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IN VIVO KINETICS OF FOLATE METABOLISM

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■ **Abstract** Investigation of the in vivo kinetics of folate metabolism provides information that contributes to a better understanding of the manner in which this vitamin is processed in vivo. Kinetic studies can yield insight into the requirements for folate, especially with respect to factors that may lead to increased requirements. This review considers the strengths and weaknesses of various approaches to the study of folate kinetics and resulting data, followed by a summary and interpretation of existing data.

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INTRODUCTION AND OVERVIEW

Folate Metabolism and Health

The requirement for folate is derived from the need to maintain tissue pools of tetrahydrofolates. These compounds act as substrates and coenzymes in the acquisition, transport, and enzymatic processing of one-carbon units for amino acid and nucleic acid metabolism and metabolic regulation (18, 91). Folates act in the methionine cycle, in which one-carbon units are converted to methyl groups for use in the remethylation of homocysteine to form methionine for use in cellular methylation reactions (as S-adenosylmethionine) and protein synthesis. Folates also support nucleic acid synthesis through their roles as carbon donors in the synthesis of purines and thymidylate. Thus, a deficiency of tetrahydrofolate pools can lead to impaired homocysteine remethylation, methionine synthesis, cellular methylation processes, and nucleotide synthesis. Similar metabolic effects also can occur as a consequence of mutations or genetic polymorphisms of enzymes of one-carbon metabolism and can be secondarily associated with other nutritional deficiencies, especially vitamin B12.

The public health consequences of inadequate folate nutrition are clearly apparent. Elevated plasma homocysteine concentration is an independent risk factor for coronary heart disease, venous thrombosis, carotid artery stenosis, other forms of vascular disease, and adverse outcomes of pregnancy (7, 8, 68, 75, 78). Relationships between inadequate folate status, elevated homocysteine concentration, and risk for most of these conditions are similarly strong (7, 69, 70). Inadequate folate status in women of childbearing age increases the risk of a fetus being affected by a neural tube defect or certain other forms of birth defects (7). In addition, the risk of certain forms of cancer (e.g., colon cancer) also increases when folate intake is inadequate (14).

Whereas advances in our understanding of folate nutrition have led to new recommendations for folate intake (39), scientific disagreement still remains regarding folate intakes necessary to optimize one-carbon metabolism and minimize the risk of chronic disease. The relative roles of the various folate turnover mechanisms are not fully defined, nor are the consequences of changes in folate intake or turnover. These and other quantitative uncertainties of whole-body folate metabolism also hinder the development of precise estimates of folate requirements. Particular attention is now needed regarding optimum intakes in certain population groups (e.g., pregnant women) and on genetic effects on folate requirements.

Why Study Folate Kinetics?

Investigation of the whole-body kinetics of any nutrient provides insight into (*a*) the rate and manner of turnover, (*b*) the physiology by which the compound is handled by the body, (*c*) the number of kinetically identifiable pools and their

relative masses and rates of turnover, and (*d*) the intake that is necessary to sustain body pools of that nutrient. Examination of the influence of nutritional, genetic, or physiological variables on the kinetics of vitamin metabolism provides information that complements static measurements of nutritional or metabolic indicators. Finally, the data obtained from kinetic studies may also lead to the development of mathematical models that integrate metabolic processes in cells, tissues, organs, or the entire organism to facilitate the testing of hypotheses. Such models also may allow the prediction of the consequences of changes in intake or physiological processes that cannot be directly assessed experimentally. Insights derived from kinetic analysis and modeling also may aid in the design of more effective research studies (e.g., duration of experiments, frequency of sampling, what to measure, appropriate tracer dose, etc.). Finally, knowledge of in vivo kinetics may provide improved understanding of nutritional requirements and insight into the effects of nutritional interventions such as supplementation and food fortification.

Summary of Folate Physiology Relevant to Kinetic Analysis

An understanding of the processes by which folate is handled by the body is essential to the interpretation of kinetic data and development of mathematical models. Folate exhibits complex whole-body physiology and metabolism (80, 82). Folate absorption occurs by a primarily jejunal carrier-mediated process that exhibits saturation kinetics and functions in the micromolar range of intralumenal concentration with little selectivity among various folates. At higher folate concentrations folate uptake across the brush border membrane occurs as a linear function of concentration. Newly absorbed folate molecules can be actively metabolized in intestinal mucosal cells leading to conversion of dietary folic acid to 5-methyltetrahydrofolate (5-methylTHF). The appearance of 5-methylTHF in plasma is related to the folic acid dose by a Michaelis-Menten function (49). Higher intakes of folic acid (>200 μg dose) begin to exceed the metabolic capacity of the intestinal mucosa, which leads to the appearance of small amounts of folate in unreduced form (i.e., as folic acid) in the circulation (41).

