Folic acid enrichment of bread does not appear to affect zinc absorption in young women\(^1\)\(^-\)\(^3\)

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ABSTRACT

Background: In several countries cereals are now enriched with folic acid to reduce the risk of neural tube defects. Human studies suggest that folic acid interferes with zinc absorption. This raises concerns about the zinc status of high-risk groups such as infants, pregnant women, and older persons.

Objective: We sought to determine the effect of added folic acid on zinc absorption from white bread with high and low zinc contents.

Design: Zinc absorption was measured in 15 healthy women (22–33 y), each of whom consumed 4 single meals spaced 2 wk apart in a randomized crossover design. The servings of bread (100 g) differed in zinc and folic acid contents as follows: A, 1.2 mg Zn and 17 μg folic acid; B, 1.2 mg Zn and 144 μg folic acid; C, 3.0 mg Zn and 17 μg folic acid; and D, 2.9 mg Zn and 144 μg folic acid. Meals were extrinsically labeled with \(^{65}\)Zn and absorption was estimated from whole-body retention measurements. Folate status was assessed by measuring plasma and erythrocyte folate and plasma homocysteine concentrations.

Results: Mean (±SD) zinc absorption did not differ significantly in relation to the folate content of the breads at either the low zinc content (38.8 ± 13.5% and 40.6 ± 16.5% for A and B, respectively; \(P = 0.74\)) or the high zinc content (26.7 ± 9.3% and 22.7 ± 6.6% for C and D, respectively; \(P = 0.16\)). There was no significant correlation between folate status and zinc absorption (\(r < 0.3, \ P > 0.1\)).

Conclusion: Fortification of white bread with a commonly used amount of folic acid did not appear to influence zinc absorption at either a high or a low zinc content. Am J Clin Nutr 2001; 74:125–9.

KEY WORDS Folate, folic acid, fortification, enrichment, bread, flour, zinc absorption, zinc status, bioavailability, interaction, radioisotope, whole-body counting, \(^{65}\)Zn, neural tube defects, birth defects, congenital defects

INTRODUCTION

The fortification of cereals with folic acid has been implemented in several countries, such as the United Kingdom, Canada, Australia, and recently the United States. The purpose of folic acid fortification is to reduce the risk of neural tube defects in newborns. There is convincing evidence that folic acid supplementation, when taken from the onset of pregnancy, reduces the prevalence of neural tube defects (1). An additional effect of folic acid fortification may be a lowered risk of cardiovascular diseases. Supplementation with folic acid has been found to decrease the serum homocysteine concentration, an independent risk factor for cardiovascular disease (2).

Increasing the intake of folic acid through supplementation or fortification is generally considered safe. However, concern was raised in some (3–10) but not all (11–16) studies about a potential negative effect of folic acid supplementation on zinc absorption. Decreased zinc absorption could have adverse effects, particularly in individuals with increased requirements, such as children, adolescents, and pregnant or lactating women. Furthermore, many elderly persons may already be at risk of marginal zinc status because of poor appetites and inadequate dietary intakes.

Supplementation with 400 μg folic acid every other day was found to influence zinc homeostasis in men during a long-term, parallel balance study. At this dose of folic acid, fecal zinc increased when the diet had a low or moderate zinc content, but not when the diet was high in zinc. Urinary zinc decreased during the low- and moderate-zinc diets, which partially compensated for the fecal losses (5). In another study, 800 μg folic acid/d was suggested to have a negative effect on zinc absorption (measured by \(^{65}\)Zn whole-body counting) only in subjects with a fractional zinc absorption >30%. These subjects also tended to have lower zinc intakes and lower plasma zinc concentrations (6). These studies suggest that folic acid inhibits zinc absorption and that this inhibition is more pronounced with low-zinc meals than with high-zinc meals.

The results of plasma response studies are conflicting. Plasma zinc response to a 25-mg dose of zinc was reduced to 49% in

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mean body mass index (in kg/m²) was 22.7

subject's mean (±SD) body weight was 66.3 ± 7.9 kg and their mean body mass index (in kg/m²) was 22.7 ± 3.1. All subjects were apparently healthy, and none were pregnant or lactating. None of the subjects took any vitamin or mineral supplements or donated blood during the study or in the previous 2 mo. None used medicine regularly or took oral contraceptives. Five smoked occasionally, whereas one was a regular smoker (<10 cigarettes/d for all).

