Bioavailability of folate from fortified milk products

Miriam Verwei

Promotoren

Prof. Dr. Ir. G. Schaafsma
Hoogleraar Voeding en Levensmiddelen, Wageningen Universiteit
Wetenschappelijk staf medewerker Voeding, TNO Voeding, Zeist

Prof. C.E. West, PhD DSc FRACI
Universitair hoofddocent, sectie Humane Voeding, Wageningen Universiteit
Hoogleraar Voeding in relatie tot Gezondheid en Ziekte, Radboud Universiteit Nijmegen

Co-promotoren

Prof. Dr. Ir. J.P. Groten
Hoogleraar Combinatietoxicologie, Wageningen Universiteit
Afdelingshoofd Physiological Sciences Department, TNO Voeding, Zeist

Dr. R. Havenaar
Product manager in vitro maag-darmonderzoek, TNO Voeding, Zeist

Promotiecommissie

Prof. Dr. R.-J.M. Brummer
Universiteit Maastricht
Wageningen Centre for Food Sciences

Prof. Dr. Ir. G.J. Hiddink
Wageningen Universiteit
Nederlandse Zuivel Organisatie, Zoetermeer

Prof. Dr. M.B. Katan
Wageningen Universiteit
Wageningen Centre for Food Sciences

Dr. H. van den Berg
Voedingscentrum, Den Haag

Dit onderzoek is uitgevoerd binnen de onderzoekschool VLAG.

Bioavailability of folate from fortified milk products

Miriam Verwei

Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit
Prof. Dr. Ir. L. Speelman
in het openbaar te verdedigen
op woensdag 15 september 2004
des namiddags te vier uur in de Aula

Title Bioavailability of folate from fortified milk products

Author Miriam Verwei
Thesis Wageningen University, Wageningen, The Netherlands (2004)
with abstract - with references - with summary in Dutch

ISBN 90-8504-080-9

Abstract

The gap between actual intake and recommended intake of folate can be bridged by the consumption of fortified food products. Milk is considered as a potential food matrix for folate fortification in countries (such as the Netherlands) with a high milk consumption. The aim of the work described in this thesis was to study the bioavailability of folate from milk products to establish whether milk is a suitable matrix for fortification with folic acid or 5-CH_3-H_4folate. In addition, the role of folate-binding proteins (FBP) in the bioavailability of folate from milk was investigated.
Studies with a dynamic *in vitro* gastrointestinal model showed that folic acid and 5-CH_3-H_4-folate are highly bioaccessible from fortified milk products. The bioaccessibility of folate from fortified milk products was lower in presence of additional FBP, with a more pronounced inhibitory effect for folic acid as compared with 5-CH_3-H_4folate. This was explained by the observed difference in extent of binding to FBP between folic acid and 5-CH_3-H_4-folate in the duodenal lumen. Before gastric passage, folic acid and 5-CH_3-H_4-folate were mainly bound to FBP (76-79%) while 7% was free. After gastric passage, folic acid remained bound to FBP to a similar extent (80-81%). For 5-CH_3-H_4-folate the FBP-bound fraction gradually decreased from 79% to 5% and the free fraction increased from 7% to 93%. So, while folic acid enters the proximal part of the small intestine bound to FBP, 5-CH_3-H_4-folate appears mainly to be present as free folate in the duodenal lumen.
The intestinal absorption of folic acid and 5-CH_3-H_4folate was studied using monolayers of human colon carcinoma (Caco-2) cells. Only a small difference in transport, in rate and underlying transport mechanisms, across Caco-2 cells was found between folic acid and 5-CH_3-H_4-folate. In presence of FBP, the absorption of folic acid and 5-CH_3-H_4folate was found to be lower and dependent on the extent of binding to FBP at the luminal side of the intestinal cells.
Results from a human intervention study showed that the consumption of 200 µg of folic acid added to milk significantly increased folate concentrations in serum and red blood cells. Although only two fortified milk products were tested in a human study, several milk products fortified with folic acid or 5-CH_3-H_4-folate with or without additional FBP were tested in the *in vitro* studies with the gastrointestinal model. Finally, a kinetic model was used to integrate the *in vitro* results about the kinetics of folate bioaccessibility and intestinal absorption and to extrapolate the findings to the human situation. With this *in silico* approach, the blood folate levels in humans could be predicted accurately.
In conclusion, the *in vitro* and *in vivo* studies described in this thesis show that milk is an appropriate food matrix for folate fortification. A dietary strategy with fortified milk products can be recommended to bridge the gap between actual and recommended folate intake to optimize the folate status of the population. Folic acid-fortified milk should, however, not be supplemented with additional FBP as this will lead to a lower bioavailability of folic acid.

