aforementioned genetic polymorphism and adiposity may impact folate metabolism (455, 457, 515-517). Severe vitamin B12 deficiency is known to block the uptake of folate into tissues (442). As a group, our subjects had high blood folate concentrations, compared to pre-fortification of the food supply, and high levels of folate may down-regulate folate absorption (199).

The second study described in this thesis uses a piglet model to investigate the influence of diet on microbial folate production, and represents a further step towards our long-term objective to explore the relationship between dietary fibre, the modulation of folate production by the colonic microflora, and its effects on human folate status and colon health. Piglets have been successfully used to model the related areas of human gastrointestinal physiology and digestion, microbial digestion of dietary fibre, the microbial production of energy sources for the colon, and gastrointestinal development (48-49, 51, 338-339, 483, 518). They are suitable for modeling human folate absorption because of their similarity to humans in the enzymes required for absorption of food folates across the small intestine, their small size compared to adult pigs and the greater ease of modifying the microflora of nursing animals (44-47).

Five-day old piglets were randomly assigned to be fed one of two milk-based formula diets, one supplemented with 10 g/L of oligosaccharides (IN-GOS) with a structure related to the core structure of human milk oligosaccharides and similar to a mixture of oligosaccharides known to promote increases in bifidobacteria in human infants. The control diet was supplemented with 5g/L of maltodextrin. Results showed that supplementation of the diet with IN-GOS for 28 days increased the total bacterial load, and the concentration of anaerobes in the piglet colon. As a result, piglets fed a diet supplemented with IN-GOS also had a significantly higher amount (53.1%) of folate in the colon contents. However we found no evidence that the concentration of bifidobacteria increased in piglets on the IN-GOS diet, or that there was an increase in the folate concentrations of intestinal contents. Our data indicates that in nursing piglets, the relationship between diet and folate production is mediated entirely by increases in the energy supply, in the form of fermentable substrate, for the colonic microflora. This increase in fermentable substrate increased the bacterial load, and resulted in an increased bacterial production of folate.

No differences in folate concentrations of piglet blood, liver or kidneys were found. This may be because folate was not taken up, or was absorbed in inadequate amounts to affect blood and organ folate concentrations. However, folate is known to be absorbed across the colon of piglets (35). Possibly the increase in folate production was insufficient to alter indices of folate status. A third possibility remains, that folate was indeed absorbed but remained localized in the colonocytes. Our results showing increases in the weight of colon tissue in piglets on the IN-GOS diet suggest that the additional increment of folate produced (microbiologically synthesized) supported the observed increase in colonic tissue mass. Similar cell proliferation in the piglet colon has been described by others, in response to

microbial butyrate production (50). Our results suggest that colonic folate may play a role in this trophic effect.

A limitation to this study is that the protocol did not include tracing the fate of absorbed colonic folate in the piglets, or to compare colonic folate concentrations in the two groups. Blood folate indices suggest that the piglets were folate deficient, with the result that during rapid growth folate was probably distributed to rapidly growing tissues rather than to storage in liver and kidneys (490). Our data suggest that production of folate by the piglet microflora may be related to the supply of fermentable substrate for these piglets, and the increase in total bacterial load, rather than to any prebiotic effect the IN-GOS had on the concentration of bifidobacteria.

6.2 FUTURE DIRECTIONS

Results from this thesis provide evidence that folate can indeed be absorbed across the human colon, and that increasing the bacterial load via the provision of a fermentable source of fibre, can result in a net increase in the microbial synthesis of folate. Research should be undertaken to investigate the influence of the diet and the intestinal microflora on colonic folate absorption. Given the role that adequate folate nutrition plays in the prevention of birth defects and colorectal cancer but the real possibility of negative health consequences including promotion of colon cancer at high intakes of folate, it is clear given our research findings that there is merit in continuing future studies in this area.

We suggest that there are three key areas of future research. First, we demonstrated herein that folate can be absorbed across the intact colon of humans (Chapter 4). However, as acknowledged, this observation took place following bowel preparation for colonoscopy. The total bacterial load of the colon in this instance was therefore significantly reduced, and, no doubt, there could have been a significant shift in the species of bacteria as well as in the

luminal environment in terms of pH and the concentrations of biologically active microbial metabolites adjacent to the mucosa (453).