The study was approved by the Municipal Ethical Committee of Copenhagen and Frederiksberg (KF 01–238/98) and the National Institute of Radiation Hygiene, Denmark. The subjects were given written and oral information about the study and written consent was obtained from all subjects.

**Subjects and Methods**

**Experimental design**

Each subject consumed 4 different bread meals that were served in random order and spaced 2 wk apart. Two meals had a low zinc content and 2 had a high zinc content, with or without added folic acid (2 × 2 factorial design) in amounts commonly used in fortification. The meals were extrinsically labeled with the radioisotope 65Zn. Zinc absorption was calculated by measuring whole-body retention of the isotope. A blood sample was obtained from each subject before each of the 4 test meals to measure plasma folate, erythrocyte folate, and serum zinc concentrations. Homocysteine concentrations were measured in the first and last blood samples. The habitual dietary intake of selected nutrients was assessed by using four 2-d weighed-food records spread over 2 mo. Nutrient intakes were calculated by using a national food-composition database (DANKOST 2000, version 1.4c; Dansk Catering Service, Herlev, Denmark).

**Subjects**

Fifteen women aged 22–33 y participated in this study; the subjects’ mean (±SD) body weight was 66.3 ± 7.9 kg and their mean body mass index (in kg/m²) was 22.7 ± 3.1. All subjects were apparently healthy, and none were pregnant or lactating. None of the subjects took any vitamin or mineral supplements or donated blood during the study or in the previous 2 mo. None used medicine regularly or took oral contraceptives. Five smoked occasionally, whereas one was a regular smoker (<10 cigarettes/d for all).

The study was approved by the Municipal Ethical Committee of Copenhagen and Frederiksberg (KF 01–238/98) and the National Institute of Radiation Hygiene, Denmark. The subjects were given written and oral information about the study and written consent was obtained from all subjects.

**Formulation of test meals and serving procedure**

Each test meal consisted of 2 rolls made from white (refined) wheat flour (total wt: 100 g), 30 g raspberry jam, 10 g butter, and 300 g ultrapure water. The rolls were formulated with 2 different amounts of zinc in the presence or absence of added folic acid as follows: A, low zinc; B, low zinc + folic acid; C, high zinc; and D, high zinc + folic acid. Each type of roll was prepared in one batch and stored at ∼20°C. The basic recipe (without zinc or folic acid added) was 1500 g white wheat flour, 900 g ultra-pure water, 30 g fresh yeast, 24 g butter, and 21 g NaCl. For the batches fortified with folic acid, 10.8 mg crystalline folic acid (Sigma Chemical Co, St Louis) was mixed with 3000 g flour in a food processor for 90 min at low speed. This equals 262 μg folic acid/roll (2 rolls), calculated as the value before baking, i.e., without baking loss. The zinc content was adjusted to 1 and 3 mg/serving in the low-zinc (A) and high-zinc (C) rolls, respectively, by adding a ZnCl₂ solution (prepared from ZnO and 37% HCl; Merck, Darmstadt, Germany) to the dough. After mixing, the dough was allowed to rise for 1 h at room temperature and rolls consisting of 60 ± 0.5 g dough were prepared. The subjects received the meal after fasting for 12 h and were instructed to consume it within 15 min and to alternate between eating and drinking. The meals were served on disposable material. The subjects were told not to eat or drink for 4 h after the meal.

**Isotopes and labeling procedures**

For each test meal, the rolls were extrinsically labeled with 0.04 MBq 65Zn by adding drops of the isotope solution (1 mL total) to the top of the baked rolls. The radioisotopes were purchased at Risø National Laboratory (Roskilde, Denmark) as 65ZnCl₂ in 0.1 mol HCl/L with a specific activity of 35 MBq/mg Zn. The total zinc contribution to each meal from the radioisotope was in the order of 1 μg, which represents <0.1% of the total zinc. The labeling was undertaken in the presence of an observer ∼18 h before the test meal was served. The calculated total radioactivity dose from the 4 meals for each subject was 0.56 mSv.

**Whole-body counting and calculation of zinc absorption**

Whole-body retention of 65Zn was measured in a whole-body counter on day 12 or 13 after each test meal. This time period was chosen to allow for the excretion of unabsorbed isotope. The results were corrected for background radiation in the chamber and the subject’s background radioisotope level (40K). Zinc absorption was calculated from the whole-body retention value, which was corrected for endogenous excretion from days 0 to 12 or 0 to 13. This correction was performed by using a mean retention function developed from measurements of whole-body retention over time in a group of healthy subjects after they had received an intravenous dose of 65Zn (18). When performing calculations for the second, third, and fourth meals, allowance was made for residual activity in the subjects from the preceding test meals by using the same equation. All values were corrected for physical decay.