The liver is the major site of one-carbon metabolism and has a larger mass of folate and higher folate concentration than other tissues (for reviews see 18, 36). Retention of folates in hepatic and other cells is aided by conversion to poly-γ-glutamyl derivatives and by association with specific folate-binding proteins, most of which are enzymes involved in folate metabolism. Because folate is secreted in bile and reabsorbed, enterohepatic circulation is an important aspect of folate physiology. Biliary folate concentration varies considerably with folate intake. A calculation based on a mean biliary folate concentration of $\sim$90 nmol/liter (47) and bile flow of 600–700 ml/day suggests that about 60 nmol/day enters enterohepatic circulation (33). This suggests that at least 10% of daily folate intake would enter enterohepatic circulation, which is mainly 5-methylTHF because 5-methylTHF is a poor substrate for hepatic folylpolyglutamate synthetase (80, 81, 94). Thus,

newly absorbed 5-methylTHF, and that formed from folic acid, is more extensively exported through biliary secretion than are other folates entering the liver. The cellular retention of 5-methylTHF occurs mainly in proportion to the extent of its conversion to other folates as mediated by methionine synthase. By this process, folate entering a cell as 5-methylTHF is converted to forms of folate that are more suitable for cellular retention through polyglutamylation, specific protein binding, and additional participation in one-carbon metabolism. The major form of folate in plasma and interorgan transport is 5-methylTHF, most of which originates in the liver.

The routes of folate turnover have been well documented; however, disagreement exists regarding their relative quantitative contributions. The existence of fecal folate has been attributed to microbial synthesis of the vitamin, although as discussed later, unabsorbed dietary folate, as well as folates from bile, other gastrointestinal secretions, and sloughed mucosal cells contribute to fecal losses of folate. Because of the synthesis and breakdown of folate by the colonic microflora, identification of the origin of fecal folates and catabolic products is challenging. Evidence from tracer studies indicates that fecal excretion is a significant route of folate turnover. Urinary excretion of folate constitutes a small, intake-dependent loss of body folate (19, 33, 38, 59). The kidney serves an important role in folate metabolism through its carrier-mediated reabsorption of folate from the glomerular filtrate (5, 79), and secretion of folate from renal tubular cells into urine also occurs (56).

Folate catabolism constitutes another important in vivo mechanism for the loss of folates (57, 85). The predominant catabolism of the folate molecule occurs by cleavage to yield a pterin and *p*-aminobenzoylglutamate (57, 58). Typically at least 80% of the *p*-aminobenzoylglutamate is excreted as the acetamido derivative (57), which appears to be the product of a specific arylamine acetyltransferase (55). Because of the susceptibility of THF and dihydrofolate to cleavage of the C9-N10 bond under oxidative conditions, some folate "catabolism" probably occurs by nonspecific oxidation. Recent studies suggest that specific mechanisms of folate cleavage also exist (2, 85, 86).

EXPERIMENTAL APPROACHES TO STUDY IN VIVO FOLATE KINETICS

The selection of an experimental kinetic protocol depends on the objectives of the study and resources available. The following issues must be addressed before selecting a protocol. (*a*) Is the emphasis on short- or long-term kinetics? (*b*) Is the folate intake physiological or supraphysiological, and does the dosage regimen involve a single dose (oral and/or intravenous), constant infusion, or repeated doses? (*c*) Can the study be conducted under steady state conditions? (*d*) Are isotopic techniques and analytical procedures available to achieve the objectives of the study? (*e*) What is the appropriate dosage form of folate?

Unlabeled Folates

Short-term folate kinetics often have been examined using a single folate dose administered orally or intravenously followed by serial measurement of either total folate or individual folate forms in serum or plasma (e.g., 1, 10, 48, 53, 66, 90). The need for relatively large doses to induce a consistently measurable change in plasma folate concentration is a limitation of protocols involving short-term plasma response (3, 63), although such studies have relevance to clinical therapies. Short-term kinetics observed under high-dose conditions cannot predict the short-term kinetic behavior of folate at more nutritionally relevant intakes because of the saturability of many processes of folate transport and metabolism. Also, short-term kinetic studies cannot predict the behavior of major body folate pools that exhibit much slower turnover rates.

The saturability of metabolic conversions among folates complicates the design of kinetic experiments and interpretation of published studies, and it complicates prediction of the effects of interventions such as supplement use and food fortification. Low doses of oral folic acid undergo extensive conversion to 5-methylTHF in the intestinal mucosa, but this metabolic conversion is a saturable process (49, 65). Consequently, small amounts of unmetabolized folic acid are detectable in plasma following oral folic acid doses greater than 200 μg (41). Although no adverse consequences of the presence of unmetabolized folic acid have been documented, further research is needed to assess its metabolic effects. Because of saturability of the conversion of folic acid to 5-methylTHF and known differences in short-term plasma kinetics of folic acid and 5-methylTHF (42), the short-term plasma kinetics in studies with folic acid doses would be expected to be strongly dose-dependent. The metabolic conversion of 5-formylTHF to 5-methylTHF and other reduced folates also is subject to saturation and, consequently, dose-dependent kinetics (10, 21, 51, 67, 83). Studies of the kinetics of total plasma folate following high-dose folate administration, e.g., of 5-formylTHF, also would be complicated by the fact that differences exist between the plasma kinetics of 5-formylTHF and 5-methylTHF, the main short-term metabolite (61).

