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The fate of [^{3}H]folic acid in folate-adequate rats

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To assess more fully the metabolic fate and in vivo kinetics of dietary folate, we conducted an in depth study that followed the metabolism and excretion of radiolabeled folate from a single administration, through its many forms in the organs and tissues of folate-adequate rats. Twenty-two rats were equilibrated with an amino acid diet containing 1 mg folic acid/kg diet, then given 185 kBq [^{3}H]folic acid intragastrically. The isotopic label was followed through the tissues and in urine and feces for 32 days, every 8 hours for the first 48 hours. Individual folates, as their monoglutamyl forms, were separated and measured by high performance liquid chromatography (HPLC), and the peaks counted for ^{3}H. Liver and kidney folates exhibited labeling (~0.8 kBq/g for each) by 8 hours post-dose. These organs exhibited a peak of [^{3}H]folate at 40 hours post-dose, then slowly lost label from 48 hours to 32 days, with the exception of a rise in renal radioactivity at 16 days. Heart and spleen exhibited low levels of labeling at 8 hours, a peak at 32 hours, then a gradual loss of label. Testes and muscle (hind leg) showed very low levels of labeling throughout the study. Whole blood showed labeling almost entirely associated with 5-methyl-tetrahydrofolate (THF). Urinary excretion of intact folate occurred mainly as 5-methyl-THF, although excretion of products of folate catabolism exceeded urinary excretion of intact folates. Much of the labeled dose (35%) was excreted into the urine as catabolites and intact folates by 32 days post-dose, whereas 15% of the label was lost through the feces by 32 days post-dose. Unexpected differences were observed among the specific radioactivity values among tissues (liver < kidney << testes, spleen, whole blood < heart << muscle). These may indicate different rates of clearance of labeled folates from these tissues or else the presence of kinetically slow pools comprising a significant portion of total folate in certain tissues (especially liver and kidney). The results of this study will be used to develop a compartmental model to simulate folate metabolism in folate-adequate rats.
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Keywords: folate; rat; metabolism

Introduction

The various forms of folate are integral components of one-carbon metabolism, and thus are involved in a number of functions such as the synthesis of DNA, RNA, methionine, and purines.[1] Extensive interconversion of folates occurs during this process. Many investigators have studied the rate and extent of these interconversions and the effects of different dietary levels of folate on tissue folate levels. In particular, the following represent the major observations of such studies.

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Bhandari and Gregory[2] investigated folate metabolism in the rat by following a tritium labeled dose of folic acid, 5-methyl-tetrahydrofolate (THF), or 5-formyl-THF through a number of tissues and as excreted in urine and feces during an 8 day study. Though there were no differences due to the form fed, decreases in tritium labeling of all measured folates in urine and tissues occurred with time.

Pheasant et al.[3] measured radiolabeled folate in rat tissues after dosing with a high level of folic acid and several levels of 10-formyl-folic acid. They found over 11% of the label in the liver at 48 hours post-dose, and smaller amounts in the other tissues. These values were similar to those reported by Bhandari and Gregory.[2] When the rats were administered increasing quantities of supplemental 10-formyl-folic acid, the percentage of labeled dose retained in the tissues declined, whereas the label excreted in the urine increased.[3]

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Similar fractional retention of labeled folates in tissues was reported in a study by Barford et al.[4] These rats were given a labeled dose of folic acid, and radioactivity measured at intervals thereafter. Sixteen percent of the label was retained in the liver 1 day after the dose, a value similar to those seen by Bhandari and Gregory[2] and Pheasant et al.[3] However, Barford et al.[4] observed an increase in liver radioactivity to 25% of the dose by day 5, followed by a slow loss of label from the liver. They also detected measurable radioactivity in the liver even after 70 days post-dose.

Steinberg et al.[5] observed that over 90% of an intragastric dose of labeled folate was absorbed in fasting rats, and that the liver was a major site of storage and metabolism. Much of the folate, as 5-methyl-THF, that was taken up by the tissues was eventually returned to the liver, and incorporated into polyglutamates. They also observed that major quantities of hepatic folates, as monoglutamates, were often excreted through the bile and made available for reabsorption. Recent studies have substantiated the existence, homeostatic function, and physiological importance of extensive enterohepatic circulation of folates.[6]

In a study of human subjects given a relatively large (1.8 mg) daily dose of deuterium-labeled folic acid, von der Porten et al.[7] observed that serum, RBC, and urinary labeled folate increased for 2 to 3 weeks, then decreased toward starting levels after cessation of the labeled daily dose. Kinetics observed with this high intake may differ from those at lower levels.

Much of folate catabolism occurs in metabolically active tissues through cleavage of the C9–N10 bond of the folate molecule to produce a pteridine and para-aminobenzoylglutamate (pABG).[8] Murphy et al.[8] showed that much of the label excreted after a dose of labeled folic acid was in the form of pABG and its N-acetyl form para-acetamidobenzoylglutamate (apABG). Folate catabolic products in urine measured in a subsequent study[9] with rats showed the presence of tritium labeling in small amounts of excreted folic acid, 5-methyl-THF, and a metabolite identified as 10-formyl-dihydrofolate for as long as 8 days after the labeled dose, but the major labeled constituents of urine were the folate catabolites described previously.[8] The rate of excretion of apABG subsequently has been found to be an accurate indicator of folate catabolism in rats.[10]

The purpose of the present study was designed to follow the metabolism of a labeled dose of folic acid as it was converted to other forms of folates in a variety of body tissues and excreta over a 32 day period. Our intent was to provide data that would reflect relatively long-term metabolism of [^{3}H]folic acid, including assessment of several factors not fully assessed in previous studies. Specific attention was directed toward determining the time-dependence of the in vivo distribution and interconversions of folates, the distribution and retention of labeled folates in non-hepatic tissues, and significance of folate catabolism in the overall turnover of the vitamin. The study was conducted with growing rats maintained in moderate but adequate folate status using an amino acid-based diet[11] containing 1 mg folic acid/kg diet. This concentration of dietary folate was greater than the 674 nmol folic acid/kg diet (~0.3 mg/kg) reported by Clifford et al.[12] to provide maximum growth using the same basal diet composition. We will use the results of this study to develop a compartmental model of folate metabolism distribution, catabolism, and excretion in the folate-adequate rat, which will be reported separately. A long-term goal is to determine the influence of dietary intake on folate dynamics.

