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RESEARCH ARTICLES

Bacterially synthesized folate and supplemental folic acid are absorbed across the large intestine of piglets☆

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Abstract

A large pool of folate exists in the large intestine of humans. Preliminary evidence, primarily in vitro, suggests that this folate may be bioavailable. The purpose of this study was to test the hypothesis that supplemental folic acid and bacterially synthesized folate are absorbed across the large intestine of piglets. The pig was used as an animal model because it resembles the human in terms of folate absorption, at least in the small intestine. A tracer of [^{3}H]-folic acid or [^{3}H]-*para*-aminobenzoic acid ([^{3}H]-PABA), a precursor of bacterially synthesized folate, was injected into the cecum of 11-day-old piglets. Feces and urine were collected for 3 days. Thereafter, piglets were killed, and livers and kidneys harvested. [^{3}H]-Folate was isolated from biological samples by affinity chromatography using immobilized milk folate binding proteins and counted using a scintillation counter. In piglets injected with [^{3}H]-folic acid, the feces, liver, urine and kidneys accounted for 82.1%, 12.3%, 3.9% and 1.7% of recovered [^{3}H]-folate, respectively. In piglets injected with [^{3}H]-PABA, the amount of recovered bacterially synthesized folate in the feces, liver and urine was 85.1%, 0.4% and 14.6%, respectively. Twenty-three percent and 13% of tritium were recovered in samples examined (liver, kidney, fecal and urine) from piglets injected with [^{3}H]-folic acid and [^{3}H]-PABA, respectively. Using our estimates of [^{3}H]-folic acid absorption and the total and percent monoglutamyl folate content of piglet feces, we predict that at least 18% of the dietary folate requirement for the piglet could be met by folate absorption across the large intestine.

Keywords: Folate; Large intestine; Microbial biosynthesis

1. Introduction

Less than optimal folate nutrition has been implicated as a risk factor in a number of negative health outcomes, including neural tube defects (NTD), anemia during pregnancy, low infant birth weight, colorectal cancer and cardiovascular disease [1–9]. The weight of the evidence in the case of NTD prompted public policy makers in North America to mandate the addition of supplemental folic acid to flour (140–150 μg/100 g) [10,11]. Seemingly as a result, the prevalence of NTD has fallen by at least 25% [12,13]. Most countries, however, have not adopted an elevated folic acid fortification policy. Many have chosen not to do so because of concern over the potential adverse effects of folic acid, in particular the concern that high intakes of folic acid may delay the diagnosis of vitamin B_{12} deficiency by correcting the characteristic megaloblastic anemia [14]. It is estimated that 10–30% of humans >50 years of age have a reduced capacity to absorb naturally occurring vitamin B_{12}, and ~20% of the general population in industrialized countries may be vitamin B_{12} deficient [14,15]. We hypothesize that bacterially synthesized folates may offer a complementary source of bioavailable folate. If indeed they do, and the microbial milieu of the large intestine can be manipulated to increase folate production, dietary folate requirements and the level of folic acid fortification could be decreased. Of note, the principal form of folate (5-CH_3folate) synthesized by bacteria in the large intestine does not mask B_{12} deficiency [16].

Many bacterial species, including several present in the large intestine, are capable of synthesizing folate [17–19]. Results from our laboratory and others suggest that the amount of folate in the large intestine of humans is significant and could be clinically important if bioavailable [16,20–22]. We reported, for example, the amount of folate measured in fecal solids of infants (n=22) was 41.1±

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41.0 μg/day, representing, on average, 63% of the dietary reference adequate intake level for infants <5 months of age [14,16]. Further, ~0.5 of these folates were monoglutamylated, a form of folate that can readily cross the small intestine.

