ABSTRACT Dairy products are a potential matrix for folate fortification to enhance folate consumption in the Western world. Milk folate-binding proteins (FBP) are especially interesting because they seem to be involved in folate bioavailability. In this study, folate bioaccessibility was investigated using a dynamic computer-controlled gastrointestinal model (TNO gastrointestinal model (TIM)). We used both ultrahigh temperature (UHT)-processed milk and pasteurized milk, differing in endogenous FBP concentrations and fortified with folic acid or 5-methyltetrahydrofolate (5-CH$_3$-H$_4$folate). To study FBP stability during gastrointestinal passage and the effect of additional FBP on folate bioaccessibility, FBP-fortified UHT and pasteurized milk products were also tested. Folate bioaccessibility and FBP stability were measured by taking samples along the compartments of the gastrointestinal model and measuring their folate and FBP concentrations. Folate bioaccessibility from folic acid–fortified milk products without additional FBP was 58–61%. This was lower ($P < 0.05$) than that of the 5-CH$_3$-H$_4$folate–fortified milk products (71%). Addition of FBP reduced ($P < 0.05$) folate bioaccessibility from folic acid–fortified milk (44–51%) but not from 5-CH$_3$-H$_4$folate–fortified milk products (72%). The residual FBP levels in the folic acid– and 5-CH$_3$-H$_4$folate–fortified milk products after gastrointestinal passage were 13–16% and 0–1%, respectively, of the starting amounts subjected to TIM. In conclusion, milk seems to be a suitable carrier for folate, because both folic acid and 5-CH$_3$-H$_4$folate are easily released from the matrix and available for absorption. However, our results suggest that folic acid remains partly bound to FBP during passage through the small intestine, which reduces the bioaccessibility of folic acid from milk in this model. J. Nutr. 133: 2377–2383, 2003.

KEY WORDS: • folate • fortified milk • folate-binding protein • in vitro gastrointestinal model • bioavailability
investigation of the separate processes occurring during in vivo gastrointestinal passage. An alternative method is an in vitro, dynamic, computer-controlled gastrointestinal model [TNO gastrointestinal model (TIM)] (23,24). This model was validated and applied before with other food products and compounds and showed a good correlation with in vivo data on the bioaccessibility of various nutrients and bioactive compounds (25–28). Bioaccessible folate corresponds to the folate fraction that is released from the food matrix and available for absorption in the small intestine.

In this report, we address the following research questions related to folate-fortified milk and FBP: i) what is the bioaccessibility of folate from fortified-milk products, ii) is there a difference in bioaccessibility between folic acid and 5-CH₃-H₄folate added to milk, iii) what is the stability of FBP in milk during passage in the gastrointestinal tract and iv) to what extent does the binding activity of FBP affect folate bioaccessibility. For this purpose, we investigated in the TIM system folate-fortified, pasteurized or ultrahigh temperature (UHT)-processed milk with or without additional FBP. UHT treatment destroys FBP, and as a result, folate occurs in free form in UHT milk. In pasteurized milk, FBP is only partly destroyed by the heating and a part of the folate might remain bound (29). Besides 5-CH₃-H₄folate, folic acid was also used as fortification, because this synthetic compound is used in supplements and folate-fortified food products. In addition to folate bioaccessibility, we also studied the retention of FBP after passage through the TIM system. Previous bioavailability studies have only been able to study the folate-binding capacity of milk during the gastrointestinal transit as an indication of active FBP (14). For the first time, the present study allows direct quantification of FBP, naturally present or added to cow’s milk, before and after passage through a simulated gastrointestinal model.

MATERIALS AND METHODS

Folate- and FBP-fortified milk products. The folic acid–fortified UHT and pasteurized milk products (containing 1.5 g/100 g fat) and the FBP-rich whey fraction were kindly provided by Campina (Woerden, The Netherlands) and DMV International (Veghel, The Netherlands), respectively. The milk was homogenized at 60–65°C and then pasteurized at 76°C for 15 s or UHT treated at 140°C for 15 s and finally cooled to 7°C.

