Determination of Folate Bioavailability with a Rat Bioassay\textsuperscript{1,2}

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\textbf{ABSTRACT} Variations of a recently proposed rat bioassay were examined and applied to the measurement of biologically available folate. The concentration of hepatic folate was found to be inconsistent as a response indicator, although fasting levels of plasma folate accurately reflected available dietary folate. Studies involving the control of coprophagy suggested that fecal folate significantly contributes to the folate status of the rat. Bioassays employing multiple dietary levels of dried orange juice solids or blanched cabbage without control of coprophagy were conducted to determine available folate by slope-ratio methods. The apparent bioavailability of endogenous folate in the dried orange juice and cabbage was 146 and 68%, respectively, relative to folic acid. The presence of dried orange juice solids or cabbage did not inhibit the utilization of folate pentaglutamate added to the diets. These results illustrate the usefulness and several limitations of the rat bioassay for evaluating the bioavailability of folate. However, the appropriateness of the rat as a model of human folate digestion and absorption requires further investigation. \textit{J. Nutr.} 117: 866–873, 1987.

\textbf{INDEXING KEY WORDS:}  
folate \hspace{1em} bioassay \hspace{1em} rat \hspace{1em} bioavailability

The folate nutriment of humans and animals depends on both the amount and the bioavailability of folate in the diet. The nutritional adequacy of diets cannot be determined fully without an understanding of the factors that influence the extent of intestinal absorption and utilization of the vitamin. At present, a great deal of uncertainty exists concerning the bioavailability of folate, particularly with respect to the polyglutamyl folates. Impairment of intestinal absorption of folate could occur by inhibition of intestinal pteroylpolyglutamate hydrolases (folate conjugases), intraluminal entrapment of folates or by inhibition of transport processes. Research to date has provided little systematic information concerning the effect of dietary components on folate bioavailability.

The bioavailability of folate has been investigated and reviewed by a number of researchers [1–9]. Evaluations of folate bioavailability with human subjects based on measurement of urinary or serum folate have been variable and conflicting. Tamura and Stokstad [8] and Babu and Srikantia [2] measured urinary folate excretion in replete subjects in response to test foods and found the apparent bioavailability of folate to be low in orange juice concentrate, lettuce and cabbage but high in bananas, lima beans and liver. The large quantities of test foods administered in these studies may have affected the utilization of the vitamin. Studies involving the measurement of plasma folate levels after the consumption of foods fortified with folic acid suggested that the food matrix may influence the rate or extent of absorption of folate [10–12].

Studies of folate bioavailability have been conducted with the chick and the rat as animal models. The chick bioassay has been used by Graham et al. [13] to examine folate bioavailability in selected fortified foods. Previous studies from this laboratory employed chick bioassays to examine the effects of dietary fiber on the bioavailability of folic acid [5] as well as for assessment of folate bioavailability of various foods or the activity of synthetic folates [6, 14]. An advantage of the chick as an animal model is its apparent lack of susceptibility to variation in its folate status induced by nonfolate components of the diet [5].

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The development of a folate bioassay with rats was reported by Keagy and Oace (15); this assay provided an alternative to the avian procedure. This method has been used to evaluate the effect of various forms of dietary fiber on folate utilization (3, 16). A similar rat bioassay was later reported by others (17). The application of rat bioassay to the assessment of available folate in foods or food components has not been reported. These bioassay procedures were significant because they showed that a dose-response relationship could be achieved in the rat without the need for an oral antibiotic to suppress intestinal microorganisms.

Potential limitations of animal bioassays center mainly on the role of the intestinal microflora and the extent of enhancement or inhibition of their folate synthesis by dietary components. Certain fiber sources have been shown to contribute to the folate status of the rat by this mechanism (3). The role of coprophagy on the folate status of the rat and the response of the bioassay has not been determined.

The purpose of these studies was to examine further the response of a rat bioassay procedure and to extend its application. The major objectives of this research were 1) to examine variations of a recently proposed rat bioassay (15) for measurement of biologically available folate, 2) to determine the bioavailability for rats of naturally occurring folate in selected foods (dried orange juice solids and cabbage) and 3) to determine the influence of these tested foods on the bioavailability of synthetic polyglutamyl folate added to the diets.