Methods and materials

Animals and diets

Twenty-two male Harlan Sprague Dawley (Indianapolis, IN USA) rats weighing 140–170 g were housed in individual plastic metabolism cages in a temperature-controlled room on a 12-hour light/dark cycle. The rats were given free access to a purified amino acid diet[11] (Dyets, Inc., Bethlehem, PA USA) containing 1 mg folic acid/kg diet (2.27 nmol/g) and 1% (w/w) succinylsulfathiazole for a total of 6 weeks. The diet was adequate in all nutrients. Rats were weighed twice per week, and feed consumption was determined every other day. The protocol was approved by the Institutional Animal Care and Use Committee at the University of Florida, Gainesville, FL USA.

Protocol

After a 14-day equilibration period consuming this diet, the rats were given an intragastric dose of [3′, 5′, 7, 9-^{3}H]folic acid (5 μCi, 185 kBq, specific activity 35 Ci/mmol) (Moravek Biochemicals, Inc., Brea, CA USA) in 0.005 mol/L KH_2PO_4, pH 7.0, containing 5 mmol/L sodium ascorbate. The [^{3}H]folic acid was purified just prior to use by affinity chromatography. Briefly, the labeled folic acid was applied to an affinity chromatography column containing 2 mL of immobilized folate binding protein.[13] The column was then washed with KH_2PO_4 buffer, pH 7.4, and water. The folic acid was eluted with a 0.02 mol/L trifluoroacetic acid solution containing 10 mmol/L 2-mercaptoethanol, and collected in a 5 mL volume. The sample was evaporated to dryness under nitrogen gas with gentle heating, then reconstituted in 0.005 mol/L KH_2PO_4, pH 7.0 containing 5 mmol/L sodium ascorbate.

Urine was collected in 8-hr intervals for 48 hours post-dose, then in 24-hr intervals thereafter. Urine samples were combined into 4-day pools beginning 17 days post-dose. Fecal samples were collected in 24-hr intervals throughout the study, and combined into 4-day pools beginning 17 days post-dose. Collection tubes were covered with foil, and 3–5 mL (depending on urine volume) of a 10% (w/v) sodium ascorbate solution added to the urine tubes at the beginning of each day (or 8-hr collection period). Samples were stored at −20°C.

Rats (n = 2) were killed by exsanguination under Metafane anesthetic at 8 hours, 16 hours, 24 hours, 32 hours, 40 hours, 48 hours, 4 days, 8 days, 16 days, and 32 days post-dose. The liver, kidneys, heart, testes, spleen, and hind leg muscle were removed to ice-cold saline, weighed, and stored at −20°C until analysis. Whole blood was stored in heparinized tubes at −20°C.

Analyses

Urine samples were defrosted, centrifuged (7,000 × g, 15 min) then passed through 2-mL affinity chromatography columns containing immobilized folate binding protein.[13] A portion of the non-retained non-folate effluent (containing [^{3}H]pterins, pABG, apABG, and possibly ^3H_2O derived from C9–N10 bond cleavage) from the affinity column was kept for determination of total labeled folate catabolites by scintillation counting. Each column was then washed with KH_2PO_4 buffer, pH 7.4, and water. The folates were eluted with a 0.02 mol/L trifluoroacetic acid solution containing 10 mmol/L dithiothreitol, and collected in a 5 mL volume.

Tissues were defrosted for several minutes, and 1 to 5 g (depending on folate content and tissue mass) samples were weighed. Sample preparation followed the method described by Wilson and Horne,[14] with a minor modification that the quantity of rat plasma (source of pteroylpolyglutamate conjugase activity) specified[14] was added as a single portion, rather than at three 15-min intervals, and the samples were incubated in a 37°C water bath for 1 hour. Briefly, each sample was minced, added to 10 volumes of hot buffer (50 mmol/L N-[2 hydroxyethyl]piperazine-N'[2-ethanesulfonic acid] (HEPES), 55 mmol/L 2-[N- cyclohexylamino]-ethanesulfonic acid (Ches), 10 mmol/L 2-mercaptoethanol, 2% (w/v) sodium ascorbate), pH 7.85, and incubated in a 100°C water bath for 10 minutes, then cooled in an ice bath. Samples were then homogenized and centrifuged at 15,000 x g for 20 min. To maximize extraction, the pellet was resuspended in 10 mL of the same buffer and centrifuged again. The supernatants were combined, warmed to 37°C, rat plasma added, and the tubes incubated for 1 hour at 37°C, followed by heating for 5 minutes at 100°C, cooling in an ice bath, and centrifugation (15,000 x g, 20 minutes). After preparation, the tissue extracts were applied to affinity columns and eluted, as described above for urine samples. This separated folates from many of the non-folate components of the extracts and served as an effective purification to facilitate HPLC analysis. The effectiveness of this procedure for deconjugating folates was periodically assessed by analysis of portions of the extracts to which 1 μmol/L pteroyltriglutamate (B. Schircks Laboratory, Jona, Switzerland) had been added. Essentially full conversion to folic acid (pteroylmonoglutamate) was detected in HPLC analysis, which indicated effective deconjugation by rat plasma pteroylpolyglutamate hydrolase activity.