The 5-CH₃-H₄folate–fortified products were prepared in our own laboratory with the same pasteurized milk product (Campina) as used for the folic acid–fortified products. Folic acid and (6-S)-5-CH₃-H₄folate (sodium salt) to fortify the milk products were a gift from Eprova (Schaffhausen, Switzerland). The stock solutions of folic acid and 5-CH₃-H₄folate for folate fortification were calibrated spectrophotometrically at wavelengths of 283 nm [ε = 27.6 mmol/L cm⁻¹] and 290 nm [ε = 31.7 mmol/L cm⁻¹], respectively, to check their purity (30). Folate fortification was performed by the addition of 142 μL of folic acid (1.02 g/L) or 178 μL of 5-CH₃-H₄folate (0.81 g/L) as stock solutions (in 0.1 M phosphate buffer containing 10 g sodium ascorbate/L) into 360 g of milk. Sodium ascorbate was added to the stock solutions to stabilize the folate compounds during storage in the freezer until fortification of the milk products. Together with the folate fortification, 1.4–1.8 mg of sodium ascorbate was added to the 360 g of milk [containing 7.2 mg of natural ascorbic acid (31)]. The FBP fortification was designed to achieve a molar ratio of folate to FBP of 1:1, because 1 mol of FBP binds 1 mol of folate at pH 7.2 (32,33). FBP was added as 0.432 g of concentrated FBP-rich whey powder (735 mmol FBP/kg whey) to 360 g of milk. After addition of folate and FBP, the milk was stirred and placed in the dark at 20°C for 1 h before the experiments in TIM were started.

The dynamic in vitro computer-controlled gastrointestinal model. The TIM system (Fig. 1) has been described by Minekus et al. (23,24). The gastric small-intestinal model comprises four connected compartments that represent the stomach, duodenum, jejunum and ileum, respectively. Each compartment consists of a glass outer wall with a flexible inner wall. The flexible wall was surrounded by water at 37°C to squeeze the walls, which ensures mixing of the food with the secreted enzymes by peristaltic movements in the gastrointestinal tract. The pH was continuously measured in the four compartments and regulated by addition of hydrochloric acid or sodium bicarbonate. The pH values, as well as the gastric emptying and small-intestinal passage of the food, were controlled according to preset curves based on information on human in vivo conditions (23,24). Artificial oral fluid and gastric juice with lipase (150,000 U/jl; Rhopont lipases F-AP 15; Amano Pharmaceuticals, Nagoya, Japan) and pepsin (2200 U/ml; P7012; Sigma, St. Louis, MO) were gradually added into the gastric compartment (24,28). Bile (porcine bile extract; P8631; Sigma), pancreatic juice (Pancrex V powder; Paines & Byne, Greenford, UK) and electrolytes were gradually added into the duodenal compartment (23,24,28). The jejunal and ileal compartments are connected with semipermeable hollow-fiber membranes with a cut-off of 5 kDa (Cobe Hemophan Hemodialyzers; Hospal, Dransfeld, Germany), which mimic the absorption of digested products and water (Fig. 1, points N, O and P). The nonabsorbed fractions were collected after passage through the ileocecal valve as ileal delivery (Fig. 1, point H).

TIM experiment with milk products. In preliminary experiments, we established that folate recoveries of ~100% were achieved when membranes with a cut-off of 5 kDa were used and precautions were taken against folate oxidation and/or degradation, such as nitrogen flushing, protection against sunlight and addition of sodium ascorbate after sampling, in the 5-h experiments in TIM. Because polyglutamates become deconjugated in vivo to absorbable short-chain glutamates by an enzyme associated with the jejunal brush border (34), we checked the membranes in TIM for passage of mono- and polyglutamates with liver homogenate as a source of polyglutamates. We found that both mono- and polyglutamates could pass through the membranes. Because the membranes do not discriminate between mono- and polyglutamates, the incorporation of conjugate enzymes in the TIM system is not necessary in measuring the bioaccessible folate fraction after the jejunal and ileal compartment. Therefore, external deconjugation of the collected samples from the TIM system was performed before folate analysis by HPLC.
Six different milk products (Table 1) were tested in duplicate for folate bioaccessibility in TIM. Appropriate software and working protocol were used to simulate the human gastrointestinal conditions after consumption of milk products.