MATeRIALS AND METHODS

Folate compounds and tested foods. Folic acid (pteroylmonoglutamic acid) used for dietary supplementation in these studies was obtained from Sigma Chemical Co. (St. Louis, MO). Pteroylpentaglutamic acid was synthesized in this laboratory by the solid-phase procedure of Krumdieck and Baugh (18) and then purified with ion-exchange chromatography. The concentration of folate compounds used for diet fortification and for analytical standards was determined spectrophotometrically (19).

Cabbage was purchased locally, finely chopped and blanched under flowing steam at atmospheric pressure for 10 min at 100°C. After blanching the cabbage was homogenized with an equal volume of distilled water, freeze dried and finely powdered. The efficiency of blanching in inactivating endogenous folate conjugase was confirmed by a negative conjugase assay (20). Orange juice solids used in these experiments consisted of 100% vacuum-dried orange juice (Crystals International, Inc., Plant City, FL).

Protocols of rat bioassays. The bioassays were performed according to the general methods of Keagy and Oace (15) with the addition of pair feeding and evaluation of plasma folate as a response variable.

The objectives of Experiment 1 were to evaluate the usefulness of fasting plasma folate as a response criterion and to determine the effect of the prevention of coprophagy on the results of the bioassay. Male weaning CrI:CD(SD)BR Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, MA), 40–50-g initial body weight, were housed individually in stainless-steel cages with wire-mesh bottoms and fed ad libitum a basal diet containing no added folate for a period of 28 d. The composition of the basal diet, which was also used as the basis for the formulation of the standard and experimental diets, is summarized in Table 1. The use of 3% (wt/wt) cellulose in all diets was a modification of the originally proposed diet (15). During the last 3 d of the depletion period, half of the rats were fitted with tail cup devices similar to those described by Barnes et al. (21), as we have recently described (22), to prevent coprophagy.

Before the end of the depletion period, the rats were divided randomly into groups of 10 each and pair fed one of five diets containing graded amounts of folic acid or 15% (wt/wt) orange juice solids [substituted for wheat starch] as the source of added dietary folate for a repletion period of 12 d. Pair feeding consisted of feeding the rats the amount of food consumed by the group receiving the unfortified diet. Half of the rats from each group were fitted with tail cups for the last 3 d of the depletion period and for the entire repletion period.

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**Table 1**

<table>
<thead>
<tr>
<th>Component</th>
<th>Percentage by weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein, vitamin-free³</td>
<td>20.0</td>
</tr>
<tr>
<td>Wheat starch</td>
<td>66.7</td>
</tr>
<tr>
<td>Cellulose [Alphacel]³</td>
<td>3.0</td>
</tr>
<tr>
<td>Corn oil</td>
<td>5.0</td>
</tr>
<tr>
<td>Mineral mixture⁴</td>
<td>4.0</td>
</tr>
<tr>
<td>Vitamin mixture³,⁵</td>
<td>1.1</td>
</tr>
<tr>
<td>DL-Methionine²</td>
<td>0.2</td>
</tr>
</tbody>
</table>

¹Folic acid fortification was performed by adding 25 mL of appropriate aqueous solutions per kilogram of diet. All diets received equivalent amounts of water before blending. Test materials [orange juice solids or dried cabbage] were substituted for wheat starch.

²U.S. Biochemicals Corp., Cleveland, OH.

³ICN Nutritional Biochemicals, Cleveland, OH.

⁴Mineral mixture [Teklad Test Diets, Madison, WI] was the Wesson-Modified Osborne-Mendel mixture that provided (mg/kg diet) calcium carbonate, 8400; copper sulfate pentahydrate, 15.6; ferric phosphate, 588; manganese sulfate, 8; magnesium sulfate, 3600; potassium aluminum sulfate, 4800; potassium dihydrogen phosphate, 12,400; potassium iodide, 2; sodium chloride, 4200; sodium fluoride, 20; tricalcium phosphate, 5960. Zinc carbonate was added to provide 20 mg Zn/kg diet.