Urine and tissue samples were analyzed by HPLC with diode-array UV detection at 280 nm and a separation method based on that described by Horne et al.,[15] but substituting acetonitrile for ethanol in the mobile phase. Briefly, 100 μL to 1 mL samples were injected and analyzed using a Beckman Ultrasphere IP column (octadecylsilica, 3.9 mm x 25 cm, Beckman Instruments Inc, Fullerton, CA USA). The mobile phase consisted of 10 mmol/L tetrabutylammonium phosphate (TBAP) (solvent A) and 10 mmol/L TBAP:acetonitrile (50:50, v/v) (solvent B), and was run with a gradient program from 8% solvent B to 60% solvent B over 35 min at a total flow rate of 1 mL/min. One-minute fractions were collected and counted for tritium. The folates were identified by retention times and UV absorption spectra recorded with the diode array detector, as compared with standards. Spectra were identical to those reported previously under similar chromatographic conditions.[13] 10-Formyl-THF and 5,10-methenyl-THF were measured separately, but the data combined and expressed as 10-formyl-THF because the presence of the methenyl form may have been an artifact of the analysis. A typical chromatogram, as monitored at 280 nm, is shown in *Figure 1*. Because of the susceptibility of 5,10-methenyl-THF to nonenzymatic hydrolysis at neutral pH,[16] this folate is generally not detected in tissue samples. Its presence in these samples suggests that it was formed as an artifact of the preparative conditions, either during the brief acid exposure encountered in elution from the affinity columns or during storage or defrosting of the tissues prior to extraction. Studies of the recovery of 10-formyl-THF added to tissues at the time of homogenization indicated little or no conversion to 5,10-methenyl-THF in this analysis. Thus, the presence of this compound may be attributable to the conditions of tissue handling. All extractions and HPLC analyses were performed in duplicate.

Quantification was done relative to individual standards prepared from commercial folic acid, 5-formyl-THF, 5-methyl-THF using published molar absorptivities[17] to determine the concentration of these folates in stock solutions. For 10-formyl-THF, THF, and DHF, the relative absorptivity values reported by Selhub[18] were used to determine the exact concentration of the standard by comparison to the response of a folic acid standard of known concentration. Standards of 5,10-methenyl-THF and 10-formyl-THF were prepared from commercial 5-formyl-THF.[16] Folic acid, 5-formyl-THF, 5-methyl-THF, THF, and DHF were obtained from Sigma Chemical Co. (St. Louis, MO USA).

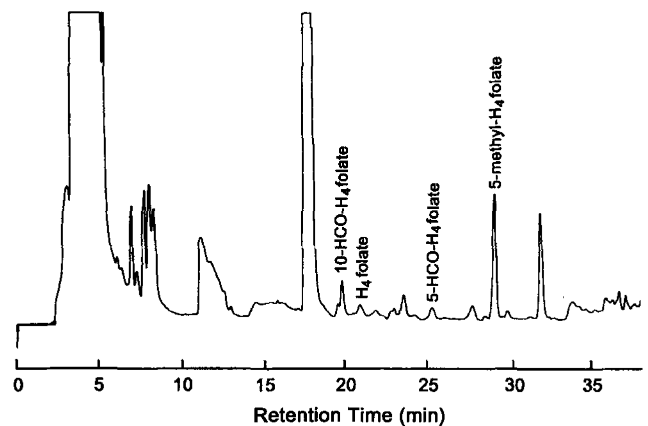

Figure 1 A typical chromatogram showing the separation of folate monoglutamates in liver. The Y-axis represents the absorbance at 280 nm. In addition to the peaks identified, retention times for folates not detected in this sample were: 5,10-methenyl-THF, ~20.5 min; DHF, ~26.5 min.

Fecal samples were defrosted then homogenized in HEPES/Ches buffer, pH 7.85, with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY USA). After centrifugation (7000 x g, 15 min), a 1 mL portion was decolorized by adding 25 μL of 3 mol/L KOH, incubating at 70°C for 1 min, adding 100 μL of 30% H_2O_2, waiting 15 min, then adding 25 μL of 3 mol/L HCl.[19] A portion of the decolorized fecal homogenate was counted for radioactivity.

Results

Twenty-two rats started the study weighing 140 to 170 g (151.1 ± 8.3 g) (mean ± SD) and two finished 6 weeks later weighing 337 to 345 g (341 ± 5.7 g), which constituted a mean weight gain of 4.2 g/d over the course of the study. The rats consumed a mean of 15.4 g food/day/rat.

Following a dose of [^{3}H]folic acid, labeled folates were excreted rapidly in the urine during the first 48 hours, then more slowly for the remainder of the study (*Figure 2*). Urinary folates were almost entirely 5-methyl-THF, with very small amounts of 5- and 10-formyl-THF. By 32 days post-dose, 35% of the tritium label had been excreted in the urine.

Labeling of the non-folate fraction of the urine increased rapidly during the first 48 hours, which was followed by a slower steady rate of excretion of tritium throughout the rest of the study (*Figure 2*). The non-folate fraction of urine was comprised of folate catabolites derived from cleavage of the C9–N10 bond. No attempt was made to quantify these catabolites individually in this study. Consequently, compensation for the distribution of tritium on the folate molecule and the resulting differences in specific radioactivity of catabolic products and the parent compound was not necessary. Also, no correction was made for the potential loss of ^{3}H from the C9 position of the folate molecule during