A focus of our on-going work in this area is on mechanisms to deliver folate, or its precursor *p*-aminobenzoic acid, past the ileocecal junction to the colon without disturbing the microflora. The development by our group of a colon-targeted capsule delivery system opens the way to the study of colonic folate absorption in the presence of an intact microflora (519). We are currently completing the laboratory work for a recently completed clinical trial where we used these pH-dependent caplets to deliver a physiological dose of ^{13}C-labeled [6S]-5-formyltetrahydrofolic acid to the colon of healthy adults to measure folate absorption as determined by the appearance of $^{13}C_5$-methyltetrahydrofolic acid in plasma. This delivery mechanism will enable us to study factors affecting colonic folate production and absorption.

The second key area of future research is investigation of the impact of manipulation of microbial biosynthesis of folate in the colon on folate metabolism in the colonocyte specifically. Most simply this could be accomplished by using the piglet model described in Chapter 5 and feeding either the prebiotics we worked with herein (*i.e.*, 5g/L inulin plus 5g/L galactooligosaccharides) or others, to see what impact colonic microbial folate production has on the folate content of the colonocyte – including mitochondrial and cytosolic fractions. In Chapter 5, we did not see an impact of feeding prebiotics on indices of folate status in piglets, but there is significant evidence in the literature that folate depletion of specific tissues can occur in the absence of systemic folate depletion (20-21, 267, 520-521). This may be particularly true in our animal model where it appears that the piglets may have been folate deficient. It would also be of interest to examine key regulatory enzymes involved in folate metabolism within the colonocyte to gauge the impact of prebiotics on them specifically.

The partitioning of colonic folate between the colon and the systemic circulation, its influence on colonic health and particularly its relationship to colorectal cancer are important topics currently not well understood. The influence of high folate status such as in supplement takers, on colonic folate absorption should be clarified in view of the finding of increased recurrence of adenoma in supplement takers (261). Our study protocol did not permit assessment of the amount of absorbed folate remaining in the colonic mucosa, though our data showed variability in colonic folate metabolism of the absorbed folates.

The relationship of microbially produced folate to the dual role of folate in both folate deficiency and overnutrition, and as a risk factor in the progression to colorectal cancer should be pursued (17, 449). Data regarding the benefit of fruit, vegetable and fibre intake in regard to colon cancer risk have been contradictory (450). The role of microbial folate in colorectal cancer is not well understood, but several possible routes of influence for the intestinal microflora on colorectal cancer development have been reviewed, including microbial folate production (522). Bifidobacteria have been shown to suppress azoxymethane- induced colonic aberrant crypt foci in rats, and the bifidogenic long-chain oligosaccharide inulin also has this property (394, 523). Further, butyrate, a product of intestinal bacteria, is effective in promoting apoptosis, associated with colon cancer inhibition, in colonocytes (524).

A third logical area of future research is to investigate the impact of high folate status and folic acid supplementation specifically in humans, on folate absorption across the colon. There is evidence from *in vitro* studies that long-term exposure of human colon-derived epithelial cells to high folate results in down–regulation of folate absorption at the level of transporter RNA and protein (199). Colonic mucosal folate levels correlate well with plasma folate at normal levels of folate nutrition, however, in overnutrition this correlation is lost (269). Given the very high intake of folic acid in North American it would be interesting to

know whether high circulating folates may down regulate folate transporters in the colon and thereby reduce the availability of bacterially synthesized folate. Could colonocytes under normal circumstances preferentially use bacterially synthesized folate in which they are bathed continuously versus systemic sources of the vitamer?

The active mechanism, *in vivo*, for folate transport across the colon is unclear: early work suggests a mechanism similar to that in the small intestine, which has been shown to be mediated by the proton-coupled transporter (36-38, 134, 162). However, along the length of the colon, efficiency of folate transport is likely to be affected by the viscosity of intestinal contents, by luminal pH at the mucosal surface and by the expression of two transporters, the PCFT and RFC (160, 162). Production of short chain fatty acids by a healthy microflora tends to lower pH and so may influence the activity of the expressed transporters, since the pH optimum for the PCFT is pH 5.5 and that of the RFC, near neutral at pH 7.4 (158, 161, 285). Studies of the effect on colonic folate absorption of changing pH and of butyrate at physiological concentrations may help to clarify the role of folate in the intestinal trophic response to butyrate.

Our data show that colonic folate enters the systemic circulation. The continual influx of low amounts of colonic folate around the clock, between short periods of high folate uptake across the small intestine after meals, may also provision other rapidly dividing tissues than the colon, and in so doing, reduce the turnover of folate pools and its metabolic cost, in depots such as the liver and kidneys and the other potential target tissues of microbial synthesized colonic folate should be investigated. However, the significance of colonic folate absorption to optimal function beyond colonic health may be difficult to establish, in an era of folate fortification, supplementation and overnutrition. If the colonic folate reservoir plays a significant role in provisioning the colon and other rapidly dividing tissues, then future assessments of folate

requirements should address tissue-specific needs for folate and include consideration of folate absorbed across the colon.