Contents

Chapter 1	General introduction	9
Chapter 2	Folic acid and 5-methyltetrahydrofolate in fortified milk are bioaccessible as determined in a dynamic in vitro gastrointestinal model	27
Chapter 3	Bioaccessibility of folic acid and 5-methyltetrahydrofolate decreases after the addition of folate-binding protein to yogurt	43
Chapter 4	Bioaccessibility of folate from several liquid and solid food products	57
Chapter 5	The binding of folic acid and 5-methyltetrahydrofolate to folate-binding proteins during gastric passage differs in a dynamic in vitro gastrointestinal model	69
Chapter 6	Effect of folate binding protein on intestinal transport of folic acid and 5-methyltetrahydrofolate across Caco-2 cells	87
Chapter 7	Transport of folic acid and 5-methyltetrahydrofolate across Caco-2 cells occurs via the reduced folate carrier and multi-drug resistance proteins	101
Chapter 8	Bioavailability of folic acid from fortified pasteurized and UHT-treated milk in humans	117
Chapter 9	General discussion: integration of in vitro and in vivo results	133
	Summary	153
	Samenvatting	157
	Curriculum vitae	163
	List of Publications	165
	Dankwoord	169
	List of abbreviations	173

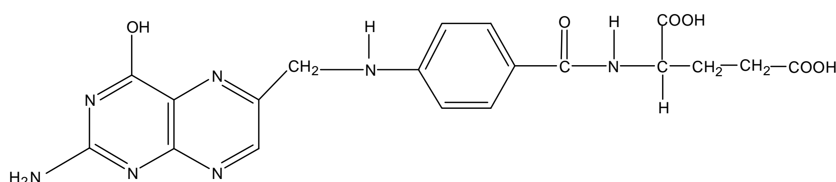
1

General Introduction

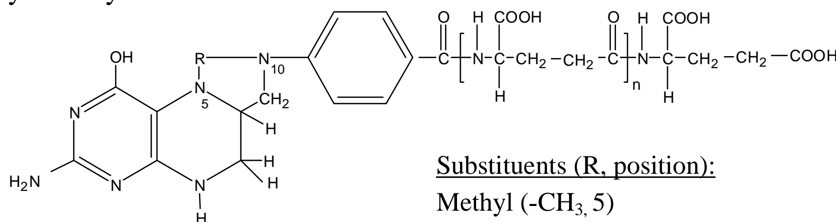
Role of folate in human health

Folate is the generic term for a class of B vitamins that have a chemical structure and nutritional activity similar to that of pteroylmonoglutamic acid (PGA or folic acid) (1). Folic acid is hardly present, if at all, in natural products, but is a synthetic form, used in supplements and food fortification. Folic acid is fully oxidized and consists of p-aminobenzoic acid (PABA) linked to a substituted pteridine ring, together forming pteroic acid, and one residue of glutamic acid (monoglutamate) (Figure 1.1A). Natural folates are mainly the reduced form tetrahydrofolate (H_4folate) and its methylated or formylated derivatives with a number of glutamyl residues (1-7) attached to the pteroyl group (Figure 1.1B). Folic acid is only active in the human body after reduction to tetrahydrofolate (2). This is carried out by the enzyme dihydrofolate reductase (DHFR) that reduces folic acid to dihydrofolate (H_2folate) and also reduces dihydrofolates to tetrahydrofolate (Figure 1.2). The main function of folate is the transfer of one-carbon moieties, such as methyl and formyl groups, in the body. Tetrahydrofolates can be converted to 5-methyltetrahydrofolate (5MTHF or 5-CH_3-H_4folate) via 5,10-methylene-tetrahydrofolate (5,10-CH_2-H_4folate) by the enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR). 5-CH_3-H_4folate is the methyl group donor in the remethylation of homocysteine to methionine by the enzyme methionine synthase (MS). Methionine is an essential amino acid that is converted to S-adenosylmethionine (SAM) which is an important methyl donor for many reactions that occur in the cell.