Rong et al. [23] provided the first direct evidence in vivo that bacterially synthesized folates could be absorbed across the intact large intestine and incorporated in tissues. In this latter study, [^{3}H]-PABA was injected directly into the cecum of rats, and bacterially synthesized [^{3}H]-folate was recovered from the livers despite prevention of coprophagy [23]. Indirect evidence of folate absorption across the large intestine comes from several [24–29] but not all [30] studies in which increased plasma, liver and/or colonic tissue folate concentrations were observed among rats fed diets containing a source of indigestible carbohydrate to promote intestinal fermentation. A valid criticism of this work, including our own, is that it is difficult to extrapolate data from rats to the human condition. Rats, for example, are coprophagic, and so folate synthesized by microorganisms in the large intestine and excreted in the feces could be absorbed across the small intestine. There are also well-characterized differences between the rat and human with respect to folate absorption, at the level of the small intestine [31]. For example, only intracellular folate conjugase, the enzyme responsible for deconjugation of polyglutamyl folates, exists in the rat small intestine [31]. In contrast, both the brush border and intracellular folate conjugases have been described in the pig with strikingly similar characteristics to those identified in the human intestine [31,32]. As it now appears likely that the mechanism for folate absorption is similar in the small and large intestine, it seems logical to proceed with work in this area using an animal model that more closely resembles the human in terms of folate absorption — the pig [32,33].

The purpose, then, of the present study was to determine whether supplemental folic acid and bacterially synthesized folate are absorbed across the large intestine of piglets and are incorporated into the piglets' tissues.

2. Methods and materials

3′,5′,7,9-[^{3}H]-Folic acid, potassium salt was purchased from Amersham (Buckinghamshire, UK), and 3′,5′-[^{3}H]-PABA was purchased from Moravek Biochemicals (Brea, CA). Except where indicated, all anesthetics (including gases) were provided by CDMV (Hyacinthe, Quebec), and other chemicals were purchased from Sigma (St. Louis, MO).

2.1. Animals

Piglets were used as the animal model in this experiment because of their similarity with humans in regard to folate absorption, smaller size compared to adult pigs and the relative ease of manipulating the microflora of nursing animals. The latter consideration is important for future experimentation in this area. Locally purchased male Yorkshire piglets (5 days old, $n=13$) were individually housed in cubicles containing a wood chip bedding with a 12-h light/dark cycle and a controlled room temperature (23–24°C). Heat lamps were installed in each cubicle to provide a supplementary source of heat. The piglets were acclimated for 6 days prior to study initiation. Commencing on arrival at the animal care facility, the piglets were fed four times daily ad libitum quantities of a freshly prepared milk-based formula in manual feeders. Piglets had free access to tap water. The milk-based diet met all the nutrient requirements for piglets as described by the National Research Council [34], contained 1.4 μg folate/g and was free of antibiotics. Detailed description of this diet can be found in a previous publication [16].

2.2. Surgery and sample collection

Piglets were monitored for diarrhea four times daily and were weighed each day (±10 g) using a MBS 2010 Digital Baby Scale (My Weigh, Vancouver, BC, Canada). The experimental protocol was reviewed and approved by the Animal Care Committee at The Hospital for Sick Children. When piglets were 10 days old, they were shaved with an electric shaver around the tail, anus and urethra. Pediatric urine collection bags (Precision Dynamic, San Fernando, CA) were then attached to the skin using tape for a 24-h cold fecal and urinary collection. The quantity and form of folate found in the cold fecal samples were previously reported [16]. Once piglets were 11 days old, they were anesthetized, initially using acepromazine (1 mg/kg), and then the anesthesia was maintained using halothane, oxygen and nitrous oxide (2 L/min oxygen, 2 L/min nitrous oxide and halothane 1.5%). Administration of gases lasted no more than 50 min. A small abdominal incision was made and the cecum was exteriorized. A tracer dose of [^{3}H]-folic acid (1.70 TBq/mmol) or [^{3}H]-PABA (1.51 TBq/mmol) was injected into the cecum of each of the piglets using a 27-gauge needle. Tracer doses of either [^{3}H]-folic acid or [^{3}H]-PABA provided 1% of the adequate daily intake of folate for human infants (65 μg/day for 0–5 months) or the molecular equivalent of PABA, respectively. The point of injection was sutured to prevent leakage of the tracer dose into the abdominal cavity. The abdominal wall incision was closed by two layers of sutures. Acetaminophen (100 mg, Tylenol, McNeil Consumers Healthcare, Guelph, ON) was administered via a dropper prior to surgery and every 6 h for 48 h thereafter.