The whole-body counter, located at the National University Hospital in Copenhagen, consisted of a lead-lined steel chamber with 4 plastic scintillator blocks (NE110; Nuclear Enterprises Limited, Edinburgh), 2 placed above the subject and 2 placed below. The counting efficiency and the setting of the energy window were established by measuring 3 water-filled phantoms, each containing 0.04 MBq 65Zn. The phantoms weighed 55, 66, and 77 kg and had outlines of typical humans of corresponding weights. A slight decrease in counting efficiency was observed with increasing weight. A linear interpolation was used to calculate the actual counting efficiency for each individual subject according to her weight. The overall counting efficiency for the 66-kg phantom was 0.2 counts·s⁻¹·Bq⁻¹ 65Zn. The energy
The intraassay CVs were 1.0% and 1.6% for hemoglobin and hematocrit, respectively, and the corresponding interassay CVs were 1.7% and 2.4%, respectively. Blood collected in heparin-containing tubes was used for plasma and erythrocyte folate analyses. For erythrocyte folate, 50 μL blood was incubated with 1 mL ascorbic acid (L-ascorbic acid; Merck) solution (0.5% wt:vol in distilled water) for 20 h in the dark at room temperature. Erythrocyte and plasma folate concentrations were analyzed fluorometrically (Delfia 1232; Wallac OY, Turku, Finland) with a time-resolved fluororinmanonassay kit (Delfia Folate Kit A072–101; Wallac OY) (20). The interassay CVs were 6.5% and 3.4% for plasma and erythrocyte folate, respectively. Plasma homocysteine concentrations were analyzed fluorometrically with a fluorescence polarization immunoanalysis kit (IMX Homocysteine kit 3D3920–20; Abbott Laboratories, Chicago) (21). An internal control was included (12.3 μmol/L with an average laboratory value of 12.5 μmol/L; intraassay CV, 1.9%; interassay CV, 4.1%).

### Statistical analyses

Values are expressed as means ± SDs. To compare fractional zinc absorption, we performed pairwise comparisons (paired t tests) between the 2 high-zinc meals and also between the 2 low-zinc meals. Pairwise comparisons were performed because of the inherent inverse relation between zinc dose and fractional absorption. Absolute zinc absorption values for all 4 meals were compared by using a repeated-measures two-factor analysis of variance with an interaction term. When significance was reached, we performed a modified t test according to the Bonferroni method. MICROSOFT EXCEL 2000 software (Microsoft Corp, San Francisco) and the STATISTICAL ANALYSIS SYSTEM (SAS) statistical package, version 6.12 (SAS Institute Inc, Cary, NC) were used for the statistical analyses.

### RESULTS

The zinc and folate contents of the test meals are shown in Table 1. We had added 262 μg folic acid to each serving of rolls, and 144 μg folic acid was recovered in the rolls after baking. After we accounted for the endogenous folate content (17 μg folate equivalents) of the unenriched rolls, the recovery of added folic acid was calculated to be 48%. Consequently, the baking loss was ≈52%.

Addition of folic acid did not influence zinc absorption from either the low-zinc or the high-zinc meal (Table 1). Fractional zinc absorption was higher from the 2 low-zinc meals (38.8–40.6%) than from the 2 high-zinc meals (22.7–26.7%), whereas in absolute amounts, zinc absorption was higher from the high-zinc meals. There was no significant interaction between zinc content and addition of folic acid.

The subjects’ habitual daily intakes of energy and selected nutrients were 9.9 ± 1.2 MJ, 12 ± 2% of energy as protein, 26 ± 3% of energy as fat, 58 ± 5% of energy as carbohydrate, 23 ± 5 g fiber, 9.3 ± 2.0 mg Zn, and 308 ± 60 μg folate. Dietary zinc and folate intakes met the Nordic Nutrient Recommendations of 7 mg and 300 μg, respectively (4). The subjects’ concentrations of serum zinc, plasma and erythrocyte folate, and plasma homocysteine were within reference ranges for the Danish population (Table 2). Zinc absorption was not affected by folate status, because neither erythrocyte folate nor plasma folate correlated with fractional zinc absorption (r < 0.3, P > 0.1).