A) Pteroyl-L-glutamic Acid (PGA) or folic acid



B) Polyglutamyl tetrahydrofolates



Substituents (R, position):
Methyl (-CH_3, 5)
Formyl (-CHO, 5 or 10)
Formimino (-CH=NH, 5)
Methylene (-CH_2-, 5 and 10)
Methenyl (-CH=, 5 and 10)

Figure 1.1 Chemical structures of folic acid (A) and tetrahydrofolates differing in the presence of substituents and the number of glutamyl residues (B).

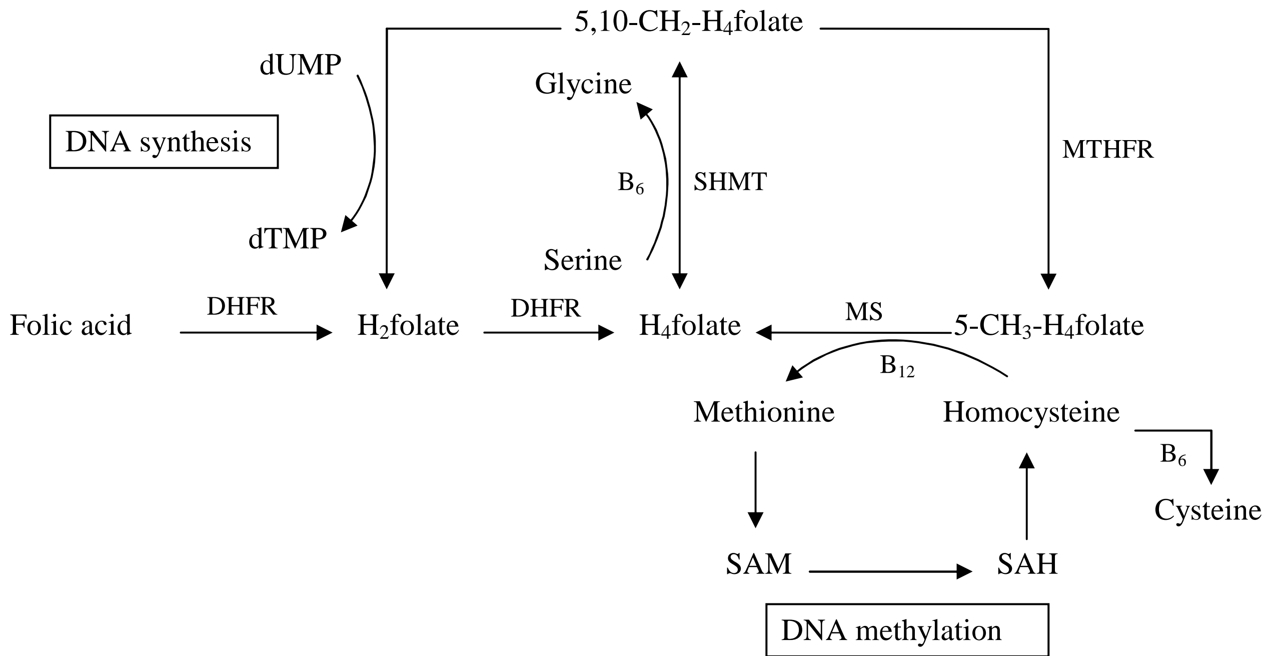


Figure 1.2 Overview of the main metabolic pathways of folate. See description in text for abbreviations.

As illustrated in Figure 1.2, folate plays a role in DNA synthesis, serine and glycine metabolism, methionine biosynthesis and DNA methylation. Due to the important role of folate in these processes, folate deficiency leads to several physiological disorders such as megaloblastic anemia (3) which is a result of impaired cell division. A relative folate deficiency is also found to be associated with an enhanced risk for neural tube defects (4,5). Because folate is involved in the metabolism of homocysteine, elevated plasma homocysteine levels are found in people with a low folate status. There is increasing evidence for the relation between high levels of plasma homocysteine and an enhanced risk for cardiovascular diseases (6,7). Furthermore, a low folate status may be causally related to certain types of cancer, particularly colon cancer (8). Moreover, evidence is accumulating that folate is also important for the regulation of gene expression by means of DNA methylation. The important role of folate in several processes in the human body and in the prevention or reduction of diseases emphasizes the need for an adequate folate intake of the whole population.