After surgery, piglets were returned to their cubicles, and 24-h fecal and urinary collections were completed for 3 days. Fecal and urine bags were changed every 8 and 4 h, respectively, and stored at 4°C until the end of the 24-h collection period. Thereafter, samples were pooled and stored at −80°C. To preserve urine and to prevent bacterial growth, 400 μl of chloramphenicol (31 mmol/L) and 2.5 mmol of sodium ascorbate were added to each bag used to collect urine. After the 3-day collection, the piglets

Table 1
Tritium and [^{3}H]-folate recovered in the excreta and organs of piglets injected with [^{3}H]-folic acid[a]

	[^{3}H]-Recovered		[^{3}H]-Folate recovered	
	Bq	%[b]	Bq	%[b]
Liver	74,163±93,227[c]	2.8±3.6[c]	69,690±87,852	2.7±3.4
Kidney	9934±15,497	0.4±0.6	9461±14,608	0.4±0.6
Urine	25,258±17,911	1.0±0.7	22,695±15,975	0.9±0.6
Feces	489,198±339,921	18.9±13.1	465,827±321,597	18.0±12.4
Total recovery	598,553	23.1	567,673	22.0

[a] $n=7$; a tracer dose of [^{3}H]-folic acid (1.70 TBq/mmol) was injected into the cecum of each piglet. This dose provided ~1% of the dietary reference adequate intake level of folate for the full-term human infant.
[b] The recovery of tritium and [^{3}H]-folate in each of the biological samples was calculated as a percent of the [^{3}H]-folic acid dose injected into the cecum.
[c] Values are mean±S.D.

were killed using an overdose of sodium phentobarbitol (1 mol/L), and the liver and kidneys were removed, cleaned and weighed.

2.3. Preparation of samples for analysis

Liver, kidney and fecal samples were homogenized in 10 volumes of HEPES/CHES buffer (50 mmol/L HEPES, 50 mmol/L CHES, pH 7.85), containing 100 mmol/L sodium ascorbate and 10 mmol/L 2-mercaptoethanol [35,36]. Homogenates were boiled for 15 min and cooled in an ice bath, and the supernatant fraction extracted by centrifugation (40,000×g, 20 min, 4°C) and stored at −80°C until further analysis. A subsample of pellets ($n=3$) produced by centrifugation of liver homogenates were resuspended and counted for tritium (LS6500 Beckman Scintillation Counter, Beckman Coulter, Fullerton, CA) to ensure the efficiency of our extraction procedure. In all cases, the tritium found in pellets did not exceed background. For urine collections, the supernatant fraction was extracted by centrifugation (2700×g, 10 min, 4°C), filtered (0.45-μm disposable syringe filter, Pall Corporation, Ann Arbor, MI) and then stored at −80°C until further analysis. At all times, samples were protected from light using aluminum foil.

2.4. Analyses

Folates in the supernatants were isolated by affinity chromatography using immobilized bovine milk folate binding proteins in columns (10 ml capacity) as previously described by Kim et al. [16]. Columns were freshly packed each week. Biological samples were thawed and loaded onto the affinity columns, then washed sequentially with water to remove any nonfolate material. The folate that was bound to the column was eluted by a wash buffer (pH 3.34) containing equal portions of eluant A (112 mmol/L potassium phosphate, 240 mmol/L phosphoric acid), eluant B (800 ml/L acetonitrile) and water. The eluant was then counted for tritium using a Quench curve correction.