Radioisotopic Studies

The use of tracers in metabolic studies has the conceptual advantage that the investigator can be assured that any labeled compound in blood, tissues, or excreta is derived from the administered dose. The development of [^{3}H]folic acid of high specific activity led to many metabolic and kinetic studies, most of which involved the administration of a single dose of [^{3}H]folic acid or other [^{3}H]folate forms. Additional studies have been conducted using [^{14}C]folic acid, but its lower specific radioactivity limits its use in some applications. Typical radioisotopic protocols used in studies of folate kinetics involve administration of a single dose of [^{3}H]folic acid or other folate, followed by serial determination of radioactivity in urine, feces serum/plasma, and/or tissue radioactivity in animals (4, 45, 58, 76, 87) or humans (44, 46, 72). Because of the sensitivity of liquid scintillation spectrometry in

quantification of [^{3}H]folates and their metabolites, studies with [^{3}H]folates permit the use of single doses that induce little or no perturbation of folate pools.

A novel approach to radioisotopic tracer studies involves the use of accelerator mass spectrometry to determine ratios of $^{14}C/^{12}C$ of carbon derived from plasma, erythrocytes, urine, and feces, which permits the use of a single very small dose (35 μg, 100 nCi) of [^{14}C]folic acid in human subjects (9, 15). The extremely low detection limits for this method, coupled with a large dynamic range, permit investigation of both short-term and long-term kinetics following the administration of a radiologically insignificant dose of this radiotracer. Kinetic protocols that involve sufficient sampling for short-term and long-term observations have yielded preliminary but thorough data on human folate kinetics (9, 15). This powerful technique is not widely applicable because of the limited availability of the instrumentation and the strict need for a dedicated laboratory to minimize contamination from extraneous carbon sources. Also, studies to date have not involved separation and individual analysis of folates and catabolic products.

Stable-Isotopic Studies

The use of stable isotopes in labeling organic compounds for in vivo use has been an important tool since pioneering work in the 1930s (95). The synthesis of organic compounds such as folate containing one or more atoms of a stable isotope of a constituent element or elements (e.g., ^{2}H, ^{18}O, ^{13}C, and/or ^{15}N) can provide a potentially useful tracer. The incorporation of stable isotopes into the folate molecule ideally yields no significant change in chemical or metabolic properties or significant loss of the isotropic atoms during metabolism, separations, or chemical derivatization reactions necessary for gas chromatography–mass spectrometry analysis. The use of deuterium (^{2}H) in labeling folates requires caution because ^{2}H must be located at positions not subject to chemical release (e.g., by either acid-catalyzed exchange or other dissociation) and that do not alter rates of metabolic/physiological processes.

The labeling of folate molecules suitable for in vivo use has mainly involved the introduction of ^{2}H or ^{13}C on the central benzene ring or glutamate tail (23, 24, 26, 29, 30, 34, 50, 62). Many of the original applications of stable-isotope–labeled folates addressed issues related to folate absorption and bioavailability in humans, and many such protocols require the use of two labeled forms of the vitamin (e.g., 6, 25, 26). The measurement of labeling of folates and catabolic products generally involves gas chromatography–mass spectrometry (e.g., 88), and liquid chromatography–mass spectrometry procedures also have been reported (60a). These methods are used to determine the molar ratio of labeled and unlabeled forms of the analyte after correction for the background that exists from the natural abundance of the isotopes. A characteristic of such methods is their modest sensitivity, which yields a working detection limit of approximately 0.1 mole percent excess above natural abundance. Consequently, applications of stable-isotope–labeled folates may lack the sensitivity of radioisotopic methods when used in

single-dose protocols, especially when labeled compounds are administered in the physiological range. Alternatively, protocols that use long-term, repeated administration of the labeled folate tracer(s) allow kinetic analysis of rates of labeling of body folate pools. An advantage of chronic administration is that kinetics can be evaluated during the tracer administration phase of the protocol (33, 84) and after replacing the tracer with an equivalent chronic dose of unlabeled folate (27). The precision of quantification is improved by the use of tracers that have more than one heavy atom, ideally two or more, because there is a lower background signal from natural abundance. Thus, a singly labeled folate tracer, such as the [benzoyl carbonyl-$^{13}C_1$]folate (60) or [7-$^{2}H_1$]folate (64), would not be ideal for in vivo studies.

Two other matters should be considered regarding the appropriateness of using stable-isotope–labeled folates for in vivo analysis: the potential for isotope effects and the possibility of loss of deuterium atoms by proton exchange during metabolism or analysis. Isotope effects include all differences in physical, chemical, or metabolic properties between the labeled and unlabeled forms of the compound of interest. Depending on the site of labeling, the introduction of heavy isotopes into the folate molecule has the potential to alter the rate of processing (e.g., reduction, polyglutamylation, and/or catabolism) or alter physical properties (e.g., pKa values) that could potentially affect transport or coenzymatic or protein binding properties. In addition, it is essential that no labeling occur at sites of enzymatic oxidation/reduction processes because of the kinetic difference expected in enzymatic reactions involving hydride transfer. To date, no significant isotope effects have been identified for the stable-isotope–labeled folates that have been used as tracers in studies of folate bioavailability and whole-body kinetics, which include [glutamate-$^{2}H_4$], [3′,5′-$^{2}H_2$], and [glutamate-$^{13}C_5$] folates examined in the authors' laboratory. Direct comparisons of in vivo short-term absorption, metabolism, and excretion have been made between [glutamate-$^{2}H_4$] and [3′,5′-$^{2}H_2$] folic acid species (31) and between [glutamate-$^{13}C_5$] and [3′,5′-$^{2}H_2$] folic acid species (71) in human subjects. The equivalent metabolic and analytical recovery observed between these ^{13}C- and ^{2}H-labeled folates (71) is strong evidence of the appropriateness of quantitative metabolic studies with these compounds when administered by the same oral route; these data indicate that chemical or metabolic isotope effects, if any, are not of quantitative importance. Several alternative labeled forms of folates have been reported, including [2′,3′,5′,6′-$^{2}H_4$] and [benzoyl-$^{13}C_6$] folates (23, 50, 73). Although little or no information has been published with which to evaluate the latter compounds, one would predict that isotope effects would be minimal.