Recommended and actual intake of folate

The recommended daily allowances (RDA) is defined as the amount of nutrient that is needed to cover the needs of 97.5% of the healthy population. The RDA of dietary folate is 300 µg for adults (>19 y) in the Netherlands (9). This advice is based on the prevention of megaloblastic anaemia and not on the prevention of neural tube defects or cardiovascular disease. For optimal reduction of the plasma homocysteine concentrations, to decrease the risk for cardiovascular diseases, a folate intake higher than the actual RDA is required (10). In order to prevent neural tube defects, Dutch women

who want to become pregnant are advised, irrespective of the dietary intake level, to use a daily supplement of 400 µg folic acid from 4 weeks before, till at least 8 weeks after conception. For pregnant women the adequate intake level is 400 µg/day. The RDA established for the Dutch population equals the RDA for the Scandinavian population (300 µg), but is lower than that established in the USA and Germany (both 400 µg) and higher than the advised daily intake in the United Kingdom (200 µg). The variation in RDA levels between several countries is due to the fact that each country has its own approach and correction factors based on the interpretation of available scientific data on which the recommendations are based (11).

Dietary folate equivalents are used to convert all forms of dietary folate, including synthetic folic acid in fortified products, to an amount that is equivalent to food folate (12,13). The dietary folate equivalents differentiate between food folate and supplemental folate because the bioavailability of folate (folic acid) from fortified products and supplements is estimated to be 1.7 and 2 times, respectively, higher than that of dietary folate from natural food products (12,13).

The dietary folate intake of a representative sample of the Dutch population (n=6218, the 1992 Dutch National Food Consumption Survey) was calculated to be 182 µg/day according to Konings et al. (14) and 251 µg/d according to Bausch-Goldbohm et al (15), respectively, based on HPLC analysis and microbiological analysis of the dietary folate content. The average dietary intake of adult populations in Europe was found to be 291 µg/day for men and 247 µg/day for women (11). Thus, the actual dietary intake levels are often lower than recommended, and therefore, there is room for enhancing the folate intake.

Strategies to fill the gap between actual and recommended folate intake

In many countries, the actual folate intake was found to be lower than recommended (11,14). An enhanced folate intake can be realized following one (or a combination) of the following strategies: 1) consumption of folate-rich food products such as orange juice and spinach, 2) taking folate supplements (e.g. tablets), or 3) consumption of folate-fortified food products. Which strategy or combination of strategies would be the most effective and lead to an optimal folate intake is currently under debate in several European countries.

The first option to enhance the folate intake is an increased consumption of foods naturally rich in folates such as vegetables and (citric) fruits. The folate content of food products should be accurately established to determine the dietary folate intake of the population. Accuracy in the determination of folate content in foods is dependent, in part, on the completeness of extraction of folates from the food matrix and their stability during extraction. Folic acid is more stabile than the reduced folate derivatives. The order of stability of the reduced folate compounds in water is: 5-CHO-H_4-folate > 5-CH_3-H_4folate > 10-CHO-H_4folate > H_4folate (16). All folate compounds are susceptible to oxidative degradation, resulting in splitting of the molecule into biologically inactive forms such as p-aminobenzoyl-glutamate. This process is enhanced by (UV-)light and heat and can be reduced in presence of sufficient amounts of antioxidants, e.g. ascorbic acid and thiols (17). Complete extraction of folate from the food matrix can be realized using the so-called tri-enzyme treatment of the food samples. After incubation with protease and amylase to extract folate from the

food matrix, the samples are incubated with γ -glutamyl hydrolase (conjugase) to enzymatically deconjugate the folate polyglutamates to monoglutamates (18). After extraction, the folate content of the food products can be measured with several analytical techniques, e.g., HPLC, microbiological assay or radio-protein-binding assay. As most of these methods only measure folate monoglutamates, complete deconjugation is important to prevent underestimation of the folate content. Next to analytical variability, the determination of the dietary folate intake is also influenced by the (seasonal) variation in folate content of foods and the potential loss of folate during thermal processing.

A diet high in folate-rich food products has shown to improve the folate status, including a lower plasma homocysteine (19,20). Such a diet has additional benefits as it is likely to have also a high content of various other vitamins and minerals. This strategy has been shown to be effective under controlled conditions, i.e. in an experimental setting. However, the compliance to a diet with high amounts of fruits and vegetables appears to be low in the Netherlands, as in many other countries. Therefore this dietary strategy seems difficult to apply for the whole population and for a longer time period. A complementary approach to improve the folate status of the whole population might be a combination of dietary change in combination with the consumption of fortified food products and/or supplements with folic acid or 5-CH_3-H_4folate.