At the beginning of each experiment, a test portion of 300 g of milk was put into the gastric compartment of TIM. During digestion, total dialysate was collected for 0–1, 1–2, 2–3, and 3–5 h after passage through the semipermeable hollow-fiber membranes (Fig. 1, point N) connected to the jejunal and ileal compartments. Total ileal delivery (Fig. 1, point H) was collected over a period of 0 to 5 h. The dialysate contained the absorbable (bioaccessible) fraction, whereas the ileal delivery material corresponded to the nonabsorbable (nonbioaccessible) fraction. After the 5-h experiment, the residues from the ileal dialysate and eluted over the FBP affinity columns were prepared according to Konings (35) with FBP as a competitive inhibitor.
H4folate, the recoveries were incomplete and lower with in-
H2O849Folate bioaccessibility
calculated by the formula:

\[
\text{Recovery} = \frac{(\text{Folate}_\text{duodenal} + \text{Folate}_\text{ileal delivery}}
\quad + \frac{\text{Folate}_\text{endogenous}}{(\text{Folate}_\text{cod} + \text{Folate}_\text{endogenous}}) \times 100
\]

where Folate\text{duodenal} is the folate content in jejunal plus ileal dialysate, Folate\text{ileal delivery} is the folate content in the total material collected behind the ileocecal valve, Folate\text{endogenous} is the folate content in the bile and pancreatic solutions secreted into the duodenal compartment (Fig. 1, point K).

Folate bioaccessibility was expressed as fraction of intake and calculated by the formula:

\[
\text{Folate bioaccessibility} = \frac{\text{Folate}_\text{duodenal}}{(\text{Folate}_\text{cod} + \text{Folate}_\text{endogenous}) \times 100.}
\]

The data were analyzed by ANOVA, after Barlett’s test for homo-
genosity of the variances. ANOVA was performed to test the overall difference between the foods. When the F test was significant, pairwise tests on individual means were performed using the least

cratic run effect on the folate bioaccessibility.

The endogenous folate content (Folate\text{endogenous}) in bile and pancreatic juice was 1.3–1.4% of the folate in the food (Folate\text{cod}). Approximately 10% of the folate intake (Folate\text{endogenous}) was found as residue (Folate\text{endogenous}) in the TIM system at the end of the experiments. Dividing the folate content in the TIM samples by the folate intake gave recov-
eries of 80–90% in the 12 experiments performed. No difference was found between folate acid and 5-CH3-H4folate concern-
ing stability during passage through TIM (Table 3). The differences in folate bioaccessibility between the six milk prod-
cucts could therefore not be explained by differences in total reco-
vies.

The kinetic profile of folate bioaccessibility was measured by collecting samples from jejunal and ileal dialysates during 0–1, 1–2, 2–3 and 3–5 h after starting the experiment. The maximum concentration of folate acid and 5-CH3-H4folate in jejunal dialysate was in the samples collected between 1 and 2 h and in the ileal dialysate between 1 and 3 h (Fig. 2).

### TABLE 3

Folate content in jejunal dialysate, ileal dialysate, jejunal plus ileal dialysate (=bioaccessible fraction) and ileal delivery (=non-bioaccessible fraction) samples collected between 0–5 h from the in vitro gastrointestinal model