Dueker et al. (22) have discussed issues of tracer selection and protocols for human metabolic studies with labeled compounds. Much of their discussion focused on potential problems of certain folate tracers, especially with respect to the potential for loss of label by exchange processes during separations and preparation for analysis. They discussed the potential for the acid-catalyzed loss of deuterium atoms from [3′,5′]folate or catabolic products. Because acidic conditions are used

chromatographically in isolating folates and catabolic products and acidic conditions are encountered during derivatization prior to gas chromatography–mass spectrometry analysis, the potential for loss of deuterium from 3′,5′-labeled folates is of concern. Indeed, acid-catalyzed exchange from D_2O is actually a facile means of labeling folic acid (34). Whereas Dueker and associates (22) are correct with respect to this caution regarding loss of label from folate tracers, such concerns do not override the several lines of evidence (NMR and mass spectral) that indicate that these tracers can be effectively used to obtain valid kinetic and metabolic data when used for in vivo studies. In particular, gas chromatography–mass spectrometry analyses of urinary folates and p-aminobenzoylglutamates from in vivo studies using [3′,5′-$^2H_2$] folic acid as a tracer have not found loss of label to be a problem, as would be evidenced by changes in labeling distributions of $^2H_1$ and $^2H_2$ species (27, 33). In addition, short-term studies using simultaneously administered [3′,5′-$^2H_2$] and [glutamate-$^{13}C_5$]folates support the equivalence of these tracers (6). Although it is prudent for investigators using any folate tracer labeled with deuterium to be cognizant of these potential concerns, we strongly disagree with the statement by Buchholz et al. (9) that studies with deuterium-labeled folate tracers "suffer from problems of poor sensitivity, isotope exchange, and isotope effects."

CURRENT UNDERSTANDING OF FOLATE KINETICS

Many in vivo studies have shown the existence of multiple folate pools that can be characterized as either rapid turnover or slow turnover (4, 15, 27, 33, 44, 45, 58, 72, 87, 90). The following discussion summarizes the published literature, emphasizing primarily human studies, with the objectives of identifying areas of consensus and new insight and needs for further research.

Short-Term Kinetics

An understanding of short-term distribution, metabolism, and transport of absorbed folates is essential to a complete understanding of folate kinetics, but studies focused only on short-term events are not very useful in terms of predicting whole-body folate turnover and metabolism. Thus, minimal attention is given to short-term kinetics in this review. A limitation of many long-term studies is that insufficient sampling time points have been obtained to allow precise characterization of the rapid-turnover folate pools.

One notable aspect of short-term kinetic studies involved the measurement of plasma folate clearance rate following an intravenous injection of folic acid (13, 37, 46, 54). Chanarin et al. (13) observed that the clearance rate of serum folate following folic acid injection (based on *Streptococcus faecalis* assays) was greater in vitamin B12–deficient megaloblastic anemia patients than in normal controls. Metz et al. (54) reported that elevated plasma clearance rate after folic acid injection

is an indicator of folate deficiency, and they correctly speculated that the increased clearance rate seen in vitamin B12 deficiency was due to a consequential folate deficiency. The increased plasma clearance rate caused by both folate deficiency and vitamin B12 deficiency appeared to be due to enhanced retention of injected folic acid by folate-deficient tissues. Herbert & Zalusky (37) extended these observations through a comparison of *S. faecalis* and *Lactobacillus casei* assays (specific for nonmethylated and total folate, respectively) of serum folate after injection of folic acid. They observed faster clearance of serum folate as assessed by *S. faecalis* assays than by *L. casei* assays in vitamin B12 deficiency. These results were interpreted as a "piling up" of folate in a metabolically unavailable form (37), which is consistent with the "methyl trap" phenomenon.

Long-Term Folate Kinetics

Several studies have been reported in which physiological doses of folate tracers have been used in humans (15, 16, 27, 33, 44, 84). Krumdieck et al. (44) administered to a single female subject four consecutive 10-μCi (80 μg) doses of [^{14}C]folic acid over 48 h, then collected periodic urine and feces samples. These investigators reported biphasic kinetics, with an initial rapid decline of urinary radioactivity with a half-life ($t_{1/2}$) of 31.5 h ($k = 0.693/t_{1/2} = 0.022\ h^{-1}$) followed by a long, first-order decline with $t_{1/2} = 100$ days ($k = 0.0069\ days^{-1}$). A similar biphasic decline occurred for fecal radioactivity, although there were insufficient observations to assess the rapid-phase kinetics accurately. We analyzed Krumdieck et al.'s data describing the slow decline of fecal radioactivity and found $t_{1/2} = 94$ d ($k = 0.0074\ d^{-1}$), which suggests that folate pools from which urinary and fecal folates and their catabolic products are derived exhibit similar slow-phase turnover kinetics. Fecal and urinary excretion of total radioactivity were of similar magnitude.