The second option to increase the folate intake is the daily use of supplements (tablets). The experiences with the campaign ‘prevention of neural tube defects’ in the Netherlands, and in many other (European) countries indicate that compliance to the advice of taking folate supplements is low (21,22). Therefore, the consumption of folate-fortified food products might be an alternative strategy to enhance the folate intake at the population level (third option). This strategy has been shown to be effective in increasing the average folate intake. Mandatory folic acid fortification of grain products in the USA and Canada since 1998 has led to a substantial increase in folate status and lowering of homocysteine levels at the population level (23,24). Despite these positive findings, several countries, including the Netherlands, do not allow the introduction of food products fortified with folic acid. The reason is the potentially negative effects of folic acid (9). The Health Council of the Netherlands has accepted the Tolerable Upper Intake Level of 1 mg folic acid set by the EU Scientific Committee as well as by the US Institute of Medicine (9). This upper level is based on findings that excessive folic acid intake may mask the diagnosis of vitamin B_{12} deficiency in elderly, and especially could induce neurologic damage (25).

The potential negative effect of folic acid might be circumvented by the use of natural folate, 5-CH_3-H_4folate, as a fortificant. 5-CH_3-H_4folate is unlikely to mask vitamin B_{12}-defiency and no upper level exists for the consumption of natural folate (25). Contrary to folic acid, 5-CH_3-H_4folate needs no reduction by DHFR (Figure 1.2) before being incorporated in the active folate pool. A disadvantage of the use of 5-CH_3-H_4folate as fortificant in food products is its instability compared with folic acid. The beneficial effects of folic acid have been extensively studied. Whether 5-CH_3-H_4folate is as effective as folic acid in increasing the plasma folate levels and prevention of neural tube defects seems likely, but remains to be demonstrated. In long-term bioavailability studies (24 wk), 104 (26) or 167 (27) subjects received daily a folic acid (100 µg), 5-CH_3-H_4folate (113 µg) or placebo supplement. These studies showed that 5-CH_3-H_4folate was as effective as folic acid in

increasing plasma and red blood cell (RBC) folate levels and in reducing homocysteine levels in healthy persons. These findings were confirmed by a 24 week-study of Lamers et al. (28). In this study, the homocysteine levels were as effectively lowered by 5-CH_3-H_4folate (416 µg) as by folic acid (400 µg). In addition to these long-term studies, the plasma folate responses in 13 men were studied after a single oral dose (capsule) of 500 µg 5-CH_3-H_4folate or folic acid (29). The plasma folate responses were found to be similar after 5-CH_3-H_4folate or folic acid consumption indicating an equal short-term bioavailability. Thus, these recent studies (26-29) show that 5-CH_3-H_4folate can be an adequate alternative to folic acid for fortification purposes.
If food fortification would be allowed in the Netherlands, the next step could be to establish the most suitable food matrix or food product for fortification to enhance the folate status of the population. In this respect, milk products should be considered as candidate product for fortification with folic acid or 5-CH_3-H_4folate as milk products are consumed by a large part of the Dutch population. Milk is also expected to be a potential food matrix for folate fortification due to the presence of folate-binding proteins (FBP). Whether the milk matrix is a suitable carrier for folic acid or 5-CH_3-H_4folate to enhance the folate status of the population is dependent on the bioavailability of folate from milk.