<table>
<thead>
<tr>
<th>Milk product</th>
<th>Jejunal dialysate</th>
<th>Ileal dialysate</th>
<th>Jejunal plus ileal dialysate</th>
<th>Ileal delivery</th>
</tr>
</thead>
<tbody>
<tr>
<td>UHT plus folic acid</td>
<td>48.4 ± 1.2a</td>
<td>12.9 ± 1.0a</td>
<td>61.2 ± 2.2a</td>
<td>6.9 ± 0.8a</td>
</tr>
<tr>
<td>UHT plus folic acid plus FBP</td>
<td>38.6 ± 2.1b</td>
<td>11.9 ± 0.6a</td>
<td>50.5 ± 2.7b</td>
<td>22.0 ± 2.9bc</td>
</tr>
<tr>
<td>Pasteurized milk plus folic acid</td>
<td>37.1 ± 4.3b</td>
<td>21.2 ± 3.7b</td>
<td>58.3 ± 0.6a</td>
<td>16.5 ± 5.2abc</td>
</tr>
<tr>
<td>Pasteurized milk plus folic acid plus FBP</td>
<td>34.1 ± 0.7b</td>
<td>9.8 ± 1.2c</td>
<td>43.9 ± 1.99</td>
<td>29.0 ± 4.9c</td>
</tr>
<tr>
<td>Pasteurized milk plus 5-CH3-H4folate</td>
<td>61.4 ± 3.1c</td>
<td>9.3 ± 1.7a</td>
<td>70.8 ± 1.5c</td>
<td>4.3 ± 0.2a</td>
</tr>
<tr>
<td>Pasteurized milk plus 5-CH3-H4folate plus FBP</td>
<td>60.3 ± 3.6c</td>
<td>11.7 ± 0.6a</td>
<td>72.0 ± 3.1c</td>
<td>6.0 ± 4.2a</td>
</tr>
</tbody>
</table>

1 Values are means ± range, n = 2. Abbreviations: 5-CH3-H4folate, 5-methyltetrahydrofolate; FBP, folate-binding protein.
2 Means in column without at common letter differ significantly, (P < 0.05).
The analyzed FBP content decreased after passage through the TIM system. As expected, no FBP was found in the dialysate membrane. After folic acid fortification, 13–16% of the FBP naturally present in the milk products was recovered in the ileal delivery. In the 5-CH$_3$-H$_4$folate–fortified pasteurized milk, however, 0–1% was recovered after passage through the gastrointestinal tract.

**DISCUSSION**

The folate bioaccessibility from fortified milk products measured with the in vitro gastrointestinal model was 60–70%. This is in agreement with published observations (8,38–39) concerning the bioavailability of dietary folates varying between 40 and 70% as reported in previous studies on human subjects (38) and in a rat bioassay (39). Results indicate that fortification of milk with 5-CH$_3$-H$_4$folate leads to higher folate bioaccessibility (~70%) (P < 0.05) than that resulting from fortification with folic acid (~60%, Table 3). The higher bioaccessibility of 5-CH$_3$-H$_4$folate is mainly a result of the significantly higher (P < 0.05) folate content found in the jejunal dialysate collected between 1 and 2 h (Fig. 2). This difference in release from the food matrix, including endogenous FBP, could be explained by the lower binding affinity of FBP for 5-CH$_3$-H$_4$folate compared with folic acid at the pH range of 5–7.4 (40). A lower binding affinity could result in a higher release during gastric passage and less recombination in the duodenal part of the small intestine. In addition, FBP did not affect 5-CH$_3$-H$_4$folate bioaccessibility, whereas the inhibitory effect of added FBP on the bioaccessibility of folic acid was 11–14% (Table 3). In Figure 3, the level of FBP as measured in the folic acid–fortified milk products (Table 1, products 1–4), is plotted against the bioaccessibility of folic acid from the fortified UHT and pasteurized milk products. It appears that the FBP concentration of the milk products is inversely related to the bioaccessible folic acid fractions.

FBP did not affect 5-CH$_3$-H$_4$folate bioaccessibility; this could indicate that the 5-CH$_3$-H$_4$folate–FBP complex is less stable in the intestine than the folic acid–FBP complex. After gastrointestinal passage, the retention of FBP from the folic acid–fortified milk products was 13–16%. However, only 0–1% FBP retention was found after passage of 5-CH$_3$-H$_4$folate–fortified milk products. An explanation for the higher FBP retention after gastrointestinal passage of the folic acid–fortified milk products could be that the FBP-folate complex is more stable than the free FBP molecule (41). Remarkably, no relative difference was found between the FBP-fortified and nonfortified milk products, which could indicate the presence of a nondegradable FBP fraction.