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**TABLE 1**

<table>
<thead>
<tr>
<th>Test meals</th>
<th>Folate&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Zinc&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Zinc absorption&lt;sup&gt;3&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μg</td>
<td>mg</td>
<td>% (mg)</td>
</tr>
<tr>
<td>A: Low zinc</td>
<td>17 ± 2</td>
<td>38.8 ± 13.5</td>
<td>(0.47 ± 0.16)&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>B: Low zinc + folic acid</td>
<td>144 ± 13</td>
<td>40.6 ± 16.5</td>
<td>(0.49 ± 0.20)&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>C: High zinc</td>
<td>17 ± 2</td>
<td>26.7 ± 9.3</td>
<td>(0.78 ± 0.27)&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>D: High zinc + folic acid</td>
<td>144 ± 13</td>
<td>22.7 ± 6.6</td>
<td>(0.68 ± 0.20)&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup>Endogenous folate was analyzed in unfortified bread (A and C), n = 2. Folate in B and D (n = 5) includes endogenous folate and added folic acid.

<sup>2</sup>n = 2 for each kind of bread.

<sup>3</sup>For % absorption, there were no significant differences between A and B or between C and D (paired t tests). For mg absorption, values with different superscript letters are significantly different, *P* < 0.05 (ANOVA followed by multiple comparisons). The differences between A and D and between B and D were marginally significant (*P* = 0.05).

<sup>4</sup>Zinc absorption was correlated with fractional zinc absorption (r = 0.83, *P* < 0.001).

<sup>5</sup>Zinc absorption was correlated with fractional zinc absorption (r = 0.78, *P* < 0.001).

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Dietary analyses

Two rolls from each batch were pooled, freeze-dried, homogenized, and analyzed in duplicate for zinc content by using atomic absorption spectrometry (Spectra AA 200; Varian, Mulgrave, Victoria, Australia) after microwave digestion. A reference material [Standard Reference Material 1548, total diet material (mixed food sources); National Institute of Standards and Technology, Gaithersburg, MD] was analyzed for zinc content in the mixed food sources; National Institute of Standards and Technology material [Standard Reference Material 1548, total diet material (mixed food sources); National Institute of Standards and Technology, Gaithersburg, MD]. The zinc and folate contents of the test meals (bread rolls) per serving and zinc absorption were determined on a Cobas Minos ABX automatic cell counter (Groupe Hoffmann, Montpellier, France).
TABLE 2
Serum zinc and blood indexes of folate status\textsuperscript{1}

<table>
<thead>
<tr>
<th></th>
<th>Serum zinc\textsuperscript{2}</th>
<th>Plasma folate\textsuperscript{2}</th>
<th>Erythrocyte folate\textsuperscript{2}</th>
<th>Plasma homocysteine\textsuperscript{1}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\mu mol/L)</td>
<td>(\nnol/L)</td>
<td>(\nnol/L)</td>
<td>(\mu mol/L)</td>
</tr>
<tr>
<td>(\bar{x} \pm SD)</td>
<td>15.4 ± 1.3</td>
<td>13.1 ± 3.8</td>
<td>436 ± 84</td>
<td>6.4 ± 1.9</td>
</tr>
<tr>
<td>Range</td>
<td>14.3–17.0</td>
<td>6.2–28.1</td>
<td>305–645</td>
<td>4.0–12.7</td>
</tr>
<tr>
<td>Reference range\textsuperscript{4}</td>
<td>10.0–19.0</td>
<td>1.7–23.5</td>
<td>225–676</td>
<td>5.0–12.0</td>
</tr>
</tbody>
</table>

\textsuperscript{1}n = 15.
\textsuperscript{2}Average of 4 blood samples (1 obtained at each of the 4 test meals).
\textsuperscript{3}Average of 2 blood samples (1 obtained at the beginning and 1 obtained at the end of the study).
\textsuperscript{4}Laboratory reference range for the Danish population.

DISCUSSION

Folic acid is highly bioavailable from bread, according to previous research (22). In the present study, we added folic acid to test meals (bread rolls) in amounts typically used in fortification. The zinc contents of the test meals also corresponded to realistic dietary intakes. Two different amounts of zinc (low and high) were used because previous studies suggested that folic acid inhibits zinc absorption from low-zinc diets (5, 6). Consistent with previous results (23), the fractional absorption of zinc was substantially higher from the low-zinc meals than from the high-zinc meals (Table 1) and the absolute amount of zinc absorbed was approximately 1.5-fold greater from the high-zinc meals.