Mean recovery from the affinity column, as measured with tritiated folic acid, was 85.5±0.7% (mean±S.D.). The total folate binding capacity of the columns exceeded 500 nmol/ml. Results presented in this manuscript were not corrected for percent recovery from the affinity column. Our preliminary work indicated that [^{3}H]-PABA did not bind to the affinity column and that unlabelled 5-CH_3folate did using the aforementioned experimental conditions. The reproducibility of determining the [^{3}H]-folate content from biological samples was assessed by analyzing aliquots of pooled minced liver ($n=3$) from each of three piglets. The interassay CV was 2.2%.

2.5. Statistical methods

Values in the text are mean±S.D. unless otherwise noted. In the text, the percentage of recovered [^{3}H]-folate (Bq) found in each of the liver, kidney and urine was calculated by dividing the [^{3}H]-folate (Bq) found in each by the sum of the [^{3}H]-folate (Bq) found in the liver, kidney and 3-day collections of urine and feces. In the tables, the recovery of tritium and [^{3}H]-folate in each of the biological samples was calculated as a percent of the administered dose.

3. Results

3.1. Animal characteristics

The mean weight (kg) of piglets at 5 (2.4±0.3), 10 (3.3±0.3) and 14 (4.0±0.6) days of age was within typical reference ranges calculated from the animal husbandry literature [37]. Piglets appeared healthy and were diarrhea-

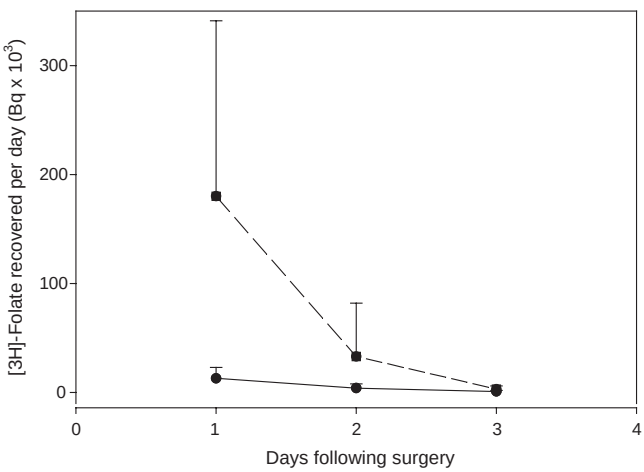

Fig. 1. Tritium and [^{3}H]-folate recovered over a 3-day period in the feces (■) and urine (●) of piglets ($n=3$) injected with [^{3}H]-folic acid.

Table 2
Tritium and bacterially synthesized [^{3}H]-folate recovered in the excreta and organs of piglets injected with [^{3}H]-PABA[a]

	[^{3}H]-Recovered		[^{3}H]-Folate recovered	
	Bq	%[b]	Bq	%[b]
Liver	1197±251[c]	0.1±0.01[c]	1017±578	0.1± 0.03
Urine	48,081±30,490	2.1±1.3	41,666±26,302	1.8±1.2
Feces	246,555±189,243	10.9±8.3	243,004±187,220	10.7 ±8.2
Total recovery	295,832	13.1	285,687	12.6

[a] $n=6$; a tracer dose of [^{3}H]-PABA (1.51 TBq/mmol) was injected into the cecum of each piglet. This amount provided an equimolar dose of PABA to that of folic acid administered in Table 1.
[b] The recovery of tritium and [^{3}H]-folate in each of the biological samples was calculated as a percent of the [^{3}H]-PABA dose injected into the cecum.
[c] Values are mean±S.D.

free except for some watery stools during the first 72 h of acclimation to the diet. The mean formula and folate intake of piglets were 1.3±0.3 L/day and 395.7±79.5 μg folate/day, respectively. The dietary folate requirement for piglets weighing 3 to 5 kg is 80 μg/day [34]. The mean amount of feces and urine collected over the 3-day study period was 57.1±20.6 g and 363.9±119.7 ml, respectively. The mean weight of livers and kidneys at day 14 was 143±30.9 and 31.4±7.6 g, respectively.