Russell et al. (72) conducted a study of folate kinetics in alcoholic patients. Prior to the study these subjects received 5-mg/day folic acid supplements to attain a state of elevated folate status; consequently, their results are not comparable to those conducted with unsupplemented subjects. The $t_{1/2}$ for the slow phase of folate turnover was 63.7 days in subjects consuming ethanol and 9.6 days during periods of abstinence. These results suggest that chronic alcohol consumption greatly alters the rate of folate turnover; however, the relevance of these findings to humans of typical nutritional status is unclear. Marked effects of chronic alcohol intake in animal studies also have been reported (87).

The first investigation of long-term folate kinetics using stable-isotope–labeled folate was a pilot study (28) that involved chronic administration of [3′,5′-$^2H_2$] folic acid for 8 weeks to a female human subject consuming a controlled diet ($\sim$200 μg/day total folate intake). We reported a preliminary compartmental model comprised of a rapid and a slow pool, with provisions for urinary and other excretory routes from both pools (28). This analysis yielded a fractional catabolic rate for whole body folate of 0.0055 day^{-1} (i.e., loss of 0.55% of the whole-body folate

per day). Although urine collections were not sufficient to estimate the kinetics of the fast-turnover pool reliably, the slow-turnover pool exhibited $k = 0.0073\ day^{-1}$ ($t_{1/2} = 95$ days). Perhaps more important, these results demonstrated the efficacy of protocols with controlled intake of dietary folate and chronic administration of stable-isotope–labeled folate. Also, the agreement of these results and those of Krumdieck et al. (44) and subsequent studies support the validity of the deuterium-labeled tracer.

Stites et al. (84) extended this protocol in a study involving four healthy males who received [$^2H_4$]folic acid daily for 56 days, followed by a switch to an equivalent daily dose of unlabeled folic acid for 56 days. Compartmental modeling based on the isotopic labeling of urinary folate as in the pilot study yielded a fractional catabolic rate of $0.0081 \pm 0.0012\ day^{-1}$ (standard error of the mean), i.e., 0.8% turnover of whole-body folate per day. Estimates of the mass of whole body folate pools were probably imprecise because this analysis was based solely on urinary folate, which accounts for a small fraction of overall folate turnover, and because folate intake was not controlled.

O'Keefe et al. (59) reported a study in which three groups of healthy women (20–30 years old) were fed a controlled diet providing total folate of either 200, 300, or 400 μg/day for 8 weeks. This was the first controlled dietary study indicating that a total folate intake of 200 μg/day, approximately equivalent to the RDA of that era, was not sufficient to maintain adequate folate status. A component of the daily folate intake in this study was [$^2H_2$-folic acid], and determination of labeling of urinary folate and acetamido-*p*-aminobenzoylglutamate (ApABG, the primary catabolic product) allowed a more detailed kinetic analysis than those conducted previously (33). Turnover kinetics exhibited clear effects of folate intake. Of particular kinetic interest were the facts that the rate of labeling of urinary folate and ApABG differed and the relative rates were affected by folate intake. These results indicate different rates of labeling of the in vivo pools from which they are derived. The previous compartmental model (84) was expanded to include a rapid-turnover nonsaturable pool, a slow-turnover nonsaturable pool, and a large, slow-turnover saturable pool whose mass was governed by Michaelis-Menten kinetics (the latter two pools presumably tissue folates). Provisions were included for output of folate via catabolism from the slow-turnover pools, output through urinary excretion from the two nonsaturable pools (which is consistent with urinary folate originating from both glomerular filtration and renal secretion) (56). Provisions were also made for fecal folate excretion from slow-turnover (tissue folate) pools. Finally, a provision was made for a constant return of folate from the nonsaturable slow-turnover pool to the rapid-turnover pool to account for an approximate rate of folate recycling by enterohepatic circulation.

We acknowledge that, without additional data from direct analysis of tissue folate pools, there are major problems of identifiability of pools from the existing data and uncertainty of many rate constants for which there is no direct means of calculation. These factors limit confidence in estimates made using this compartmental model. This illustrates the dilemma that is described perfectly

by the quote from Albert Einstein: "Everything should be as simple as possible, but not simpler." We believe that this compartmental model provides a physiologically relevant starting point in the development of a compartmental model of whole-body folate metabolism. We also believe that the inclusion of a major saturable pool is essential in future compartmental modeling of folate metabolism because most tissue folate is tightly associated with major enzymes of one-carbon metabolism (18, 42, 74, 93). Overall fractional catabolic rates for whole-body folate were 0.0047 ± 0.0002 day^{-1}, 0.0061 ± 0.0004 day^{-1}, and 0.0082 ± 0.0005 day^{-1}, for folate intakes of 200, 300, and 400 μg/day, respectively. These values correspond to whole-body mean residence times (reciprocal of fractional catabolic rate) of 212 ± 8, 169 ± 12, and 124 ± 7 days, respectively (33). The inclusion of data from both urinary folate and urinary ApABG in this study strengthened kinetic conclusions and modeling precision because these two components accounted for nearly 50% of folate intake.