The extent of zinc absorption in the present study is consistent with previous research that included a range of zinc intakes from high-bioavailability diets (24). Our results showed no significant differences in zinc absorption from bread with and without folic acid fortification at either low or high zinc contents. However, a small inhibitory effect of folic acid on zinc absorption from the high-zinc meals (Table 1) and the absolute amount of zinc absorbed was approximately 1.5-fold greater from the high-zinc meals.

The mechanism for a potential folate-zinc interaction could operate over the long term, rather than at one moment in time. If a high folate content of a single meal does inhibit zinc absorption, then it is possible that a high folate intake over the long term would lead to low zinc absorption from a single meal. In accordance with the lack of effect of folic acid content on zinc absorption in this single-meal study, there was no correlation between markers of folate status and fractional zinc absorption.

Folic acid content of the fortified bread rolls used in the present study was 144 \(\mu g/100\) g. After correction for the endogenous folate content of the flour, the baking loss of added folate acid was 52%. This loss was substantially higher than the losses from bread reported by others [8–14% (25) and 30% (22)]. The baking loss of added folic acid may be related to the size and shape of the bread. The 144 \(\mu g\) folic acid in 100 g bread was higher than the amount that would be obtained by using flour fortified with 140 \(\mu g\) folic acid/100 g, the standard of the Food and Drug Administration (26). Thus, our data show that even with folic acid fortification at the high end of the recommended range, there was no significant interaction with zinc absorption.

Although several studies investigated the effect of folic acid on zinc absorption, limitations in the study designs and methodologies may have contributed to the inconsistent findings. In several studies, the plasma zinc response to an oral zinc challenge was measured to assess the effect of folic acid on zinc absorption (7, 11, 17). Some disadvantages of this method include the use of pharmacologic doses of zinc and the confounding effects of large fluctuations in the rate of gastric emptying and blood clearance and urinary excretion of zinc. These limitations are reflected by large inter- and intradividual variations (27, 28) and poor concordance with isotope methods (27). Pharmacologic doses of folic acid were also used commonly in studies of folate-zinc interaction (11, 13, 17). At unphysiologically high doses of folate, zinc, or both, other factors may be responsible for an observed interaction. These factors may include saturation or alternation of uptake mechanisms in the brush border membrane because of stressing of the absorptive mechanisms. This may explain why inconsistent results were obtained in studies with folate-to-zinc molar ratios ranging from 1:1 (17) to 1:975 (7). Another methodologic problem is the lack of sensitive and valid indexes of zinc status; this may explain why no consistent relation between folate intake or status and zinc status has been found (10, 12–14, 29). In accordance with this, several studies found no effect of folic acid supplementation on serum zinc concentrations during pregnancy (13, 14, 29).

The strengths of the present study were the use of isotope labeling and a whole-body counting technique for measurement of zinc absorption and the use of nonpharmacologic doses of zinc and folic acid. Isotope techniques generally provide more accurate and precise results than do other methods, but few published studies used isotope techniques. Milne (6) used \(\text{\textsuperscript{65}}\text{Zn}\) labeling and the whole-body counting technique for determining zinc absorption from single meals. Our data are in accordance with those of Milne, who found no significant difference in zinc absorption between a control meal providing 2.7 mg Zn (equivalent to our high-zinc meal) and the same meal supplemented with 800 \(\mu g\) folic acid (a 5.5-fold greater dose than that used in our test meals). In Milne’s study, \(\text{\textsuperscript{65}}\text{Zn}\) absorption from the control meal was plotted against the difference in absorption between the folic-acid-supplemented and control meals, and a linear correlation was found. On the basis of this finding, Milne concluded that zinc absorption is inhibited by folic acid; however, by using the author’s reasoning it can be argued equally well that folic acid promotes zinc absorption.

The long-term effect of folic acid on zinc status per se cannot be evaluated effectively in humans because reliable biomarkers of zinc status are not available. Therefore, we must rely on zinc absorption studies. On the basis of the present results obtained with radioisotope labeling and whole-body counting, it is not likely that fortification with the currently used concentrations of folic acid is cause for any concern. However, it would be relevant to study the effects of higher doses of folic acid, as are found in supplements, on zinc absorption with the same method.

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REFERENCES

2. Boushey CJ, Beresford SA, Ommen GS, Motulsky AG. A quantitative assessment of plasma homocysteine as a risk factor for vascular