Milk as potential food matrix for folate fortification

Folate and folate-binding proteins in milk

The naturally occurring form of folate in unprocessed milk, 5-CH_3-H_4folate, is present as a mixture of mono- and polyglutamates (14,30). Unprocessed cow's milk has a folate content of 5-10 µg/100 g, which is found to vary over the year as higher folate values were observed during the summer season than during the winter season (30). The natural folate content in milk is low compared to folate-rich food products such as green vegetables and citric fruit (14). Despite their low folate content, milk products contribute for 10-15% to the daily folate intake in countries with a high milk consumption, such as The Netherlands (14) and Sweden (31).
In unprocessed and pasteurized milk, the native folate is essentially bound to FBP (30,32). FBP are whey proteins with a molecular weight between 30-40 kDa (33). Folate and FBP occur in an equimolar ratio in milk as FBP levels of 160-210 nmol/L and folate levels of 110-220 nmol/L were measured (34). After pasteurization or Ultra-High Temperature (UHT) treatment of milk, the total amount of folate was reduced by 8% and 19%, respectively (35). FBP in milk were also found to be susceptible to heat treatment. Although pasteurization of milk led to a small decrease in FBP content, fermentation (yogurt) and severe heat treatment (UHT milk) of milk resulted in the detection of only traces of FBP in these processed milk products (35).
The suitability of the milk matrix for fortification with either folic acid or 5-CH_3-H_4folate to enhance the folate status of the population is largely dependent on the bioavailability of the folate compounds from milk. Therefore, the bioavailability of folic acid and 5-CH_3-H_4folate from milk and the effect of FBP, and the underlying mechanisms of this effect, on the bioavailability of both folate compounds need to be investigated. Information about the bioavailability of the individual

folate compounds from milk in absence or in presence of FBP helps in the development of an alternative dietary strategy to enhance the folate status of a large part of the population.

The effect of folate-binding proteins on folate bioavailability

Folate bioavailability is described by the proportion of the ingested amount that is absorbed from the small intestine and available for metabolic processes or storage in the body. A lot of studies have been performed in which the bioavailability of folate from fruits and vegetables is studied but no information is available about the bioavailability of folate from milk. In addition the effect of FBP on the bioavailability of folate from milk is unclear. It has been speculated that FBP protects folate from bacterial uptake and degradation which might indirectly lead to a higher folate absorption (36,37). FBP might also directly affect intestinal transport, but contradictory results have been found considering the influence of FBP on folate uptake and transport both in *in vitro* studies (using isolated rat mucosal cells, goat brush-border membrane vesicles or everted sacs of rat intestine) (38-40) as well as in an *in vivo* study with rats (41).

Whether FBP affects folate absorption is primarily dependent on the binding activity of FBP in the intestinal lumen. In a study with rats it was found that under acidic gastric conditions ($pH < 4.5$) folic acid was released from FBP and recombined in the small intestine of rats (pH 6-7) (41). This is in line with findings that the dissociation of folic acid from FBP occurs at a pH of approximately 5 and lower and is completely reversible (33,41,42), even after pepsin treatment (41). Also in a study with young goats, who received FBP-bound folic acid in goat's milk, it was found that folic acid occurred bound to FBP in the small intestine which indicates that the gastric acidity and gastrointestinal digestive enzymes had little effect on the binding characteristics of FBP for folic acid (43). The studies with animals show that the binding of folic acid to FBP is pH-dependent and reversible as folic acid and FBP recombine in the small intestine. Whether this is also true for the human gastrointestinal tract has not been investigated before. In addition, the binding characteristics of FBP for 5-CH_3-H_4folate, the naturally occurring form of folate in milk, has not been studied so far.

The effect of FBP on the bioavailability of folate has been investigated in a few studies with human volunteers. In a study with nine ileostomists it was found that FBP, present in non-fermented milk (endogenous FBP), had no effect on folate bioavailability as equal amounts of folate were absorbed from fermented and non-fermented milk products (44) In a recent study (45) the effect of additional FBP on the bioavailability of 5-CH_3-H_4folate from fermented milk was investigated. Nine ileostomists consumed fermented milk fortified with 5-CH_3-H_4folate in absence or presence of additional FBP. In almost all volunteers the addition of FBP led to a lower bioavailability of 5-CH_3-H_4folate from fermented milk (45).

Determination of folate bioavailability following a step-wise approach

Information about the bioavailability of folate from fortified and non-fortified food products is necessary to determine whether the amount of folate consumed daily is sufficient to meet the nutritional requirements. Two individual processes determine the bioavailability of folate, which are

1) the release from the food matrix due to digestion (bioaccessibility) and 2) intestinal absorption (Figure 1.3). After absorption of folate from the intestinal lumen, folate is distributed in the body resulting in a certain blood folate level reflecting the folate status of the human body.
This thesis is focused on the bioavailability of folate from fortified milk products. The bioavailability of folic acid and 5-CH_3-H_4folate from fortified milk in absence or in presence of additional FBP has been studied following a step-wise approach, as illustrated in Figure 1.3, to get more insight into the kinetics of release and intestinal transport of folate and the effect of FBP on these processes.