This basic stable-isotopic protocol was extended to a study of folate metabolism in controls and women during the second trimester of pregnancy (11, 12, 27). The subjects were fed a controlled diet plus supplemental folic acid for a period of 16 weeks, with total folate intakes of 450 or 850 μg/day. During the first half of the protocol 15% of the daily folic acid supplement was [$^2H_2$]folic acid, which was replaced with unlabeled folic acid during the second phase of the study in order to have a withdrawal period with no change of total folate intake. Although the intent was to conduct compartmental modeling based on labeling of urinary folate, ApABG, and pABG over the entire protocol, this was not feasible because the total folate intakes in the protocol differed markedly from those of most of the controls and some of the pregnant subjects, which yielded an initial non–steady state condition that precluded modeling of the early phase of the study (27). Kinetic analysis was conducted solely on data following withdrawal of the tracer, because all subjects had reached a quasi–steady state by that time. The decline in labeling of urinary folate after tracer withdrawal exhibited a biphasic pattern for all subjects (i.e., both levels of intake for pregnant women and controls), with a first-order slow phase. In contrast, the labeling of the catabolites pABG and ApABG declined in a simple first-order fashion consistent with their origin from homogenous but distinct tissue folate pools. There was no evidence of increased turnover of tissue folate pools in pregnancy, nor was there increased excretion of labeled forms of the catabolites pABG and ApABG. Differences were observed in the initial isotopic enrichment of urinary folate, pABG, and ApABG, which suggests that pABG is not simply a breakdown product of urinary folate nor is it entirely derived from the same in vivo pool as ApABG. The relatively high chronic folate dose (either 450 or 850 μg/day) yielded accelerated turnover of folate pools. Fractional catabolic rates for whole-body folate turnover were 0.077 and 0.061day^{-1} for nonpregnant women and 0.034 and 0.058 day^{-1} for pregnant women at folate intakes of 450 and 850 μg/day, respectively. When compared with data for nonpregnant women at lower folate intakes, these results illustrate that the turnover of folate pools is greatly accelerated (5- to 10-fold in this case) when folate intake is high or when

folate status is high. These data also illustrate a trend of pregnancy to yield a lower rate of folate turnover than controls at the same level of folate intake (27).

A well-designed 202-day study conducted by Clifford et al. (15) examined the appearance of ^{14}C folates in plasma, erythrocytes, urine, and feces following administration of a single oral [^{14}C]folic acid dose to one subject. After the rapid appearance of ^{14}C in plasma, the subsequent biphasic labeling yielded $t_{1/2}$ values of 8 h and 69 days for rapid and slow turnover processes, respectively. The labeling of erythrocytes began 4 days post-dose, which reflected the time for maturation and release of newly formed cells. Urinary excretion of total ^{14}C, constituting folate and catabolic products, exhibited biphasic kinetics with $t_{1/2}$ values of 4.6 h ($k = 0.15$ day^{-1}) and 63 days ($k = 0.011$ day^{-1}). Fecal excretion (11% of dose) occurred during the first 3 days, which was interpreted as excretion of unabsorbed folate. Unlike the study of Krumdieck et al. (44), Clifford et al. (15) reported that the cumulative fecal excretion of ^{14}C occurred to a much lower extent than urinary excretion. This study has been repeated with several additional human subjects, although the new results only have been reported in abstract form with few quantitative data (16). The strength of this technique is its sensitivity and precision, while the inability to differentiate labeled folate and catabolic products is a limitation.

ISSUES AND PRIORITIES IN STUDIES OF FOLATE KINETICS

The currently available isotopic tracers, experimental protocols, and analytical methods have yielded important insight into whole-body metabolism and physiology of folate. However, uncertainties in folate kinetics and whole-body processing exist, including the following.

Relationships of Folate Kinetics and Health

Although our understanding of the relationships among folate intake, folate status, genetics, and risk of disease has improved markedly in recent years, in most cases the specific mechanisms involved have not been determined. Questions that might be addressed by appropriate kinetic protocols include:

1. What is the effect of increased intake of folic acid or other folate supplements on rates of folate turnover and mass of folate pools? Does marginal vitamin B12 nutritional status, rather than outright deficiency, alter folate kinetics?
2. Does the intake of methyl donors such as choline and betaine influence the in vivo kinetics of folate?
3. What is the influence of common genetic polymorphisms of one carbon metabolism on folate kinetics? In this regard, Stern et al. (83) showed no effect of the C677T polymorphism of methylenetetrahydrofolate reductase on the short-term extent of conversion of a large dose of 5-formylTHF

to 5-methylTHF. Would the C677T effect be more pronounced at a lower 5-formylTHF dose or at low riboflavin status, and is the association of the T/T genotype of this polymorphism with elevated plasma homocysteine at marginal folate status related to differences in rate of folate turnover or mass of folate pools?