⁵Vitamin mix provided [mg/kg diet, except as noted]: retinol acetate, 9900 u; ergocalciferol, 1170 u; inositol, 55; α-tocopheryl acetate, 55; choline chloride, 825; menadione, 25; niacin, 50; riboflavin, 11; calcium pantothenate, 33; thiamin HCl, 11; biotin, 0.22; vitamin B-12, 0.015. Pyridoxine-HCl was added to provide 5.0 mg/kg diet.
After the 12-d repletion period the animals were killed by decapitation after an overnight fast (15 h). Blood was collected from the cervical vessels into heparinized tubes and livers were removed. Plasma, liver and diet samples were stored at −20°C until analyzed for folate content microbiologically as described below.

Experiment 2 was conducted to investigate the bioavailability of endogenous folate in orange juice solids and dried cabbage with slope-ratio methods without control of coprophagy. The influence of these foods on the bioavailability of supplemental folic acid pentaglutamate also was determined. Male weanling Crl:CD(SD)BR Sprague-Dawley rats (Charles River Breeding Laboratories), initial body weight 40–50 g, were housed and fed the basal diet for the depletion period as in the first experiment. The basal and experimental diets were formulated as described (Table 1). After the depletion period the rats were randomly divided into groups of 8 rats each and fed one of 13 diets containing either graded amounts of folic acid, folic acid pentaglutamate or dried foods (orange juice solids or dried cabbage) as the source of dietary folate for a repletion period of 14 d. The test diets were prepared containing 6, 12 and 18% (wt/wt) orange juice solids and 2.5, 5.0 and 9.0% (wt/wt) dried cabbage. The diets containing 12% orange juice solids and 5.0% cabbage were also prepared with supplemental folic acid pentaglutamate. The dried foods were substituted for starch in formulating the test diets. After a 14-d repletion period the animals were killed by decapitation after an overnight fast (15 h). Blood was collected from the cervical vessels into heparinized tubes and livers were excised from rats fed the basal and highest standard (3.6 nmol folic acid/g diet) diets. Plasma, liver and diet samples were stored at −20°C until they were analyzed for folate content with the microbiological assay.

**Microbiological assay of total folate.** We extracted the liver, diet and food samples for microbiological analysis as previously described (23), with minor modifications. One-gram samples were homogenized with 10 vol 0.05 M sodium acetate buffer (pH 4.9) containing 57 mM ascorbate. Liver homogenates were flushed with nitrogen gas, incubated at 37°C for 90 min to allow autolysis of hepatic polyglutamyl folates and then incubated in a boiling water bath for 10 min and centrifuged. The diet and food homogenates were flushed with nitrogen gas, incubated in a boiling water bath for 10 min and centrifuged. The supernatants were incubated with hog kidney conjugase for 1 h at 37°C under conditions chosen to yield complete hydrolysis of folic acid pentaglutamate added to these extracts. The observed recovery of folic acid added to sample homogenates before extraction was typically 85–100%. Reduced folates have been shown to be stable under these extraction conditions (23).

The sample extracts and plasma samples were analyzed for total folate by a microbiological assay (24) with *Lactobacillus casei* (ATCC 7469) and Folic Acid Casei Medium (Difco Laboratories, Detroit, MI). Calculations in the microbiological assay were performed with a nonlinear weighted regression procedure (25). The initial pH of the growth medium was adjusted to 6.2 to minimize differences in response between various folate compounds (24).

**Statistical analysis.** Differences between treatments were evaluated by analysis of variance and the Tukey method for multiple comparisons. All folate dose-response curves were plotted with supplemental folate, rather than total dietary folate, on the x-axis versus plasma folate on the y-axis. The slope and y-intercept values of all dose-response curves of rat bioassays were calculated by weighted linear regression to compensate for unequal variance in plasma folate values. Comparison of regression equations was performed by calculation of 95% confidence intervals for slopes and y-intercepts. The apparent biologically available folate in orange juice solids in experiment 1 and added folic acid pentaglutamate in experiment 2 was calculated by point interpolation from dose-response curves. Apparent biologically available folate in orange juice solids and dried cabbage in experiment 2 was determined by slope-ratio analysis (26). All statistical procedures were performed as described by Neter and Wasserman (27).

