Evaluation of nutritional status for folate is complicated by the variety of metabolic functions of this vitamin along with its complex pattern of interorgan transport and turnover (Steinberg 1984). Assessments based on plasma folate concentration are convenient and reflect long-term status but are most sensitive to recent intake of folate (Lindenbaum and Allen 1995). Measurement of erythrocyte folate concentration serves as an indirect indicator of long-term status because uptake of folate occurs mainly during erythropoiesis (Lindenbaum and Allen 1995). Plasma homocysteine has gained widespread use as a functional indicator of one-carbon metabolic status (Lindenbaum and Allen 1995). Although elevated plasma homocysteine concentration occurs during even marginally suboptimal folate status, (O’Keefe et al. 1995), this indicator also is influenced by vitamin B-6 and B-12 nutriture and age (Selhub et al. 1993). Other procedures such as the deoxyuridine suppression test and measurement of uracil incorporation into genomic DNA also provide functional information regarding folate status (Blount et al. 1997, Lindenbaum and Allen 1995). The deoxyuridine suppression test yields a specific evaluation of cellular folate status; however, this procedure is no more informative diagnostically than measurement of erythrocyte folate concentration (Tamura et al. 1990). Uracil content of DNA isolated from blood samples has been shown to be sensitive to folate depletion and repletion in human subjects (Blount et al. 1997), but the sensitivity of this procedure as an indicator of marginal folate status has not been evaluated. Moderate folate deficiency also has been shown to reduce the methylation of lymphocyte DNA in postmenopausal women (Jacob et al. 1998), which suggests the possible application of DNA hypomethylation as a functional indicator of folate deficiency. Urinary excretion of formiminoglutamate either before or after a histidine load provides additional metabolic evidence of folate and/or vitamin B-12 deficiency (Sauberlich et al. 1974), although this method has had little use recently due to problems of analytical specificity (Lindenbaum and Allen 1995). Urinary folate excretion is not viewed as a reliable indicator of folate status because it is only weakly related to plasma folate concentration at low levels of folate intake (Sauberlich et al. 1974).

This study was conducted to determine the relationship between controlled folate status and the extent of urinary
of a separate deuterated tracer, [glutamate-\(^2\)H\(_4\)]folic acid (\([\text{FH}_{\text{4}}]\)folic acid), allowed the short-term excretion study to be conducted without interference from the \([\text{FH}_{\text{4}}]\)folate tracer because of the high specificity of the gas chromatography-mass spectrometry (GCMS) analysis.

**SUBJECTS AND METHODS**

**Protocol.** The details of the protocol and diet composition have been reported previously (Gregory et al. 1998, O'Keefe et al. 1995). All subjects were nonpregnant females (\(n = 18\), age 21–27 y, weight 47–67 kg) who had normal blood chemistry and were in good health as reflected by a medical history and examination by a physician. This study was approved by the University of Florida Institutional Review Board. Informed consent was obtained from each subject. Subjects were randomly assigned to three treatment groups (\(n = 6\) per group), each group having different constant folate intake throughout the 10-wk period. One subject withdrew for personal reasons midway through the study. The protocol was conducted on an outpatient basis at the University of Florida Clinical Research Center. Adequacy of vitamin and mineral intake was ensured by administration of folate-free supplements (O'Keefe et al. 1995). The dietary intake of energy, protein, and fat and of supplemental vitamins and minerals was reported previously (O'Keefe et al. 1995). Dietary folate from food sources was \(\approx 680\) nmol/d (30 \(\mu\)g/d) for all groups. Additional folate was provided as synthetic folic acid added to commercial pasteurized apple juice consumed at breakfast and evening meals (nonlabeled folic acid, wk 1–2; \([\text{FH}_{\text{4}}]\)folic acid, wk 3–10) to yield intakes of total folate of 454, 680 or 907 nmol/d (200, 300 or 400 \(\mu\)g/d). The quantities of dietary folate, labeled and nonlabeled folic acid administered throughout the 10-wk protocol were described in detail previously (Gregory et al. 1998).

On the morning of d 70 of the controlled dietary study, all subjects were given their usual light breakfast (O'Keefe et al. 1995) and apple juice containing the \([\text{FH}_{\text{4}}]\)folic acid dose, to which 1.13 \(\mu\)mol (500 nmol) of a separate deuterated tracer, [glutamate-\(^2\)H\(_4\)]folic acid (\([\text{FH}_{\text{4}}]\)folic acid), allowed the short-term excretion study to be conducted without interference from the \([\text{FH}_{\text{4}}]\)folate tracer because of the high specificity of the gas chromatography-mass spectrometry (GCMS) analysis.