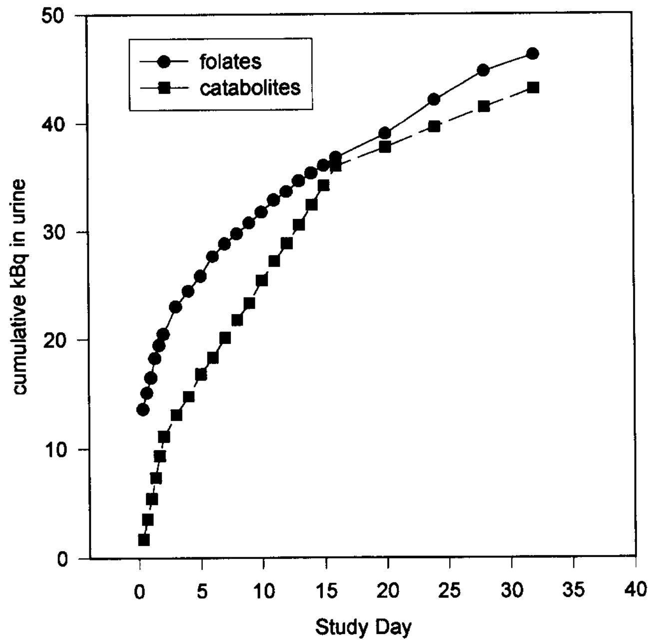

Figure 2 Cumulative excretion of (a) [^{3}H]folates and (b) [^{3}H]catabolites in the urine of growing rats fed a folate-adequate purified diet.

oxidative cleavage because the resulting ^3H_2O would be eliminated primarily by urinary excretion and, thus, detected in this analysis. Fecal tritium excretion occurred slowly for the first 3 days, then increased rapidly for 3 days, then continued at a fairly steady rate for the remainder of the study (*Figure 3*).

All of the tissue folates exhibited maximum tritium-labeling early in the study. On a per gram of tissue basis, liver and kidney contained the most label, followed by spleen, heart, whole blood, and testes, with the least labeling in muscle (*Figures 4–6*). Liver showed the highest quantity of tritiated forms of THF, 10-formyl-THF, and 5-methyl-THF by the first sample at 8 hours (*Figure 4a*). A maximum occurred at 40 hours post-dose for all liver [^{3}H]folates. Kidney also exhibited a maximum in the content of total [^{3}H]folate at 40 hours. The quantity of tritium associated with kidney folates then dropped rapidly, followed by a noticeable rise through day 16 post-dose, then a slower loss of label (*Figure 4b*). No other tissue exhibited this delayed biphasic response with the second maximum at day 16. Spleen exhibited a smaller peak of labeling within the first few days followed by another rapid increase before a slow loss of label (*Figure 4c*). This occurred before the rise in kidney labeling that occurred at day 16. Labeling in the heart was first detected at 8 hours, then decreased before exhibiting the main early peak, as seen in the other tissues (*Figure 5a*). Apparent peaks and valleys for heart may be random variation, as the scale is quite expanded. The isotopic labeling of whole blood peaked at 8 hours, then dropped, followed by a second peak and subsequent slow loss (*Figure 5b*). This may represent transfer of labeled folates from whole blood to tissues followed by loss of label from the tissues with metabolism of the folates. It should be noted that this represents combined plasma and cellular components that may exhibit quite different behavior. Testes and muscle showed a very low level of labeling throughout the study (*Figure 6 a & b*). The testes showed a small apparent peak of 5-methyl-THF within the first days, whereas labeling of muscle appeared to increase slightly as the study progressed.

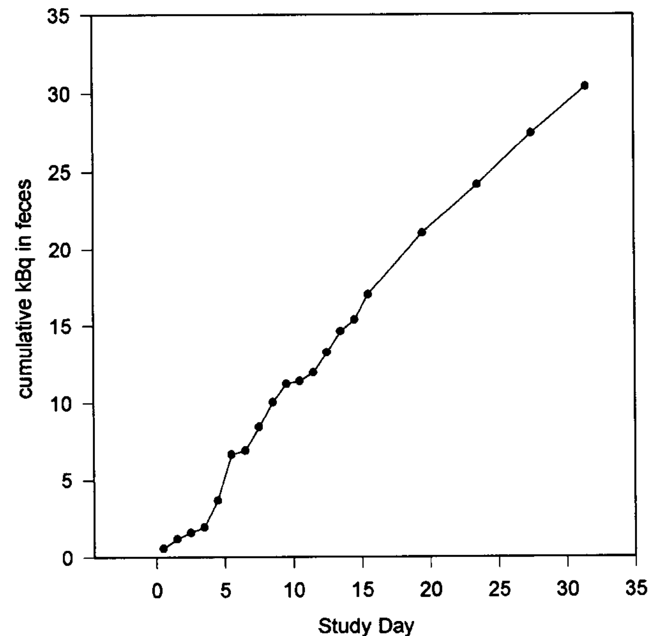

Figure 3 Cumulative excretion of tritium in the feces of growing rats fed a folate-adequate diet. (kBq.)

The design of the study involved analysis of two rats per time point. This number was chosen to minimize animal use and facilitate conducting this labor-intensive protocol. Unfortunately, this yielded a low sample number ($n = 2$) for individual observations. Irrespective of the small sample size, the variation between duplicate samples analyzed and between rats in any pair analyzed was small. This is reflected by the small standard deviations observed at each time point. The fraction of dose retained as individual folates (measured in monoglutamyl form) within each tissue at 32 days post-dose is shown in *Table 1*. This time point was chosen for presentation because it was expected to reflect most closely labeled folate that had approached equilibrium with endogenous (non-labeled) folate in tissues. Of the label present in the tissues analyzed, the greatest portion was in liver, with the predominant forms being THF, 5-methyl-THF, and 10-formyl-THF. Kidney contained greater amounts of labeled THF and 5-formayl-THF than of 5-methyl-THF. The other tissues had most of the label as 5-methyl-THF and as 10-formyl-THF.