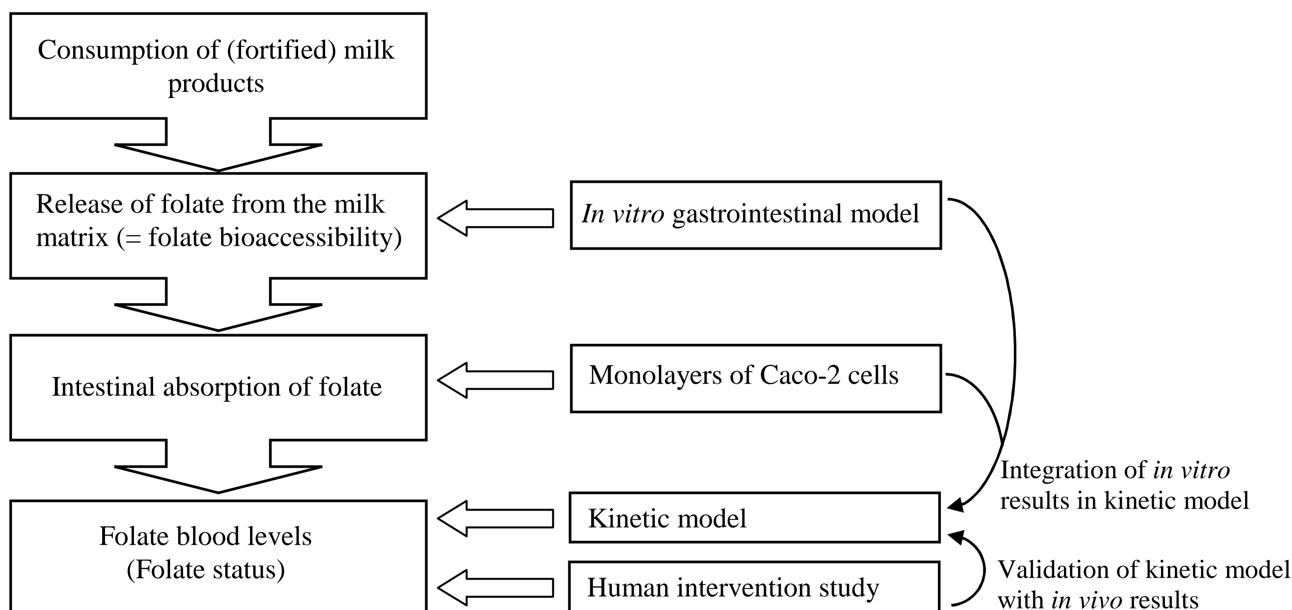


Figure 1.3 Determination of folate bioavailability following a step-wise approach.

Folate bioaccessibility

After the consumption of food products, folate can be released partly or totally from the food matrix due to digestion during passage through the gastrointestinal tract. The fraction of folate which is released from the food product in the intestinal lumen and becomes available for absorption is defined as the bioaccessible fraction. The bioaccessibility of folate from food products is influenced by the location within the food matrix and interaction with other (food and host) compounds in the lumen of the gastrointestinal tract. The food matrix can be altered due to food processing (e.g. cooking or chopping) which could enhance the bioaccessibility of folate.
In this thesis, an *in vitro* dynamic gastrointestinal model (TIM, Figure 1.4) has been used to study the bioaccessibility of folic acid and 5-CH_3-H_4folate from several products (46,47). The gastrointestinal 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 is surrounded by water at 37ºC to squeeze the walls, which ensures mixing of the food with the 'secreted' enzymes by peristaltic movements in the

gastrointestinal tract. The pH is continuously measured in the four compartments and regulated by addition of hydrochloric acid (stomach) or sodium bicarbonate (small intestine). The pH values, as well as the gastric emptying and small-intestinal passage of the food, are controlled according to pre-set curves based on literature information for human *in vivo* conditions. Gastric juice, with lipase and pepsin, and bile, pancreatic juice and electrolytes are gradually added into the gastric and duodenal compartment, respectively. The fractions which are released from the food matrix during gastrointestinal passage are collected after passage through semi-permeable hollow fibre membranes with a cut-off of 5 kDa connected to the jejunal and ileal compartments. The non-absorbed fractions are collected after passage through the ileo-coecal valve. Studies with several food compounds and food products showed a good correlation with *in vivo* data (48-51).