**RESULTS**

**Experiment 1.** This study was conducted to evaluate the relative merits of hepatic folate and plasma folate concentration as quantitative indicators in the rat bioassay. Although folate deficiency has little effect on food intake in the rat, the animals were pair-fed to minimize experimental biases caused by differences in feed consumption associated with factors such as palatability. Half of the rats of each group were maintained with tail cups to prevent coprophagy throughout the repletion period. Under these conditions, fasting levels of plasma folate were linearly related to dietary folic acid supplementation (Table 2). The regression equations of supplemental dietary folate (nmol/g) versus plasma folate (pmol/mL) were 1) with tail cups, slope 34.8 ± 7.3 (pmol/mL)/(nmol/g), y-intercept 33.0 ± 16.4 pmol/mL, correlation coefficient 0.748 ($P < 0.05$); 2) without tail cups, slope 32.2 ± 7.7 (pmol/mL)/(nmol/g), y-intercept 82.6 ± 17.2 pmol/mL, correlation coefficient 0.703 ($P < 0.05$). The hepatic folate values did not show a significant relationship to dietary folic acid concentration (Table 2). Prevention of coprophagy through the use of tail cups caused a consistent reduction in the plasma folate values for each group; however, there was essentially no difference in hepatic folate between the coprophagy-restricted animals and the controls. Although plasma folate values within each dietary group were not significantly different between groups maintained with and without tail cups, the over-
TABLE 2

Results of rat bioassay for determination of biologically available folate in orange juice solids (experiment 1)* 1,2

<table>
<thead>
<tr>
<th>Form of folate added to basal diet</th>
<th>Concentration of added folate (nmol/g diet)</th>
<th>Tail cups</th>
<th>Weight gain 3</th>
<th>Hepatic folate 4</th>
<th>Plasma folate 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Folic acid standards</td>
<td>0.0</td>
<td>+</td>
<td>61 (5)</td>
<td>20.0 (3.2)*</td>
<td>29.9 (4.2)*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>62 (19)</td>
<td>24.3 (5.4) ab</td>
<td>88.7 (25.2) ab</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>72 (12)</td>
<td>26.0 (5.2) ab</td>
<td>73.8 (14.9) ab</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>66 (9)</td>
<td>33.5 (4.9) ab</td>
<td>106 (11) ab</td>
</tr>
<tr>
<td></td>
<td>1.2</td>
<td>+</td>
<td>48 (8)</td>
<td>38.3 (7.1) ab</td>
<td>128 (32) ab</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>55 (10)</td>
<td>27.2 (3.0) ab</td>
<td>171 (23) ab</td>
</tr>
<tr>
<td></td>
<td>2.4</td>
<td>+</td>
<td>66 (7)</td>
<td>32.1 (1.2) ab</td>
<td>162 (18) ab</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>71 (6)</td>
<td>31.5 (2.6) ab</td>
<td>196 (23) ab</td>
</tr>
<tr>
<td></td>
<td>3.6</td>
<td>+</td>
<td>61 (10)</td>
<td>23.6 (2.4) ab</td>
<td>119 (16) ab</td>
</tr>
<tr>
<td>Orange juice solids</td>
<td>1.07</td>
<td>+</td>
<td>72 (4)</td>
<td>44.5 (10.9) b</td>
<td>202 (32) b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1Half of the rats within each dietary treatment were maintained with tail cups throughout the repletion period to prevent coprophagy. Values in parentheses are SEM, five rats/group. All rats were pair fed to the group receiving the basal diet, typical feed consumption was 16–18 g/d.
2Folate content (microbiological assay) of orange juice solids and the basal diet was 7.12 and 0.720 nmol/g, respectively.
3No significant difference between groups (P > 0.05).
4Values within a column with the same superscript letter were not significantly different (P > 0.05). The overall effect of the tail cup was significant at P < 0.05.

all effect of the tail cups was highly significant in analysis of variance (P < 0.01).

The y-intercepts of dose-response curves relating supplemental dietary folic acid to the fasting concentration of plasma folate for the rats with and without tail cups were significantly greater in the unrestricted controls (P < 0.05), which indicated that the recycling of fecal folate contributed to the folate status of the rats. The equivalence of slopes (P > 0.05) obtained with and without the restriction of coprophagy indicated that the absorption and utilization of the supplemental folic acid was not influenced by the use of the tail cups.