Because the rats were maintained in approximately steady-state conditions with respect to dietary folate and we observed no time-dependence of tissue total folate concentration, we pooled observations from all time points (*Table 2*). Total folate (non-labeled plus labeled) in liver was comprised predominantly of THF, 5-methyl-THF and 10-formyl-THF, whereas heart, testes, and muscle contained predominantly of 5-methyl-THF. In contrast, 10-formyl-

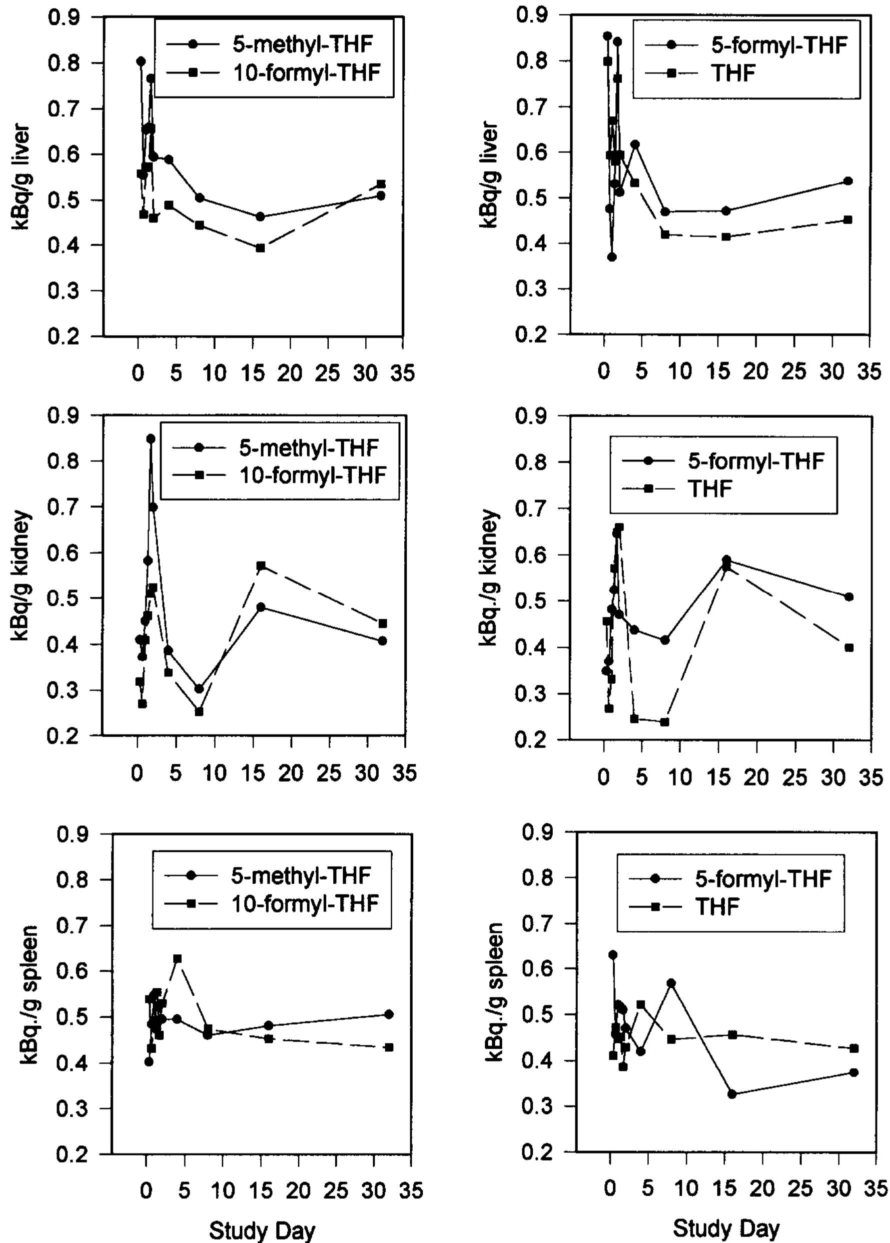

Figure 4 Tritiated folates in the tissues of growing rats fed a folate-adequate diet. (a1) liver 5-methyl-THF & 10-formyl-THF. (a2) liver 5-formyl-THF & THF. (b1) kidney 5-methyl-THF & 10-formyl-THF. (b2) kidney 5-formyl-THF & THF. (c1) spleen 5-methyl-THF & 10-formyl-THF. (c2) spleen 5-formyl-THF & THF. (kBq/g tissue).

THF was the major form in kidney and spleen. Only 5-methyl-THF was detected in whole blood. Liver had the highest concentration of total folate, with kidney exhibiting approximately half that of liver. Comparing heart, spleen, and testes (the lower folate organs), spleen had the highest overall folate concentration. However, because it was the smallest tissue mass (*Table 3*), spleen had the lowest total folate content of these organs.

The specific radioactivity of the folates in each tissue at the 32-days post-dose observation is presented in *Table 4.* Liver and kidney, the organs with the highest folate concentrations, exhibited lower specific activities than the others, with the following order: liver < kidney << testes, spleen, whole blood < heart << muscle.

Discussion

This study involved measurement of individual folates, after hydrolysis to their monoglutamyl form, and included measurement of total and radiolabeled metabolites in liver, kidney, heart, spleen, testes, muscle, heart, and urine, and radioactivity in feces of growing rats over a period of 32 days after an intragastric dose of [^{3}H]folic acid. Data of this type are essential to the development of a mathematical model of whole-body folate distribution, metabolism, and turnover. We observed that the major excretion of intact [^{3}H]folates in the urine occurred over the first 48 hours, which was in agreement with the results of Bhandari and Gregory.[2] The excretion of intact [^{3}H]folates decreased to a minimal level