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8 APPENDICES

8.1 SUPPLEMENTARY TABLE

The change in unlabeled plasma folates over time, after cecal infusion of $^{13}C_5$-5-formyltetrahydrofolic acid

Subject (MTHFR status[1])	Blood Folates at Screening		5-Formyltetrahydrofolic acid	5-Methyltetrahydrofolic acid
	Plasma folate	Red blood cell folate	slope	net increase
	(nmol/L)	(nmol/L)	(nmol/h)	(nmol)
a (CT)	34.4	1160	0.05 ± 0.04	-1.2
b (CC)	27.5	638	0.29 ± 0.07	7.5
c (CC)	50.8	1420	2.7 ± 0.2	19.5
d (CC)	21.2	1130	0.12 ± 0.04	8.7
e (CT)	79.4	702	0.22 ± 0.04	-36.7
f (CT)	75.4	1930	0.57 ± 0.03	102
Mean ± SEM	50 ± 12	1160 ± 195	0.7 ± 0.5	20 ± 20

[1]MTHFR status: methylene tetrahydrofolate reductase status for the 677C→T polymorphism.

8.2 SUBJECT CONSENT FORM FOR STUDY I

CONSENT TO PARTICIPATE IN A RESEARCH STUDY

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

Investigators:
SickKids Hospital

Deborah L. O'Connor, PhD	Principal Investigator	(416) 813-7844
Susanne Aufreiter	PhD Candidate under the supervision of Dr O'Connor	(416) 813-5777

St. Michael's Hospital

Young-In Kim, MD	Division of Gastroenterology Site Principal Investigator	(416) 864-5848
Norman Marcon, MD	Division of Gastroenterology, Co-Investigator	(416) 864-3092

After regular work hours, dial (416) 864-5431 and ask to speak to the gastroenterology fellow on call.

Research Coordinators

Maria Cirocco, RN	Gastrointestinal Research	(416) 864-6060 ext 2965
Nancy Basset, RN	Gastrointestinal Research	(416) 864-6060 ext 2964
Heather White, CRA	Gastrointestinal Research	(416) 864-6060 ext 2671

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

INTRODUCTION

We understand that your gastroenterologist has recommended that you have a colonoscopic examination. The colonoscopy will be explained later in this document. We are inviting you to participate in a research study in addition to that examination. Here we describe the objectives of the study and inform you of what you would need to do if you decide to participate.

Before agreeing to participate in this research study, it is important that you read and understand this research consent form. 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. If you have any questions after you read through this form, ask your study doctor or study personnel. You should not sign this form until you are sure you understand everything on this form. It is important that you are completely truthful with your study doctor with respect to your health history and any medication you may be taking in order to prevent any unnecessary harm to you should you decide to participate in this study.

PURPOSE OF THE RESEARCH

Folate, sometimes called folic acid, is a vitamin needed especially in growth and development. Pasta and flour are fortified with folic acid in the Canadian food supply, and adequate blood folate levels have been shown to reduce the incidence of some birth defects (Spina Bifida) and certain cancers, including colon cancer.

However, high supplementary intakes of folic acid may mask a deficiency of the vitamin B12, a vitamin needed for maintenance of blood vessels and the nervous system. Vitamin B12 is found in animal products and B12 deficiency is common among older people, and sometimes in vegetarians. Bacteria in the large intestine of humans produce large amounts of folate. The form of folate produced by these bacteria does not mask vitamin B12 deficiency.

We want to study whether the folate these bacteria produce can be absorbed through the large intestine into the blood. We will do this by putting a small amount of folate solution into your large intestine at the end of your colonoscopy. We will measure whether this folate enters your blood and urine. Three or more weeks later we will give you an injection of a small amount of folate, and will measure the appearance and gradual disappearance of this folate in your blood and urine.

Six people will take part in this study. The Research Ethics Board of St Michael's Hospital and The Hospital for Sick Children has reviewed this study

STUDY ENROLLMENT

You have been scheduled for a colonoscopy, which is an examination of the inside of your colon using an endoscope. This procedure will be described later in this document. You are being invited to participate in this study because you are a healthy adult and your gastroenterologist has recommended you have a colonoscopic examination. In order to ensure that you will be eligible for the study we will take a blood sample (about 3 teaspoons) to make sure your blood chemistries are normal and that you do not carry the common variant of an enzyme ("MTHFR") involved in folate metabolism. This is a genetic test performed by a specialized laboratory. MTHFR is a gene which in many people (around 20% of the population) may affect the metabolism of folate and blood folate levels. No additional genetic tests will be performed on your blood.