4. What is the effect of pregnancy or lactation on folate kinetics, and to what extent do any kinetic changes contribute to greater folate requirements? As discussed earlier, Gregory et al. (27) found that folate-supplemented women in the second trimester of pregnancy did not differ from controls. This confirms and extends findings of Caudill et al. (12) that indicated that there was no difference between second trimester pregnant women and controls in excretion of folate catabolites. However, Irish studies have indicated that urinary excretion of folate catabolites increases during pregnancy (38, 52). Although the studies apparently differed in the stage of pregnancy examined, possible other reasons for these disparate findings remain to be resolved. Also, further clarification of the effects of other factors such as hormones, drugs, toxicants (e.g., smoking and ethanol), and health status on folate turnover is a high priority.
5. How does the therapeutic use of antifolates alter the kinetics of tissue folate pools? How does the use of folate supplementation alter therapeutic efficacy and susceptibility to adverse effects, and how are these factors related to size and kinetics of folate pools?

Mechanisms of Folate Turnover

The efflux of folate from the body occurs by urinary excretion of intact folates, catabolic products, and fecal excretion. Fecal excretion accounts for ~200 μg/day of folate as determined by microbiological analysis (36), which is derived from unabsorbed dietary folate, folate from digestive secretions and sloughed mucosal cells, and microbial synthesis. Contradictory studies have been reported regarding the significance of fecal excretion as a route of folate turnover in humans (4, 15, 33, 44). Tracer studies with rats have shown that fecal excretion is a major route of folate turnover, approximately equivalent to urinary excretion of folate and catabolic products (4, 76). In addition, modeling of human folate metabolism suggests that fecal excretion is an important part of folate turnover (33), and measurement of total excretion of unlabeled urinary folate and folate catabolites accounts for only about half of the folate intake even under conditions in which bioavailability would be very high (11, 12, 33). Thus, balance calculations based on urinary excretion data (12, 33) and compartmental modeling (33) strongly suggest that fecal excretion from endogenous folate pools is nearly equivalent to the total excretion of urinary folates and catabolites. The relative roles of fecal excretion and folate catabolism must be resolved to achieve a complete understanding of whole-body folate turnover.

Interpretation and Modeling Issues

It is useful to consider basic kinetic principles in interpreting the aggregate data of folate tracer studies. If the body's total folate behaved kinetically as a single homogeneous pool, then the labeling and subsequent decline of labeled folates could be described by a single exponential equation (e.g., $Y = Ae^{-at}$, where a is the first-order rate constant). In addition, the isotopic enrichment or specific radioactivity of excreted folate or catabolic products as well as all forms of folate in tissues would be equivalent. We can reject this single-pool model because labeling patterns generally show two exponential components, and we have observed differences in enrichment of urinary folate and catabolic products (27, 33). Moreover, Scott & Gregory (76) determined the specific radioactivity of the hepatic folates in rats 30 days following a tracer dose of [^{3}H]folic acid and found marked differences in labeling. These data strongly suggest that not only are there large kinetic differences between slow-turnover and fast-turnover pools of body folate, but differences in turnover rates of the metabolically active tissue folate species also exist. Differences in the polyglutamyl chain length of folate pools among various organs in folate-deficient rats also suggest that tissue specificity exists in rates of folate turnover (89).

Essentially all folate clearance data obtained to date can be fit by a simple biexponential model (such as $Y = Ae^{-at} + Be^{-bt}$). Such numerical descriptions of data yield valuable quantitative information but little insight into physiological and biochemical properties and relationships among folate pools. However, on the basis of the discussion above, we strongly feel that a simple two-pool compartmental model (e.g., 4) is not appropriate for further kinetic analysis because of its lack of physiological relevance. It is also important to recall an important principle of first-order kinetics, that the rate constant (i.e., a or b in the equation above) is independent of initial concentration of the substrate. The fact that rate constants for whole-body folate turnover, which are governed mainly by the rate of large, slow-turnover tissue pools, are not independent of intake indicates that a model based solely on one or more exponential terms is an oversimplification.

Outlook for Developing Kinetically Based Diagnostic Techniques

The basis of most nutritional status assessment is the measurement of static indicators such as serum or red cell folate concentration. Plasma homocysteine concentration is a functional indicator of one-carbon metabolism, but this is not governed only by folate nutriture. Our laboratory has examined one tracer approach to assess folate status in the context of a study of controlled folate intake. After 10 weeks of controlled intake (200, 300, or 400 μg/day), a short-term study was conducted in which each human subject was given an oral dose of 500μg of [$^2H_4$]folic acid, followed by a 24 h urine collection (32). The urinary recovery of [$^2H_4$]folate, primarily as 5-methyl-[$^2H_4$]tetrahydrofolate, was significantly correlated with chronic folate intake and with serum folate. Urinary [$^2H_4$]folate recovery in a protocol such as this

would be a function of the dependence of urinary folate excretion on folate intake as well as the greater tissue retention of the folate tracer during marginal folate deficiency. We are currently examining the use of other tracers whose metabolism involves folate-dependent processes (20).