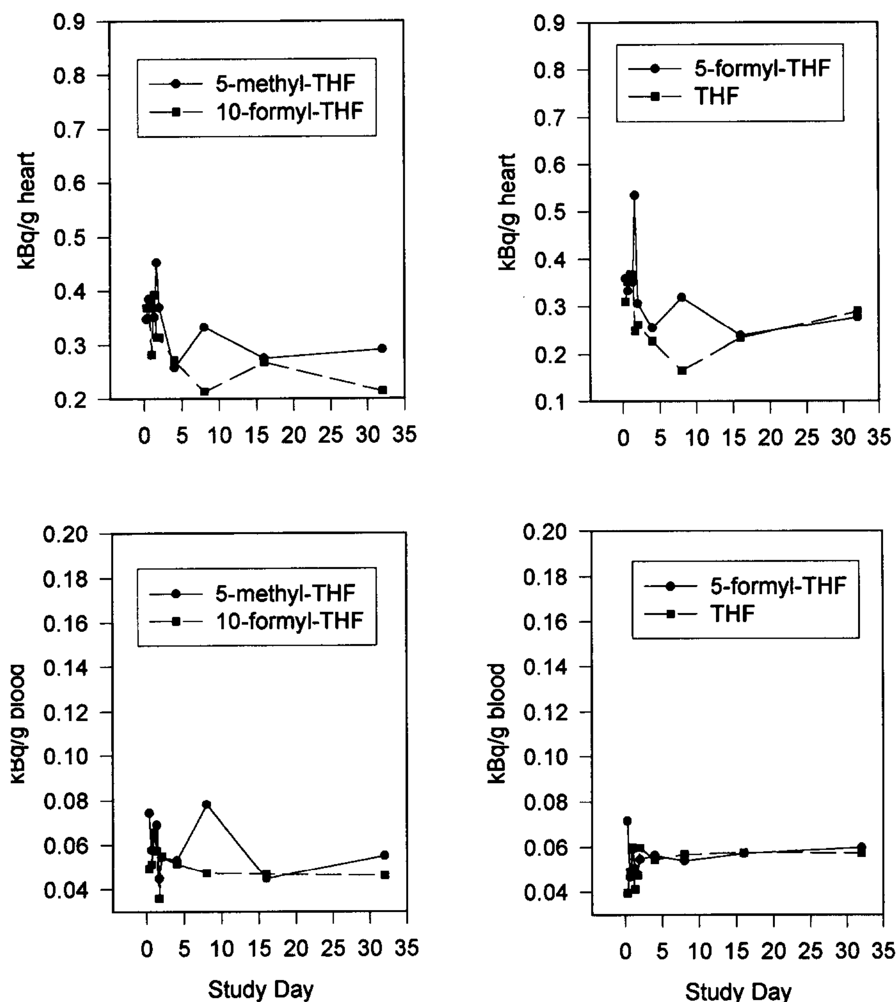

Figure 5 Tritiated folates in the tissues of growing rats fed a folate-adequate diet. (a1) heart 5-methyl-THF & 10-formyl-THF. (a2) heart 5-formyl-THF & THF. (b1) whole blood 5-methyl-THF & 10-formyl-THF. (b2) whole blood 5-formyl-THF & THF. (kBq/g tissue).

by 12 days post-dose, consistent with the observations of Murphy and Scott.[9] The absence of urinary [^{3}H]folic acid at all time points indicated complete incorporation of the dose into folate metabolism at this level of folic acid intake.

Cumulative excretion of urinary [^{3}H]folates and catabolites increased rapidly during the first 48 hours, then much more slowly for the remainder of the study (*Figure 2*). As the study progressed, labeled folate catabolites represented a higher percentage of excreted tritium, as also seen by Murphy and Scott.[8] The peak in labeled catabolites at 3 to 4 days post-dose (*Figure 2*) may correspond to the metabolism of folates from rapidly turning-over pools. The slower excretion later may represent turnover of folates from pools that exhibit slower kinetics of catabolic processes. The total tritium (folate + non-folate) excreted in urine (35% of the dose) and feces (15% of dose) follows the pattern seen by Bhandari and Gregory[2] and Pheasant et al.[3] Of the 35% of dose excreted in urine, approximately half was from intact folates, while the remainder was from catabolic products. In later stages of the study, most of the urinary radioactivity was from catabolic products. These results illustrate the significance of catabolism in the turnover and, hence, the requirement for this vitamin.

The fraction of labeled dose retained in the tissues of the rats at the end of this study followed the pattern described by Pheasant et al.,[3] Barford et al.,[4] and Bhandari and Gregory.[2] Pheasant et al.[3] found 11.4% of the labeled dose retained in the liver at 48 hours, whereas Barford et al.[4] reported 16% at 1 day, 25% at 5 to 9 days, and 15% at 13 days. Bhandari and Gregory[2] observed 14% of the dose retained in the liver at 8 hours, 10% at 4 days, and 5% at 8 days. In the current study we found 17% of the dose remaining in the liver at 32 days post-dose. The magnitude of dietary folate intake may affect the fractional retention of folates in the tissues, and this is being examined in other ongoing studies. The diet used in this study contained 1 mg folic acid/kg diet (2.27 nmol/g), which was slightly less than that contained in the commercial diet fed by Bhandari and Gregory (2) (3 nmol folic acid/g). The other two studies did not specify dietary folate levels. This current study also involved measurement of the fraction of dose retained as individual folates (in monoglutamyl form) within the tissues. The majority of the label was in the form of 5-methyl-THF and 10-formyl-THF, with lesser amounts in the forms of 5-formyl-THF, THF, and DHF.