**FIGURE 1** Urinary excretion of \([\text{FH}_{\text{4}}]\)folate over 24 h after an oral dose of \([\text{FH}_{\text{4}}]\)folic acid by nonpregnant women consuming various levels of total folate. Values are means \(\pm\) SEM; \(n = 5\) (454 nmol/d), \(n = 6\) (others). Bars designated by a different small letter are significantly different after logarithmic transformation of data, \(P < 0.05\).
m) \([^{2}\text{H}_4]\)folic acid was also added. Subjects collected all urine for the next 24 h into acid-washed brown plastic 2-L bottles containing 5 g of dry sodium ascorbate; the bottles were kept refrigerated (2–4°C) during the collection period. Immediately after the 24-h collection was completed, the total volume was measured, and 50-mL portions were transferred to polyethylene vials, saturated with nitrogen gas, then stored at -30°C until analyzed.

Folic acid sources administered. Nonlabeled folic acid was obtained commercially (Sigma Chemical, St. Louis, MO). \([^{2}\text{H}_4]\)folic acid was synthesized in this laboratory and was analyzed by HPLC, proton nuclear magnetic resonance and GCMS to verify purity and identity (Gregory and Toth 1988). The concentration of \([^{2}\text{H}_4]\)folic acid in the stock solution added to apple juice was determined spectrophotometrically using the pH 7.0 molar absorptivity coefficient of 27,600 L/(mol · cm) (Blakley 1969).

Analytical methods. Documentation of folate nutritional status. As described previously, serum and erythrocyte folate concentrations were determined by microbiological assay with \textit{Lactobacillus casei} (Tamura 1990). Total plasma homocysteine concentrations were determined by a fluorometric HPLC procedure (Vester and Rasmussen 1991).

Determination of urinary folate by HPLC and preparation of urinary folate for GCMS analysis. Urinary folate concentration was determined by HPLC after affinity chromatography (Gregory and Toth 1988, Stites et al. 1997). A portion of the urinary folate eluted from the affinity chromatography column was intentionally cleaved at the 9C-10N bond, and the resulting \(\text{para-aminobenzoylglutamate (pABG)}\) isolated and derivatized to form the \(\text{N-trifluoroacetyl-p-aminobenzoylglutamate lactam \(\alpha\)-trifluoroethyl ester (Gregory and Toth 1988).}\

![Graph A](https://academic.oup.com/jn/article-abstract/128/11/1907/4722462)

**FIGURE 2** Relationship between various indicators of folate status (classed as tertiles) and urinary excretion of \([^{2}\text{H}_4]\)folate over 24 h after an oral dose of \([^{2}\text{H}_4]\)folic acid by nonpregnant women consuming various levels of total folate; (A) serum folate; (B) erythrocyte folate; and (C) plasma homocysteine. Values are means ± SEM; \(n = 5\) (454 nmol/d), \(n = 6\) (others). Bars designated by a different small letter are significantly different after logarithmic transformation of data, \(P < 0.05\).
GCMS analysis of derivatized pABG (derived from urinary folate) was performed as previously described in electron-capture negative ionization mode with selected-ion monitoring at masses-to-charge ratios (m/z) 426, 428 and 430 (Gregory and Toth 1988, Wei et al. 1996), corresponding to nonlabeled (1H), 2H2 and 2H4 species. All analyses were performed using a Hewlett-Packard Model 5989 GCMS system (Palo Alto, CA) with methane as reagent gas. Ratios of observed peak areas from the selected-ion monitoring GCMS analysis were converted by simultaneous equations to molar ratios of these isotopomers (i.e., 2H2/1H and 2H4/1H). In this computation, all values were corrected for the natural abundance of stable isotopes. Known mixtures of pABG derivatives (prepared from known mixtures of [2H4], [2H2] and [1H]folic acid) were employed to prepare calibration standards that were used to verify the accuracy of this calculation. All standard mixtures and samples were analyzed in duplicate or triplicate.