The effect of coprophagy prevention on the apparent bioavailability of folate in orange juice solids was determined by point interpolation relative to the folic acid dose-response curves (Table 3). In this experiment, the orange juice solids were fed at only a single level (15% wt/wt). This approach necessitated the assumption of linear assay response for orange juice solids and common intercept with the folic acid response curves. In contrast to the 7.12 nmol/g total folate determined by microbiological assay of the orange juice solids, apparent available folate was 16.4 ± 3.1 and 23.2 ± 6.4 nmol/g calculated rat bioassay with and without the use of tail cups, respectively (means and SEM). These values corresponded to an apparent relative bioavailability of 230 ± 40 and 326 ± 90%, respectively, with and without the use of tail cups. The control of coprophagy was associated with a lower apparent bioavailability, although the difference was not statistically significant (P > 0.05). Both groups of rats exhibited values for apparent available folate that were significantly greater than the total folate of the orange juice solids (P < 0.05).

**Experiment 2.** Multiple levels of orange juice solids and dried cabbage were analyzed by rat bioassay with fasting plasma folate as the response criterion. The use of a multilevel analysis permitted an evaluation of the linearity of the dose-response curves and the equivalence of the y-intercepts. Coprophagy was not prevented in this experiment.

The results of this experiment (Table 4) showed linear relationships between the level of added dietary folate (as nmol/g supplemental folic acid or percentage orange juice solids or cabbage) and fasting plasma folate concentration (Table 5). No significant difference was seen in the y-intercept values of dose-response curves for folic acid, orange juice solids and cabbage (Table 5). A significant difference was observed for hepatic folate between basal and high standard groups (3.0 nmol folic acid/g), in contrast to experiment 1. On the basis of slope-ratio calculations with plasma folate as the response criterion, the concentration of apparent biologically available folate in the orange juice solids and

**TABLE 3**

Effect of using tail cups for the prevention of coprophagy on the apparent bioavailability of folate in orange juice solids (experiment 1)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Tail cups</th>
<th>No tail cups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total folate, nmol/g</td>
<td>7.12 (0.51)</td>
<td>7.12 (0.51)</td>
</tr>
<tr>
<td>Apparent available folate, nmol/g</td>
<td>16.4 (3.1)</td>
<td>23.2 (6.4)</td>
</tr>
<tr>
<td>Apparent bioavailability, %</td>
<td>230 (40)</td>
<td>326 (90)</td>
</tr>
</tbody>
</table>

1Means and SEM (in parentheses), five rats/group. Apparent biologically available folate calculated by point interpolation from the respective dose-response curves. Total folate determined by microbiological assay of orange juice solids.
**TABLE 4**

Results of rat bioassay for the determination of biologically available folate in orange juice solids and dried cabbage (experiment 2)

<table>
<thead>
<tr>
<th>Diet</th>
<th>Compound added</th>
<th>Dietary folate(^1) nmol/g</th>
<th>Plasma folate(^1) pmol/mL</th>
<th>Hepatic folate(^1) nmol/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standards</td>
<td>None</td>
<td>1.13</td>
<td>54.7 (6.6)</td>
<td>12.4 (0.8)</td>
</tr>
<tr>
<td>FA</td>
<td></td>
<td>2.22</td>
<td>127 (10)</td>
<td>—</td>
</tr>
<tr>
<td>FA</td>
<td></td>
<td>3.50</td>
<td>218 (11)</td>
<td>—</td>
</tr>
<tr>
<td>FA</td>
<td></td>
<td>4.85</td>
<td>410 (30)</td>
<td>38.3 (4.4)</td>
</tr>
<tr>
<td>PG-5</td>
<td></td>
<td>3.03</td>
<td>141 (13)</td>
<td>—</td>
</tr>
</tbody>
</table>