Total folate concentrations in the tissues were generally

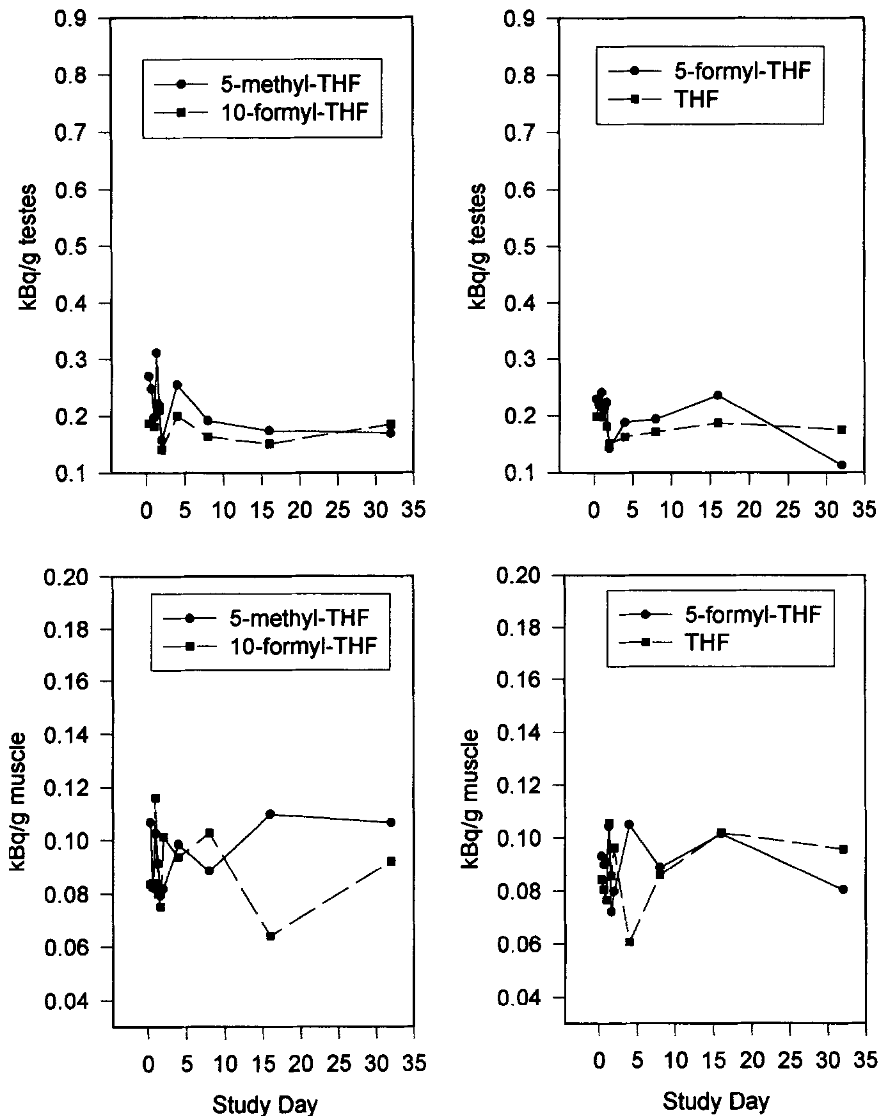

Figure 6 Tritiated folates in the tissues of growing rats fed a folate-adequate diet. (a1) testes 5-methyl-THF & 10-formyl-THF. (a2) testes 5-formyl-THF & THF. (b1) muscle 5-methyl-THF & 10-formyl-THF. (b2) muscle 5-formyl-THF & THF. (kBq/g tissue).

similar to those found by Selhub,[18] Varela-Moreiras and Selhub,[20] and Clifford et al.[21] Selhub[18] and Varela-Moreiras and Selhub[20] reported for rats receiving an 8-mg folic acid/kg diet 23 to 27 nmol folate/g liver with 7 nmol/g as 10-formyl-THF, 8 nmol/g as THF, 2.2 nmol/g as 5-formyl-THF, and 5.4 nmol/g as 5-methyl-THF. We found similar values of 28.9 nmol folates/g liver, with 8.1 nmol/g as 10-formyl-THF, 10.1 nmol/g as THF, 3.3 nmol/g as

Table 1 Fraction of dose retained in tissues 32 d after administration

	10-formyl-THF	THF	5-formyl-THF	DHF	5-methyl-THF	Total
Liver	5.79 ± 0.77[1]	3.05 ± 0.57	2.98 ± 0.35	2.26 ± 0.36	3.35 ± 0.36	17.4 ± 1.42
Kidney	1.14 ± 0.04	0.67 ± 0.03	0.69 ± 0.20	0.44 ± 0.02	0.55 ± 0.02	3.49 ± 0.15
Heart	0.38 ± 0.03	0.16 ± 0.04	0.21 ± 0.05	0.17 ± 0.02	0.22 ± 0.01	1.14 ± 0.05
Spleen	0.32 ± 0.01	0.17 ± 0.05	0.14 ± 0.03	0.13 ± 0.01	0.20 ± 0.01	0.96 ± 0.06
Testes	0.75 ± 0.07	0.31 ± 0.04	0.23 ± 0.02	0.25 ± 0.09	0.35 ± 0.02	1.89 ± 0.12
Whole blood	0.29 ± 0.05	0.15 ± 0.01	0.16 ± 0.03	0.16 ± 0.03	0.15 ± 0.04	0.91 ± 0.13
Muscle[2]	4.02 ± 0.27	3.0 ± 0.21	4.0 ± 0.36	3.0 ± 0.35	6.5 ± 0.04	20.5 ± 1.32

[1]Mean ± SD, $n = 2$.
[2]Based on an assumed muscle mass of 35% of body weight (Millward, 1970).

Table 2 Total (unlabeled & labeled) tissue folates of rats 32 d post-dose[1]

	10-formyl-THF	THF	5-formyl-THF	DHF	5-methyl-THF	Total
			nmol/g			
Liver	8.1 ± 0.04[2]	10.1 ± 0.53	3.3 ± 0.2	0	7.4 ± 0.30	28.9 ± 1.5
Kidney	4.07 ± 0.08	1.2 ± 0.7	0.51 ± 0.02	0.18 ± 0.06	2.01 ± 0.03	7.97 ± 1.3
Heart	0.14 ± 0.07	0	0.10 ± 0.004	0	0.38 ± 0.03	0.62 ± 0.11
Spleen	0.57 ± 0.09	0.42 ± 0.08	0.07 ± 0.03	0	1.11 ± 0.13	2.17 ± 1.02
Testes	0.42 ± 0.16	0.21 ± 0.02	0.09 ± 0.02	0	0.47 ± 0.04	1.19 ± 0.53
Muscle	0	0	0.014 ± 0.002	0	0.13 ± 0.02	0.14 ± 0.02
Whole blood	0	0	0	0	0.32 ± 0.03	0.32 ± 0.03

[1]Values of zero represent concentrations below those detectable by the methods used.
[2]Mean ± SD, *n* = 22.