Statistical analysis. All data are presented as means ± SEM. Absolute excretion of [2H4]folate was calculated from 24-h urine volume, concentration of total urinary folate, and molar ratios of isotopomers and expressed as a percentage of the administered dose. Differences among dietary groups with respect to urinary excretion were evaluated using one-way ANOVA, with multiple comparisons using the Student-Newman-Keuls procedure. Variability of [2H4]folate excretion, relative to the group mean, was substantially greater at the 907 nmol/d level of folate intake, which necessitated logarithmic transformation to normalize distributions and variances in ANOVA. The relationship between [2H4]folate excretion and serum folate, erythrocyte folate, and plasma homocysteine concentration was evaluated by one-way ANOVA for log-transformed data partitioned approximately as tertiles of each value (e.g., [homocysteine] <8, 8–12, and >12 μmol/L). Finally, the strength of relationships among all indicators of folate nutritional status was evaluated using the Pearson product-moment correlation procedure. All analyses were performed as described by Glantz (1992) using SigmaStat Version 1.0 software (Jandel, San Rafael, CA). Differences with P < 0.05 were considered to be significant.

RESULTS

As previously reported, the 10-wk dietary regimen of this study yielded clear differences in the folate nutritional status of the subjects (Gregory et al. 1998, O’Keefe et al. 1995). The group receiving 434 nmol total folate/d (200 μg/d) was in marginally inadequate and declining folate status as judged by serum and erythrocyte folate and plasma homocysteine concentration. Groups receiving 680 and 907 nmol/d were in adequate status according to these criteria.

Mean values for total folate excretion and [2H4]folate enrichment of urinary folate are presented in Table 1. Total urinary folate excretion increased markedly at the 907 nmol/d intake. There was no significant difference in [H4] isotopic enrichment of urinary folate, although the expected downward trend with increasing folate intake was observed.

Under the conditions of this protocol, the 24-h excretion of urinary [H4]folate was low, with all mean values <1%/24 h (Fig. 1). The observed levels of [H4]folate excretion were readily measurable by the procedures used in this study. Differences in mean excretion and within-group variability are illustrated in the data of Figure 1, presented as mean (± SEM) values determined before transformation. ANOVA of log-transformed data indicated that the urinary excretion of [H4]folate was significantly affected by folate intake (P = 0.0157), with [H4]folate excretion at the 907 nmol/d intake significantly greater than at the lower two intake levels.

The relationship between urinary [H4]folate excretion and other indicators of folate status was also examined. In view of the relatively small number of subjects in this study, values for each indicator (serum and erythrocyte folate and plasma homocysteine concentration) were classified as terciles, analogous to the approach used by others to facilitate examination of such relationships (Selhub et al. 1993, Tucker et al. 1996). Again, ANOVA was performed after logarithmic transformation. In this analysis, a significant relationship was found between serum folate concentration and [H4]folate excretion (P = 0.0380), with [H4]folate excretion significantly greater for subjects with serum folate concentration >10 nmol/L than for those with concentrations <6 nmol/L (Fig. 2A). There was no significant relationship between [H4]folate excretion and erythrocyte folate concentration (Fig. 2B). Also, no significant differences in [H4]folate excretion were observed as a function of plasma homocysteine concentration (Fig. 2C).

As shown in Figure 2C, variation in [H4]folate excretion increased with decreasing homocysteine concentration, which made detection of a significant relationship difficult with the small number of subjects employed.

The strength of the overall linear relationships among [H4]folate excretion and all other criteria, as evaluated by the Pearson product-moment correlation procedure, yielded the results shown in Table 2. Significant correlations were observed between folate intake and all outcome variables examined. The linear correlation of [H4]folate excretion and other outcome variables was significant only in the case of serum folate. Serum folate was significantly correlated with erythrocyte folate concentration, whereas the relationship between

### Table 2

<table>
<thead>
<tr>
<th>Variable</th>
<th>Serum folate</th>
<th>Erythrocyte folate</th>
<th>Plasma homocysteine</th>
<th>[H4]Folate excretion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Folate intake</td>
<td>0.683</td>
<td>0.549</td>
<td>−0.5779</td>
<td>0.603</td>
</tr>
<tr>
<td>Serum folate</td>
<td>−0.00249</td>
<td>−0.0226</td>
<td>−0.0151</td>
<td>0.0104</td>
</tr>
<tr>
<td>Erythrocyte folate</td>
<td>−0.0519</td>
<td>−0.0328</td>
<td>−0.2600</td>
<td>0.0180</td>
</tr>
<tr>
<td>Plasma homocysteine</td>
<td>−0.364</td>
<td>0.0151</td>
<td>−0.0831</td>
<td>0.751</td>
</tr>
<tr>
<td>[H4]Folate excretion</td>
<td>−0.0364</td>
<td>0.0180</td>
<td>−0.288</td>
<td>0.263</td>
</tr>
</tbody>
</table>

1 Values shown are Pearson product-moment correlation coefficients, with P-values shown in parentheses. Coefficients with P < 0.05 were considered to indicate a significant linear relationship. Positive correlation coefficients indicate that both variables increase together, whereas negative values indicate an inverse relationship.
erythrocyte folate and plasma homocysteine concentration was not significant.