3.2. Piglets injected with [^{3}H] folate

[^{3}H]-Folic acid injected into the cecum of piglets ($n=7$) was absorbed across the large intestine and incorporated into the liver and kidneys and excreted in the urine (Table 1). Recovered [^{3}H]-folate accounted for 96% of the total radioactivity recovered. Twenty-two percent of the total [^{3}H]-folic acid injected into the cecum of piglets was recovered in the liver, kidney, urine and fecal samples. The tritiated folate found in the feces, liver, urine and kidneys accounted for 82.1%, 12.3%, 3.9% and 1.7% of the total recovered [^{3}H]-folate, respectively. The greatest quantity of recovered radioactivity in the feces was found in samples collected in the first 24 h (82.4%) while the lowest amount was recovered on day 3 (1.6%) (Fig. 1). Likewise, the greatest quantity of recovered radioactivity in the urine was found in samples collected in the first 24 h (14.6±11.3 kBq or 72.5%) while the lowest was recovered on day 3 (1.3±0.8 kBq or 0.6%).

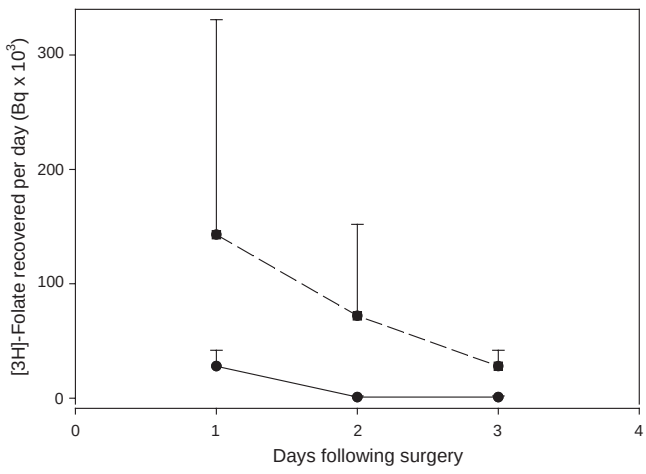

Fig. 2. Tritium and [^{3}H]-folate recovered over a 3-day period in the feces (■) and urine (●) of piglets ($n=3$) injected with [^{3}H]-PABA.

3.3. Piglets injected with [^{3}H] PABA

[^{3}H]-PABA injected into the cecum of piglets ($n=6$) was converted into [^{3}H]-folate by the intestinal microflora, absorbed across the large intestine and incorporated into the liver or excreted into urine (Table 2). Only trace amounts of [^{3}H]-folate, below detection limits in most samples, were found in the kidneys. Tritiated folate accounted for 97% of the total radioactivity that was recovered. The total [^{3}H]-folate that was recovered accounted for 12.6% of the total [^{3}H]-PABA administered. Eighty-five percent of all [^{3}H]-folate recovered was found in the 3-day fecal collections. Smaller quantities of [^{3}H]-folate were recovered from the urine (14.6 %) and liver (0.4 %). The greatest quantity of radioactivity in the feces was recovered during the first day of collection (54.4%) while the lowest amount was recovered on day 3 (18.9%) (Fig. 2). Likewise, the greatest quantity of recovered radioactivity in the urine was found in samples collected in the first 24 h (32.0±18.2 kBq or 92.7%) while the lowest was recovered on day 3 (1.0± 1.4 kBq or 2.9%).

4. Discussion

Our findings show that supplemental folic acid and bacterially synthesized folate can be absorbed across the large intestine of piglets and incorporated into their tissues. In piglets that were injected with [^{3}H]-folic acid into their cecum, over 14% of recovered [^{3}H]-folate was found in the liver and kidneys; a further 4% of recovered [^{3}H]-folate was found in the urine (Table 1). Similarly, 0.4% of the recovered [^{3}H]-folate in piglets injected with [^{3}H]-PABA, a bacterial folate precursor, was found in the liver. A further 15% of recovered [^{3}H]-folate was found in the urine.