If you are female and able to get pregnant, a urine pregnancy test will be done. If the test is positive, you will not be able to enter the study. Additionally, if you are taking birth control pills, you are also ineligible to enter the study as some oral contraceptives affect folate metabolism.

If you choose not to participate in the research study you will still receive the necessary treatment, including colonoscopy, and follow-up that you require by your physician, without penalty.

DESCRIPTION OF THE RESEARCH:

If you decide to participate in the study, the study nurse at the Gastroenterology Clinic at St Michael's Hospital will call you with some simple questions concerning your past medical history and your use of medications, vitamins and minerals. She will also arrange for you to obtain the screening blood and urine tests at the hospital.

During the study, we will need your participation for two periods of 3 days each, the first period of three days taking place at the time of your colonoscopy, the second period of 3 days taking place 3 weeks or more after the first (colonoscopy) study period.

Colonoscopy Study Period:
Day 1. The weekend before the colonoscopy 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. You will begin fasting at 8 p.m.

Day 2. This day includes having a colonoscopy. You will come to the Gastroenterology Clinic at St Michael's Hospital and you will be at the hospital for about 8 hours. You will need to collect all of your urine on the day of the colonoscopy in collection bottles provided by us.

A colonoscopy is the insertion of a flexible tube-shaped scope a bit larger than the size of a finger into your rectum. The scope has a lens and a light which allows the doctor to see a video image of your colon on a screen. The scope also has a working channel, which is a hollow tube incorporated in the scope, which allows the doctor to introduce instruments, such as catheters to specific areas of the colon. The scope is gently pushed inside your colon so the physician can examine the inside of the entire length of your large bowel (colon). This procedure is generally not painful but may be uncomfortable while the scope is passed through the colon due to bloating. Intravenous sedation will be provided if you find the procedure uncomfortable.

For the study, once the colonoscope has reached the top of the large bowel (the cecum), and no abnormalities are seen, a tube (an irrigation catheter) will be threaded into the colonoscope. Through this tube a saline (salt) solution of 100 milliliters (about 7 tablespoons), containing 400 micrograms of folate in a natural form, is transferred into your cecum. This is the same amount of folate found in most vitamin supplements that you can buy in the store. The 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. We will measure the amount of this label in your blood and urine.

At the time of the infusion of folate into the caecum, and every ½ hour after that for 4 hours, blood samples of 5 mL (one teaspoon) will be taken, to measure the uptake of folate into your blood. These will be taken via a second IV in your other arm.

All meals and snacks for this day will be provided to you for 24 hours after the colonoscopy. You should not take any vitamin supplements for the next 24 hours.

If you receive sedation during the colonoscopy procedure, you will require someone to drive you home at the end of this day.

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.

Folate Injection Study Period (Three weeks after the colonoscopy):

Day 1. The day before the study visit 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. You will begin fasting at 8 p.m.

Day 2.- ***Takes place at the Clinical Investigation Unit at The Hospital for Sick Children***
This day includes having an IV injection of folate solution. You will be at the hospital for about 5 hours.

You will need to come to Clinical Investigation Unit at the Hospital for Sick Children. There we will insert an IV into each of your arms.
You will need to collect all of your urine on this day in collection bottles provided by us.

For the study, we will inject through your IV 100 micrograms of labeled folate dissolved in 1 mL (about a fifth of a teaspoon) of salt solution with label. This is about ¼ 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 into the IV, and every ½ hour after that 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.

All meals and snacks for this day will be provided to you for 24 hours after the injection. You should not take any vitamin supplements for the next 24 hours.

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.

POTENTIAL HARMS

Colonoscopy/Endoscopy:
If you agree to be in this study, you will be exposed to risks associated with the standard colonoscopy, which include a risk of over sedation, of possible perforation (creating a small hole) in the colon wall, and possible minor bleeding from biopsy or infection. These conditions occur in less than 1% of patients and may resolve spontaneously; however if they are very severe, you may require antibiotics or an operation to correct the problem. Additionally, you may experience some abdominal discomfort and bloating described earlier.

Infusion of folate and blood draws:
There are no known harms associated with the folate infusion. 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 St Michael's Hospital on 2 occasions. Study obligations also require you to take time away from your regular schedule.

POTENTIAL BENEFITS

There are no direct benefits to volunteers from participation in this study. However, your participation is appreciated as the results will be used to better understand if folate made by bacteria in the large intestine can be taken up by the body.