Orange juice solids

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
<td>6%</td>
<td></td>
<td>1.55</td>
<td>142 (8)</td>
<td>—</td>
</tr>
<tr>
<td>12%</td>
<td></td>
<td>2.03</td>
<td>116 (15)</td>
<td>—</td>
</tr>
<tr>
<td>18%</td>
<td>PG-5</td>
<td>3.21</td>
<td>212 (9)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.26</td>
<td>182 (10)</td>
<td>—</td>
</tr>
</tbody>
</table>

Cabbage

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>3%</td>
<td></td>
<td>1.22</td>
<td>79.7 (3.1)</td>
<td>—</td>
</tr>
<tr>
<td>5%</td>
<td></td>
<td>1.80</td>
<td>120 (6)</td>
<td>—</td>
</tr>
<tr>
<td>5%</td>
<td>PG-5</td>
<td>2.66</td>
<td>166 (16)</td>
<td>—</td>
</tr>
<tr>
<td>9%</td>
<td></td>
<td>2.64</td>
<td>156 (13)</td>
<td>—</td>
</tr>
</tbody>
</table>

\(^1\)Diets were prepared with and without the addition of folic acid pentaglutamate [PG-5]. Standard diets were fortified with folic acid [FA]. Means and SEM (in parentheses), eight rats/group.

\(^2\)Determined by microbiological assay. Folate content of the basal diet was 1.13 nmol/g. Liver folate was determined only on rats fed the basal diet and the diet containing 3.0 nmol folic acid/g.

Dried cabbage was 10.4 ± 3.3 and 14.8 ± 2.5 nmol/g, respectively, which corresponded to apparent bioavailability values of 146 and 68%, respectively (Table 5). The apparent bioavailability of folate in orange juice solids was not significantly different from 100% \(P > 0.05\), while that of cabbage was significantly < 100% \(P < 0.05\).

An additional aspect of this experiment was an evaluation of the relative bioavailability of exogenous folic acid pentaglutamate in the basal diet and in diets containing orange juice solids or dried cabbage, as shown in Table 6. For all three diets, the bioavailability of added folic acid pentaglutamate was not significantly different from 100%, relative to folic acid. These results indicate that folic acid pentaglutamate added to the basal diet was utilized by the rats and that the presence of orange juice solids or dried cabbage in the test diets did not adversely affect the deconjugation and subsequent utilization of this polyglutamyl folate.

**DISCUSSION**

In view of the importance of animal bioassays for the evaluation of nutrient bioavailability, it was the pur-

**TABLE 5**

Slope-ratio analysis for determination of the bioavailability of endogenous folate in orange juice solids and dried cabbage\(^1,2\)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Folic acid standards</th>
<th>Orange juice solids</th>
<th>Dried cabbage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose-response curve(^2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>y-intercept, pmol/mL</td>
<td>53.0 (11.5)</td>
<td>55.1 (4.8)</td>
<td>54.6 (4.8)</td>
</tr>
<tr>
<td>Slope, (pmol/mL)/(nmol/g)</td>
<td>80.0 (6.1)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Slope, (pmol/mL)/(% wt/wt)</td>
<td>—</td>
<td>8.37 (2.58)</td>
<td>12.9 (1.8)</td>
</tr>
<tr>
<td>Available folate, nmol/g</td>
<td>—</td>
<td>10.4 (3.3)</td>
<td>14.8 (2.5)</td>
</tr>
<tr>
<td>Apparent bioavailability(^3), %</td>
<td>—</td>
<td>146 (47)</td>
<td>67.9 (11.6)</td>
</tr>
</tbody>
</table>

\(^1\)Values are means and SEM (in parentheses). Regression equations for groups fed orange juice and cabbage included data for the basal diet. All dietary values for available folate are expressed per gram of orange juice solids or dried cabbage.

\(^2\)The slope of each regression equation was significantly different from zero at \(P > 0.05\). Correlation coefficients were standards, 0.930; orange juice solids, 0.764; cabbage, 0.860.

\(^3\)Apparent bioavailability was calculated relative to the total folate content determined by microbiological assay (orange juice solids, 7.12 nmol/g; dried cabbage, 21.8 nmol/g).
pose of this study to evaluate the applicability of several variations of a recently proposed bioassay procedure (15) to the determination of biologically available folate. Orange juice solids and dried cabbage were selected for analysis because of previous reports of incomplete bioavailability of folate from these foods (2, 6, 8, 9). These studies were also designed to investigate the relative bioavailability of exogenous polyglutamyl folate in the basal diet and in diets containing orange juice solids or dried cabbage.