The percent incorporation of [^{3}H]-folate into the liver and kidney of piglets injected with [^{3}H]-PABA in our experiment is similar to that reported by Rong et al. [23] who injected [^{3}H]-PABA into the cecum of adult rats ($n=9$) fitted with sling suits to prevent coprophagy. In this latter experiment, 0.9% of recovered [^{3}H]-folate was found in the

liver and kidneys of rats. In contrast, a smaller percentage of [^{3}H]-folate was excreted in the urine of rats (0.4%) in the Rong et al. [23] study, compared to that reported for piglets herein. Total recovered [^{3}H]-folate synthesized by intestinal bacteria was 6.9% and 12.6% in the Rong et al. [23] and the present study, respectively.

Using our estimates of [^{3}H]-folic acid absorption reported herein (i.e., 18% from liver, kidney and urine), published estimates of the total (~301.3 nmol) and percent monoglutamyl folate (29.3%) content of piglet feces and an assumed bioavailability of mono- vs. polyglutamyl folate of 85% and 50%, we predict that approximately 18% of the dietary folate requirement for the piglet could be met by folate absorption across the large intestine [14,16].[1] With almost certainty, this is an underestimate of the actual percent absorption and percent tissue incorporation of folate. First, and foremost, not all of the tissues of piglets were analyzed. The goal of this first very labour-intensive study was to ascertain whether folate is absorbed across the large intestine. For this reason and the practicalities of preparing whole carcass and organ homogenates from large radio-labelled animals, we confined our tissue analyses to the liver and kidney. While the liver and kidneys contain significant depots of folate, the carcass contribution to total body folate is likely to be considerable. Estimates in growing rats fed either one (1 mg/kg) or four times their dietary folate requirement (4 mg/kg) suggest that the liver and kidneys account for only 39% and 50% of whole-body folate, respectively [38]. Interestingly, the carcass of growing rats fed 1 and 4 mg/kg of folic acid was shown to contain 47% and 38% of whole-body folate, respectively.

Further, we may have underestimated percent absorption of the folate as the 3-day urine collections, averaging 121 ml/day, could have been incomplete. While the literature regarding the usual urine output of piglets is quite limited, available data suggest that 7- to 10-day-old piglets (3–5 kg) excrete approximately 820-ml urine/day [39]. This estimate is similar to the predicted urine output of 657 ml/day for human infants <1 month of age [40]. By regular visual inspection, the urine bags appeared firmly attached to the skin of piglets; however, it is possible that there was leakage between the bag and skin as our male piglets rested or slept on their stomachs.

It is also possible that while piglets were under anesthesia that there was passive backward movement of the [^{3}H]-tracer through the ileocecal sphincter and absorption across the terminal ileum. If this occurred we could have over-estimated the absorption of folate across the large intestine. A number of precautions were taken to prevent this from happening including the following: (1) the [^{3}H]-tracer was injected into the mid-distal portion of the cecum; (2) the total volume administered was small (<150 μl); (3) the cecum was full as fasting prior to surgery was limited to 1 h.

It should be noted that a greater proportion of recovered [^{3}H]-folate was found in the liver of piglets injected with [^{3}H]-folic acid compared to [^{3}H]-PABA. We suspect that difference in the metabolism of folic acid and bacterially synthesized folates account, at least in part, for this observation. Folic acid, a synthetic form of the vitamer, is not found in nature and when consumed orally is generally thought to be reduced and methylated in the enterocyte prior to absorption [41,42]. The fraction of folic acid that escapes this process is transported to the liver where they are reduced and a proportion methylated. Wright et al. [43] recently speculated that absorbed folic acid may be reduced and methylated in the liver, and not in the mucosa. In contrast to folic acid, bacterially synthesized folates are reduced and most are methylated prior to absorption.