DISCUSSION

This study was conducted to determine whether short-term excretion of labeled (i.e., $^{13}$H) folate from a bolus oral dose would be reflective of folate nutritional status. The 10-wk study previously described (O’Keefe et al. 1995) provided an excellent opportunity to test this hypothesis. Folate intake was primarily in the form of synthetic folic acid, which exhibits high and consistent bioavailability in the context of this protocol (Pfeiffer et al. 1997). In view of the relationships among $^{13}$H$_4$ folate excretion, folate intake and serum folate, we conclude that $^{13}$H$_4$ folate excretion does reflect folate status.

Indicators of folate status that reflect the functional adequacy of various folate-dependent metabolic processes are required. We believe that measurement of short-term isotopic excretion in the present protocol does provide a functional measure of one aspect of the in vivo physiologic handling of folate, i.e., cellular uptake and retention. The urinary excretion of folate is influenced by many factors. Intestinal absorption is a key factor. Malabsorption of the oral $^{13}$H$_4$ folate acid dose would yield low percentage excretion and would be potentially associated with inadequate folate status. Thus, the protocol described here involved the use of an oral, rather than an injected dose. It should also be noted that a recent study has shown that deuterated forms of folic acid exhibit quite different short-term kinetics in vivo when administered orally vs. intravenously presumably because of the hepatic first-pass effect experienced after intestinal absorption (Rogers et al. 1997). Modeling studies of long-term folate metabolism from this 10-wk study (based on the $^{13}$H$_4$ folate acid tracer) have indicated that the fraction of ingested folate that appears in urine increases in proportion to folate intake (Gregory et al. 1998), which is consistent with observations of this study regarding folate intake and $^{13}$H$_4$ folate excretion. This finding is also consistent with results in rats showing that reduced urinary excretion of labeled folate in folate deficiency is related, in part, to the enhanced uptake and retention of labeled folate molecules by nonhepatic tissues (Eisenga et al. 1992).

Urinary excretion of folate with increasing intake is likely also related to saturation of the renal tubular reabsorption system as plasma folate concentration increases (Birn et al. 1993), although nonspecific pathways also appear to play a role in renal folate reabsorption (Muldoon et al. 1996). The relationship between folate intake and urinary total folate excretion is due in part to this effect. Additionally, the in vivo retention of folate is a function of saturable processes. Specific binding to several intracellular folate-binding proteins is one aspect (Wagner 1995). Additionally, metabolic trapping occurs by polyglutamylations of folates in tissues catalyzed by folicpolyglutamate synthetase. Product inhibition of this enzyme by the long-chain polyglutamyl folates that predominate in tissues (Cichowicz and Shane 1987) would reduce cellular retention of newly absorbed monoglutamyl folate molecules after cellular uptake when dietary intakes and tissue folate stores are high. Thus, we conclude that the 24-h urinary excretion of $^{13}$H$_4$ folate is a functional indicator of folate status that is related to the concentration dependence of multiple folate transport/reten tion processes.

In summary, this isotopic procedure adds to the growing list of functional indicators of folate status. Disadvantages include the needs for complete urine collection and two analytical methods (i.e., measurement of urinary folate concentration by HPLC and isotopic enrichment by GCMS). It is likely that the quantitative HPLC step could be eliminated through the use of a stable isotopic internal standard in GCMS to allow calculation of total urinary folate in a manner analogous to that reported by Santhosh-Kumar et al. (1995). The HPLC and GCMS methods are not inordinately difficult, although they are fairly labor intensive. The complete urine collection required reduces the applicability of this method in surveys of nutritional status but not in controlled research studies. For future application of this method, we recommend, for reasons of economy and ease of synthesis, that the labeled folate dose be prepared as $^{3}$H$_5$-$^{13}$C$_2$ folic acid (Gregory 1990), rather than the $^{13}$H$_4$ folic acid preparation used in this study or the $^{13}$C$_6$ folic acid preparation recently developed (Pfeiffer et al. 1997). The current lack of commercial availability of $^{13}$H$_4$ folic acid may also limit application of the method; however, a single synthesis typically yielding 0.5–1.0 g of product would suffice for many trials at the dosage level described here.

LITERATURE CITED