It must be recognized that the determination of total folate in the tested materials is critical to the accuracy of the bioavailability studies. The microbiological assay employed in this study yielded consistent recovery values for added folic acid. The absence of positive or negative drift in the assays, along with the acceptable recovery values, supported the validity of the analysis. We have also found that treatment of cabbage and orange juice extracts with hog kidney conjugase under the conditions of this analysis yields complete hydrolysis of polyglutamyl folates to their respective monoglutamates (R. Engelhardt and J. F. Gregory, unpublished data), which exhibit full activity in the microbiological assay. The values obtained for total folate in the orange juice solids and dried cabbage were consistent (on a dry weight basis) with published data. For orange juice solids, the observed value of 7.12 nmol/g was within the range of 5.5–10.4 nmol/g previously determined (8, 9, 28, 29). The observed 21.8 nmol/g total folate in dried cabbage is in general agreement (dry weight basis) with values reported for raw cabbage (13.7–38.6 nmol/g; refs. 8, 28, 30, 31) and cooked cabbage (7.2–25.8 nmol/g; refs. 8, 28). The steam blanching treatment used on the cabbage of this study would be expected to induce only small thermal losses of folate.

An important finding in these studies was the fact that fasting plasma folate concentration was a sensitive indicator of available dietary folate in short-term bioassays. Hepatic folate levels were inconsistent between experiments 1 and 2. Previous studies of rat bioassays (15, 17) have indicated significant relationships between dietary and hepatic folate concentrations, which would provide a viable response criterion in studies of folate bioavailability. The inconsistent response of hepatic levels of folate to dietary concentrations in this study is unexplained. The levels of endogenous folate in the basal diet of experiments 1 and 2 (0.72 and 1.12 nmol/g diet, respectively) were comparable to that reported by Keagy and Oace (1.02 nmol/g) and somewhat higher than reported by Hoppner and Lampi (0.17 and 0.26 nmol/g). It is unclear whether the concentration of endogenous folate in the basal diets of this study was responsible for the inconsistent response of hepatic folate. Variation in the folate status of the rats before the experiment may have been involved. Because fasting plasma folate has been found to be a consistently sensitive indicator of available dietary folate in this and preliminary studies in our laboratory, plasma folate would appear to be preferable to hepatic folate as the primary response criterion in bioassays such as these. It is recognized that plasma folate fluctuates postprandially and that the time of sampling is critical in this type of analysis. Blood collection after an overnight fast was chosen to minimize errors in interpretation caused by differences in the rate of intestinal absorption and tissue uptake of the dietary folate.

The results of experiment 1 indicated that the prevention of coprophagy significantly reduced the folate status of rats, presumably by preventing the recycling of fecal folate. This observation is important because it suggests that components of test diets that would stimulate the synthesis of folate by intestinal microorganisms could cause overestimation of dietary folate in conventional bioassays. The analysis of orange juice solids in these studies may be an example of such overestimation, although conclusive evidence of stimulation of microbial folate synthesis in the intestine was not obtained in these studies. In experiment 1 the prevention of coprophagy did significantly reduce the concentration of apparent biologically available folate (16.4 vs. 23.2 nmol/g), as would be predicted. The apparent available folate in the orange juice solids was significantly higher than the total folate, however, which yielded apparent bioavailability > 100% regardless of the prevention of coprophagy.

Orange juice solids from the same lot were analyzed
in experiment 2 by the slope-ratio method. A linear
dose-response curve was obtained with y-intercept
equivalent to that of the folic acid standard curve, which
supports the validity of the bioassay. However, the re-
results of experiment 2 (Table 5) yielded apparent bio-
logically available folate values that were again greater
than the concentration of total folate, although the dif-
ference was not statistically significant in this case
($P > 0.05$). It would appear that components of the
orange juice solids tend to yield an overestimation of
available folate when determined by this rat bioassay
procedure. The actual bioavailability of the endogenous
folate in the orange juice solids could not be evaluated
accurately with these bioassay methods, although the
results suggest effective utilization by the rats.