As described above, much of the work investigating whether a clinically relevant amount of folate can be absorbed across the large intestine was done using the rat. It is difficult, in most instances, to extrapolate this work to the human condition because rats are coprophagic and, hence, microbially synthesized folate can be absorbed across the small intestine. Second, there are well-characterized differences between the rat and human with regard to the mechanism of folate absorption across the small intestine [31]. Available in vitro data suggest that the mechanism for folate absorption across the large intestine is similar to that of the small intestine [32]. Given these observations, it made sense to continue animal work in this area using a model that resembles the human in terms of folate absorption—the pig [31,33]. Despite similarities in folate absorption, we acknowledge that differences in folate metabolism exist between the human and the pig [44,45]. For example, the plasma of pigs, unlike that of humans, contains appreciable quantities of high-affinity folate binders and tetrahydrofolate [44,45]. It has been proposed that the high concentrations of high-affinity folate binders in the plasma of pigs permit the existence of tetrahydrofolate, a very labile form of folate. It remains to be seen whether these differences limit our ability to extrapolate the data from the present study to the human condition. These differences do, however, underscore the need to directly assess folate absorption across the large intestine in humans, perhaps using stable isotopes.

Data, albeit indirect, do exist in the literature to suggest that bacterially synthesized folate in the large intestine may impact the folate status of humans. For example, we conducted an observational study to assess the effect of total dietary fibre intake on the folate status of young women (n=224, [46]). Results from this study suggest that consumption of total dietary fibre, particularly soluble fibre, was positively associated with serum folate concentrations

[1] % [^{3}H]folate recovered in tissues and urine (18%)×daily quantity of folate (unlabelled) found in stools (301.3 mmol or 132.9 μg)=A. % Absorption of folate across the large intestine=[A×% monoglutamyl folate in stools (29.3%)×bioavailability of monoglutamyl folates (85%)+A×% polyglutamyl folate in stools (70.7%)×bioavailability of polyglutamyl folates (50%)]/dietary requirement for folate (80 μg/day)×100.

even after controlling for folate intake and other confounding variables ($P<.001$). Likewise, Wolever et al. [47] in a randomized controlled study of type 2 diabetics showed serum folate was significantly higher in subjects treated with miglitol vs. metformin. Miglitol is an α-glucosidase inhibitor that improves glycemic control by competitive inhibition of carbohydrate digestion. In contrast, metformin promotes glycemic control by improving insulin sensitivity and reducing hepatic glucose output. Enhanced microbial growth secondary to an increase in the quantity of fermentable substrate reaching the colon was proposed as the mechanism for the observed increase in serum folate content among young women consuming higher levels of fibre in the former study and among miglitol users in the latter study.

In addition to the total bacterial load and net folate production in the colon being affected by diet, there is also evidence from animal studies to suggest that manipulation of the profile of microorganisms in the large intestine can alter folate production [25,27,29]. For example, we showed in a series of experiments that rats fed diets containing human milk solids, known to be bifidogenic, had at least a seven- and a one-fold increase in cecal ($P<.0007$) and colonic ($P<.04$) *Bifidobacterium* density, respectively, compared with rats fed diets containing cow or goat milk solids or diets prepared without milk solids [27]. Most subspecies of bifidobacteria are known folate synthesizers [48]. Further, plasma folate concentrations of rats were positively correlated with the *Bifidobacterium* concentrations in both the cecum ($r=.69$, $P<.0007$) and colon ($r=.57$, $P<.02$).

In conclusion, folic acid and bacterially synthesized folate are absorbed across the large intestine and incorporated into the liver and kidneys of piglets. Using our estimates of [^{3}H]-folic acid absorption and a published estimate of the total and percent monoglutamyl folate content of piglet feces, we predict that approximately 18% of the dietary folate requirement for the piglet could be met by folate absorption across the large intestine. These findings should be confirmed in animal studies where whole-body analysis of [^{3}H]-folate uptake is assessed.

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