5-formyl-THF, and 7.4 nmol/g as 5-methyl-THF. The differences in total intake, duration of feeding, and the absence of succinylsulfathiazole from the diets used by these investigators indicate that these similar patterns should be viewed with caution. The diets used by Clifford et al.[21] were of the same composition as those used here. For rats fed a diet containing 1 mg folic acid/kg of diet, total tissue folates (nmol/g) for liver, kidney, spleen, and heart as 11.6, 5.4, 1.2, and 0.55, respectively. Our values for these tissues were 28.9, 6.1, 2.1, and 1.3 nmol/g tissue, respectively. In addition to dietary intake level, age, and size of the rats might account for some of the differences, as the rats used by Varela-Moreiras and Selhub[20] were fed for 25 weeks, and those used by Clifford et al.[21] were 50 g rats (initially) fed for 25 days. Ours were 150 g rats (initially) fed for 32 days.

This study followed a tritium label through folate metabolism and catabolism in the growing rat. We have shown on a more quantitative basis than in previous studies the extent of distribution, metabolism, and excretion of intragastrically administered folic acid under conditions of controlled and known folate intake. Bhandari and Gregory[2] reported that a radiolabeled tracer dose of folic acid was almost completely absorbed. This study monitored labeling in various pools of tissue folates and their excretory forms over 32 days, much longer than most previous studies. Labeled folates were measured repeatedly in most major organs and tissues, rather than in a single tissue or at a single time point. In addition, this study provides detailed kinetic data regarding folate content (labeled and total) in tissues, not just whole body retention calculated from the dose and analysis of excreta. Approximately 26% of the dose remained in liver, kidneys, heart, spleen, and testes after 32 days. With 35% measured in the urine, 15% in the feces, and 26% in these selected tissues, there remained approximately 24% of the dose unaccounted for. Approximately 20% of the dose was found in the muscle, based on an assumed muscle mass of 35% of body weight.[22] This left approximately 4% of administered [^{3}H]folate apparently remaining in the tissues not sampled, including skin, hair, brain, bone, and the GI tract, among others. Some of the folate may also have been lost during sample collection, and during sample preparation, although these results suggest that such losses were minimal.

To our knowledge this is the first isotopic tracer study that has examined the specific radioactivity of folates in various tissues. The unexpectedly large differences observed among the tissues (*Table 4*) may be due to either or both of the following proposed mechanisms: (a) The main folate pool in these tissues varies in rate of turnover and, thus, the tissues exhibit different rates of excretion and/or catabolism of the residual [^{3}H]folate and differences in the rate of its dilution by non-labeled dietary folate. (b) These data may also indicate the presence of kinetically very slow pools in liver and kidney that do not fully equilibrate with the [^{3}H]folate over the time frame of this study. Regardless of the mechanism responsible, such differences become readily apparent at the later stages of a single-dose study of

Table 3 Range of tissue weights at the time of sacrifice

	Weight Range	Weight Average
	g	
Liver	8.1–13.1[1]	10.0 ± 1.3
Kidney	1.7–2.6	2.1 ± 0.2
Heart	0.86–1.44	1.05 ± 0.14
Spleen	0.54–0.80	0.68 ± 0.09
Testes	2.6–3.6	3.2 ± 0.3
Muscle[2]	75–121	90 ± 13

[1]Mean ± SD, *n* = 22.
[2]Total muscle mass was assumed to be 35% of body weight (Millward, 1970).

Table 4 Specific activity of tissue folates of rats 32 d post-dose[1]

	10-formyl-THF	THF	5-formyl-THF	DHF	5-methyl-THF	Total
			kBq/nmol			
Liver	0.12[2]	0.053	0.14	0	0.069	0.109
Kidney	0.21	0.33	1.0	17.1	0.20	0.32
Heart	3.07	0	2.7	0	0.76	2.48
Spleen	1.49	1.01	4.1	0	0.45	1.12
Testes	0.79	0.81	1.3	0	0.36	0.73
Muscle	0	0	6.8	0	0.82	3.62
Whole blood	0	0	0	0	0.17	0.96

[1]Values of zero represent concentrations below those detectable by the methods used.
[2]Mean ± SD, *n* = 22.

this type. These results indicate distinct differences in the kinetics of long-term folate turnover among tissues.

The biphasic nature of the tissue [^{3}H]folate retention and cumulative excretion curves of this study indicate the presence of both faster and slower turnover pools within the body. The urinary excretion of label shows a biphasic pattern of rapid and slow rates of loss. In this regard, the presence of label in selected tissues 32 days post-dose indicates the presence of at least one slow turnover pool in the tissues. As mentioned previously, the specific radioactivity data presented in *Table 4* strongly suggest kinetic differences within these slow-turnover pools. The rapid appearance and disappearance of tissue [^{3}H]folate at early time points post-dose indicates the presence of rapid turnover pools in these tissues. The presence of a peak in urinary catabolite labeling also indicates the presence of rapid turnover pools in the tissues. These data, along with the near absence of [^{3}H]folate acid in tissues and excreta, also show that the [^{3}H]folic acid was fully and rapidly incorporated into folate metabolism.

These data are being used to develop a compartmental model of folate metabolism in the growing rat. Additional studies at higher and lower levels of dietary folic acid will permit detection of any differences in metabolism of folates that may occur with changes in dietary folate intake. The model will be of use in these and further studies with the rat and for interpolating data for humans and other species where tissues and excretory products are less readily available.

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