Conflicting data have been reported concerning the
bioavailability of folate in orange juice. Tamura and
Stokstad (8) observed 35% bioavailability of endoge-
nous folate in orange juice (range 29–40%), along with
impaired utilization of supplemental folic acid hepta-
glutamate. These results were confirmed in later studies
that suggested that the low pH of the intestinal
contents induced by the administration of large doses
of orange juice reduced folate absorption (9). Rhode et
al. (32) reported that the bioavailability of folate in or-
ange juice was equivalent to that of oral folic acid sup-
plements for human subjects.

The bioavailability of endogenous folate in dried cab-
bage was found in experiment 2 to be 68 ± 12% (mean
and SEM) based on the microbiologically determined
total folate content and available folate calculated by
slope ratio. The incomplete bioavailability of folate in
blanched cabbage is consistent with the previous ob-
servation from this laboratory of 40% bioavailability
of folate in blanched cabbage in chick bioassays (6).
Similar results were reported from studies with human
subjects by Tamura and Stokstad (8), who reported 47%
bioavailability folate in cooked cabbage (range 0–97%).

Despite the limitations of the rat bioassay, some val-
uable information was obtained from these studies. The
results indicated that fasting plasma folate levels were
consistently sensitive as an indicator of folate status
in the rat. These studies also provided useful data con-
cerning the lack of inhibitory effect of two foods (cab-
bage and orange juice) on the bioavailability of long-
chain polyglutamyl folates. Because the diets in exper-
iment 2 were prepared with and without added folic
acid pentaglutamate, the difference between the plasma
folate response would accurately represent the bio-
availability of folic acid pentaglutamate in these diets.

It must be recognized that the interpretation of stud-
ies such as this is limited by assumptions inherent in
the procedure. Ideally, test materials should be ana-
alyzed in the bioassay over at least three levels of ad-
dition to the basal diet so that dose-response curves
such as those of experiment 2 can be evaluated (26).
However, multilevel analysis of several test foods in a
single bioassay is often precluded by the length, ex-
pense and laborious nature of the procedures. The cal-
culation of apparent bioavailability by point interpo-
lation from a standard dose-response curve requires the
assumption that the folate status of the animal is based
solely on the utilization of the vitamin from the test
food. Factors in the diet that would positively or neg-
atively affect the folate nutriture of the animal, e.g., by
influencing folate biosynthesis by the intestinal mi-
icroflora, would yield inaccuracy in the estimation of
available folate. Qualitative changes in intestinal mi-
icroflora, large differences in rate of folate absorption,
dietary interference with the enterohepatic circulation
of folates and possible alterations in intestinal transi-
time could also be factors affecting the accuracy of
bioassay methods such as this. Another problem is the
necessary assumption that the utilization of folate in-
herent in the ingredients of the basal diet is not influ-
enced by the components of the test diets. The inability
to verify this assumption experimentally contributes to
the ambiguity of the analysis. Such factors, as illus-
trated by the results of this study, limit the usefulness
of animal bioassays in evaluating the bioavailability of
folate in foods. Evaluation of the biological activity of
pure folate compounds with the rat bioassay method
could be performed with less experimental uncertainty.
Overestimation of biologically available vitamin B-6 in
several foods has been reported in rat bioassays (33) that
were similar to the protocol used in this study. This
suggests that the problem is not unique to bioassays of
available folate.

In summary, the results of these studies have illus-
trated usefulness and limitations of this rat bioassay
method. One must also consider the merits of the rat
as an animal model. Both rats and humans are capable
of fully utilizing dietary mono- and polyglutamyl fol-
ates (Table 6; ref. 8). The intestinal pteroylpolygluta-
mate hydrolases of rats and humans have been shown
to differ in their physical properties, subcellular loca-
tion and mode of action (34–36). It is unclear whether
dietary factors inhibiting the bioavailability of polyglu-
tamyl folates in the human would necessarily elicit
such an effect in a rat bioassay. Further characterization
of folate bioavailability in these species, along with
more complete characterization of folate digestion and
absorption processes, are needed to resolve these ques-
tions.

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