A study team member will discuss the results of the research with participants on request, once the study is completed.

CONFIDENTIALITY

The results of the tests described will be used for research purposes only in the context of this study.

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

The Research Ethics Board of St Michael's Hospital, the SickKids Clinical Research Office Monitors, employees of the granting agency funding the study (NSERC), or the regulator of the study may see your health records to check on the study. For example, people from Health Canada Products and Food Branch, if necessary, may look at your records as they pertain to this study, in order to assure that the study is conducted properly and that certain federal government rules about research are being followed.

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. Following completion of the research study, the data will be kept as long as required by the SickKids and St Michael's Hospital "Records Retention and Destruction" policy. The data will then be destroyed according to these policies.

PUBLICATION OF RESULTS

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. You will remain completely anonymous.

PRESERVATION OF SAMPLES FOR ANALYSIS

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). These samples will be stored at SickKids and will be destroyed in five years or prior if the samples are depleted.

REIMBURSEMENT

We will pay for your expenses for being in this study such as meals, babysitters, parking and getting you to and from St Michael's Hospital. If you stop taking part in the study, we will pay you for your expenses for taking part in the study so far.

ALTERNATIVES TO PARTICIPATION

If you choose not to participate in this study, you will receive a standard screening colonoscopy.

COMPENSATION FOR INJURY

If you become ill or injured as a result of participation in this study, you will obtain medical treatment in the usual manner. In no way does signing this consent form waive your legal rights nor does it relieve the investigators, sponsors or involved institutions of their legal and professional responsibilities.

PARTICIPATION

It is your choice to take part in this study. You can stop at any time. The care you get at the hospital 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 be 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 study sponsor may decide to stop the study. This could happen even 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 staff of the study, any people who gave money for the study, and the hospital are still responsible, legally and professionally, for what they do.

We will give you a copy of this consent form for your records.

CONFLICT OF INTEREST

None of the people involved in this study have a conflict of interest, meaning that they will not benefit personally, financially or in some other way from doing this study.

WHO DO I CALL IF I HAVE QUESTIONS OR PROBLEMS?

The person to call about information on this study is Susanne Aufreiter. You can call Susanne at (416) 813-5894 or pager (416) 442-0350 during regular office hours.

Should you have any questions or concerns during the study or about your treatment or in the event you experience a study-related injury, please contact your study doctor or any of the study investigators listed on the front page.

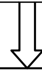
If you have any questions regarding your rights as a research participant in this study you may contact the Chair of St Michael's Hospital Research Ethics Board, Dr Julie Spence, at 864-6060 Ext 2557, or the SickKids Research Ethics Manager at 416-813-5718.

COLONOSCOPY STUDY:
TIMELINE FOR PARTICIPATION:
TWO STUDY PERIODS: 3 DAYS + 3 DAYS

Participation for Study Period 1: St. Michal's Hospital

DAY 1. A weekend day before colonoscopy:

1. COLLECT 24 HOUR URINE SAMPLE



DAY 2. The day of Colonoscopy:

1. INFUSION OF FOLATE SOLUTION INTO COLON
2. BLOOD SAMPLES TAKEN FOR 4 HOURS
3. COLLECT 24 HOUR URINE SAMPLE

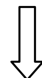
DAY 3. The day after Colonoscopy:

1. COLLECT 24 HOUR URINE SAMPLE

Participation for Study Period 2: The Hospital for Sick Children

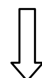
DAY 1. The day before Clinic Visit:

COLLECT 24 HOUR URINE SAMPLE



DAY 2. Clinic Visit 1:

1. **INJECTION OF 1 ml FOLATE SOLUTION**
2. **BLOOD SAMPLES TAKEN FOR 4 HOURS**
3. **COLLECT 24 HOUR URINE SAMPLE**



DAY 3. The day after Clinic Visit:

1. **COLLECT 24 HOUR URINE SAMPLE**

Study title: **Folate Absorption Across the Large Intestine: Colonoscopy Study**

CONSENT:

By signing this form, I agree that:

1) The study has been explained to me and all my questions have been answered to my satisfaction.
2) The possible harms and benefits (if any) of this study have explained to me.
3) I know about 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 health care at St Michael's Hospital or 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. No information about me will be given, unless required by law.
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.

I hereby consent to participate in this study and will be given a copy of this signed consent form

___________________________________ ________________________

Printed Name of Subject *Signature & Date*

____ _______________________

Printed Name & position of person who explained consent *Signature & Date*

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