Outlook for Determining Folate Requirements Using Kinetic Protocols

A major objective in the study of folate kinetics is to attain information that may lead to greater insight into folate requirements. A minimalist view is that the daily requirement would be equivalent to the fraction of the whole body folate pool size that undergoes obligatory daily losses. Indeed, it has been possible to estimate fractional catabolic rates and pool sizes of whole-body folate in women of various levels of controlled folate status (27, 33). However, interpretation of such data is difficult without additional information regarding the relation of pool sizes and turnover to functional aspects of folate metabolism.

Any increase in the daily obligatory loss of folate could contribute to an increase in the requirement for folate intake to maintain optimal metabolic function. For example, it has been suggested that an increase in excretion of folate catabolism during pregnancy would account, in part, for a higher folate requirement (38, 52). If folate intake did not meet or exceed excretory and catabolic losses, the mass of tissue folate pools would inevitably decline. Simulation studies with a preliminary compartmental model (33) show this predicted effect (J. F. Gregory, unpublished).

It is debatable whether nutritional requirements can be predicted solely on the basis of kinetically derived data. The consensus of data indicates that at least two kinetically identifiable folate pools exist and that the mass of the slow-turnover tissue folate pool(s) greatly exceeds that of the rapid-turnover pool. At low-to-moderate folate intake, the kinetically calculated fractional catabolic rate of whole-body folates is typically 0.005–0.01 day^{-1}, which indicates a total loss of about 0.5–1% of the total body folate pool per day via excretory and catabolic and excretory processes (15, 33, 44). We have estimated that total body folate masses at intakes of 200, 300, and 400 μg/day were 64.5 ± 2.3, 71.5 ± 3.6, and 73.0 ± 2.4 μmol, respectively, with corresponding fractional catabolic rates of 0.0047, 0.0061, and 0.0082 day^{-1}, respectively (33). On the basis of these data, the minimal quantity of absorbed folates needed to maintain steady state would be 0.30, 0.44, and 0.60 μmol/day (i.e. 132, 194, and 265 μg/day). The fact that the 200 μg/day intake yielded elevated plasma homocysteine and decline of plasma folate indicates that the human subjects were not at steady state at this inadequate level of intake; thus, the estimated minimal requirement of 132 μg/day can be excluded. The indicators of folate status remained normal at the 300 and 400 μg/day intakes; thus, the kinetically derived values of 194 and 265 μg/day appear to be reasonable estimates of minimal requirements for absorbed folate.

Translation of these estimates into minimal daily requirements for dietary folate would require adjustment for bioavailability. For example, if one assumes net

bioavailability of approximately 75% (a weighted average of ∼50% available food folate and highly available folic acid), then minimal requirements would be ∼259–353 μg/day. It should be noted that the controlled diets from which these calculations were derived provided only 50 μg/day food folate, which was supplemented with either 250 or 350 μg/day folic acid, so these patterns of folate intake are not directly comparable to typical folate intakes in the United States in terms of folate bioavailability.

Because kinetic estimates of the quantity of folate needed to replace losses provide no information about functional metabolic status, it is unlikely that kinetic analysis could suffice as the sole tool in developing recommendations for nutritional allowances. For example, plasma homocysteine increases substantially 3–4 weeks after initiation of a low-folate diet (59), whereas the $t_{1/2}$ of the slow-turnover tissue pool is typically about 80–100 days at folate intakes typical of the prefortification era in the United States (15, 28, 33, 44). This suggests that methyl group synthesis and homocysteine remethylation are probably dependent on components of the tissue folate pool that are more susceptible to depletion (i.e., faster turnover) than the majority of the slow-turnover tissue folate components.

Modeling of Folate Metabolism and Folate-Dependent One-Carbon Metabolism

Several investigators have developed mathematical models of cellular one-carbon metabolism on the basis of the kinetics of individual folate pools and the rates of their interconversion mainly in cell culture systems (40, 77, 92). These models yield an integrated view of cellular metabolism and allow simulation of the effects of antifolate drugs. Can this approach be used for a whole-organ or whole-body analysis of folate metabolism? This would be an extremely complex modeling task that may not be feasible to conduct in humans or animals, given our inability to determine serially the labeling of individual folate species in most tissues in kinetic studies. The intracellular partitioning of folate-dependent one-carbon metabolism between cytosolic and mitochondrial compartments (18, 91), which was not addressed in the earlier models (40, 77, 92), further complicates such an analysis. However, such a model would be of great value in allowing simulations of the effects of variation intakes on functional tissue folate pools, effects of reduced enzyme activities owing to genetic polymorphisms, and the effects of antifolate therapies. Coburn (17) has devised a whole-body model of vitamin B6 metabolism that may serve as a useful framework.

SUMMARY

This review has discussed the current status of kinetic analysis of folate metabolism, with emphasis on investigation with human subjects. Considerable new insight has been attained through the use of stable-isotopic and radioisotopic tracers, with supporting data from studies with animal models. Additional studies are needed

to advance our understanding of the functional effects of nutritional and genetic variables and their interactions. In addition, further effort is needed to develop quantitatively sound mathematical models to allow an integrated view of existing findings and to allow better simulation of factors affecting folate nutrition and metabolism that cannot readily be determined experimentally. Finally, further investigation is needed regarding the impact of genetic and nutritional variables on the kinetics of key folate-dependent processes.

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