Our study provides the first evidence that a fraction of endogenous FBP as well as FBP added to milk can pass intact through the gastrointestinal tract. However, the TIM system cannot address the question of whether the FBP-bound folate is absorbed, because there is no corresponding folate receptor system in the TIM system as has been suggested to occur in mammals. Previous studies support the concept that FBP affect the absorption and/or retention of folate from milk in vivo, especially during the neonatal period (9,16–21,42,43). However, some results are contradictory, because enhancement of folate absorption by FBP was observed in studies performed...
with isolated rat intestinal mucosal cells (18) and intestinal brush border membrane vesicles (19), whereas Tani et al. (20) and Said et al. (21) found lower jejunal and equal ileal transport in rats of FBP-bound folate compared with unbound folate. Mason and Selhub (43) showed that FBP-bound folate is absorbed in the small intestine by a different mechanism than the absorption of unbound folate. The absorption of FBP-bound folate occurs more gradually and slowly. It has been suggested that a slower absorption rate prevents the occurrence of high plasma folate levels, which could promote rapid excretion by the kidneys (17).

The kinetic pattern, i.e., the appearance of folate in the dialysate fractions, appeared to be different for the six milk products (Fig. 2). This could be due to the amount of protein (such as FBP) in the food. Folate compounds might bind to proteins, resulting in a slower release of folate from the food matrix. Overall, similar trends in folic acid bioaccessibility between the six milk products were found between 0 and 1 and 0 and 4 h. Therefore, it is expected that the relative bioaccessibility of folic acid and 5-CH3-H4folate in the case of FBP enrichment, a higher folate bioaccessibility in the case of 5-CH3-H4folate fortification and no difference in 5-CH3-H4folate bioaccessibility between FBP-fortified and nonfortified products. Within the first hour after milk consumption, about one-half of the stomach content was delivered to the intestine. Therefore, it seems that incubation of the food at a low pH in combination with stomach enzymes is important for the release of folate from the food matrix.

Two folic acid–fortified milk products (Table 1, products 1 and 3), which showed a high bioaccessibility in the TIM system, were also studied in a human intervention study (de Jong, Verwei, M., van Vliet, T., Siebelink, E. & West, C., unpublished results). The results also indicate a high bioavailability, because an extra dose of 200 µg of folic acid added to milk, given daily for 4 wk, significantly increased serum folate and RBC folate concentrations. The folate concentrations did not differ between the folic acid–fortified and nonfortified milk products (Table 1, products 1 and 3), which showed a high bioaccessibility in the TIM system, because goat milk was compared with that of a milk-free diet. Folic acid was added to milk-free and milk-containing diets to obtain 0, 200, 400 or 600 µg folic acid/kg diet. The dose-response curve for plasma folate showed an enhancement of folate bioavailability when human or bovine milk was incorporated, but a milk factor other than FBP caused this enhancement, because goat’s milk, with the highest FBP concentration, lowered the folate bioavailability in comparison with the milk-free diet.

In conclusion, the UHT and pasteurized milk matrix seems to be a suitable carrier for supplemental folate, because folate was easily released from the matrix and highly available for absorption (60–70%). A small but significant difference was found in the bioaccessibility of folic acid (60%) and of 5-CH3-H4folate (70%). With regard to FBP fortification, our in vitro data suggest that additional FBP decrease the bioaccessibility of folic acid, in contrast to 5-CH3-H4folate, from milk products. Therefore, FBP seems to be nonbeneficial for the enhancement of folate bioavailability. The TIM system can be considered a good methodology for nutritional science for evaluation of bioaccessibility, because the model accurately and reproducibly simulates the conditions in the lumen of the stomach and the small intestine. Because active transport can be involved, the influence of FBP on folate transport should be tested in vitro (with intestinal segments or cultured mucosal cells). These in vitro studies would provide information about folate intestinal transport and would complement the studies with the TIM system. Whether FBP could possibly influence folate transport can also be addressed by investigating the FBP binding activity during gastrointestinal passage for both folic acid and 5-CH3-H4folate, because the occurrence of folate-FBP complexes at the site of absorption is relevant. This will give more insight into the mechanisms underlying the effect of FBP on folate bioavailability.

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LITERATURE CITED