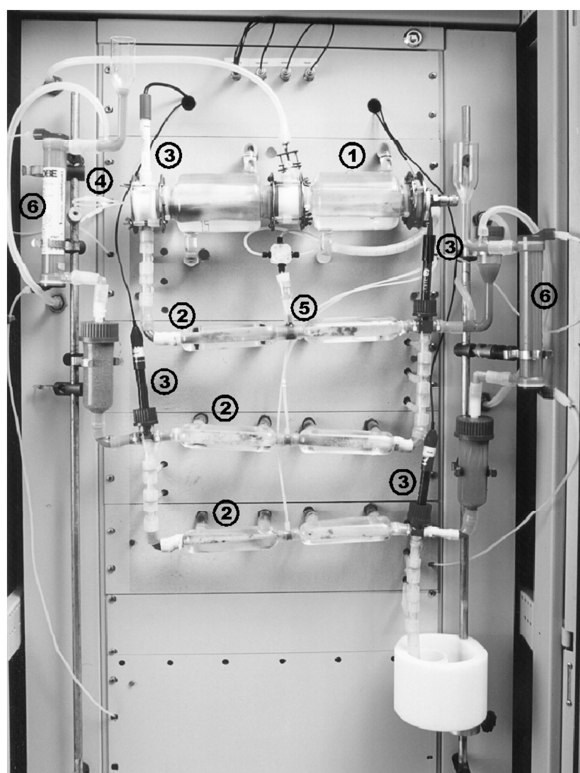


Figure 1.4
Dynamic *in vitro* gastrointestinal model (TIM).
1.gastric compartment;
2. small-intestinal compartments;
3. pH electrodes;
4. secretion of saliva, pepsin, gastric lipase, gastric acid;
5. secretion of bicarbonate, bile, pancreatic juice;
6. semi-permeable membranes

Intestinal absorption of folate

The bioaccessible fraction of dietary folate, which consists of monoglutamates or a combination of mono- and polyglutamates dependent on the food product, needs to be transported across the intestinal wall before reaching the blood. Folate polyglutamates require deconjugation to monoglutamates prior to intestinal absorption. This process can already take place during food processing by endogenous γ -glutamyl hydrolase (conjugase) in the food (52). The polyglutamates are further deconjugated in the small intestine by γ-glutamyl hydrolase, located in the mucosa cell brush border, and absorbed as monoglutamates by the enterocytes predominantly in the proximal small intestine (53). This thesis is focused on the intestinal absorption of the monoglutamates folic acid and 5-CH_3-H_4folate, which do not require deconjugation prior to intestinal absorption. Previous

research suggests that deconjugation of polyglutamates could be limiting for intestinal absorption of folate, but this research question is beyond the scope of this thesis.
The intestinal transport of folate has been characterized based upon many *in vitro* and *in vivo* studies with animals (mainly rats). The absorption of folate within physiological concentrations was found, at least partly, to occur by a pH-dependent, active, carrier-mediated system (54-57).
Folate is highly hydrophilic and, therefore, passive transcellular absorption is not expected (Figure 1.5, route A). Passive paracellular diffusion might contribute to the intestinal transport of folate, particularly at high concentrations of folate (> 10 μM) (Figure 1.5, route B). The carrier-mediated uptake of folate occurs via the reduced folate carrier (RFC), which functions as an anion exchanger (Figure 1.5, route C). The carrier can be located in both apical and basolateral cell membrane (58) and is found in nearly all cells. The folate receptor (FR) might also be involved in the absorption of folate from the intestinal lumen via receptor-mediated transport (Figure 1.5, route D). Folate receptors are structurally similar to the (s-)FBP found in milk and are also called membrane-associated FBP (mFBP). Receptor-mediated absorption is unidirectional and follows internalization of the receptor-folate complex by a process termed endocytosis (59,60). In normal tissues, the distribution of the receptor is limited to the apical membrane of some epithelial cells. High levels are found in placenta, kidney, choroids plexus, ovary and lung alveolar (61,62). Very low levels may be present in gut mucosal cells and, therefore, the involvement of the folate receptor in the absorption of folate from intestinal lumen is expected to be low. Next to these uptake mechanisms, efflux transporters (such as multi-drug resistance proteins (MRP)) could also contribute to the net transport of folic acid and 5-CH_3-H_4folate (Figure 1.5, route E) (63,64).

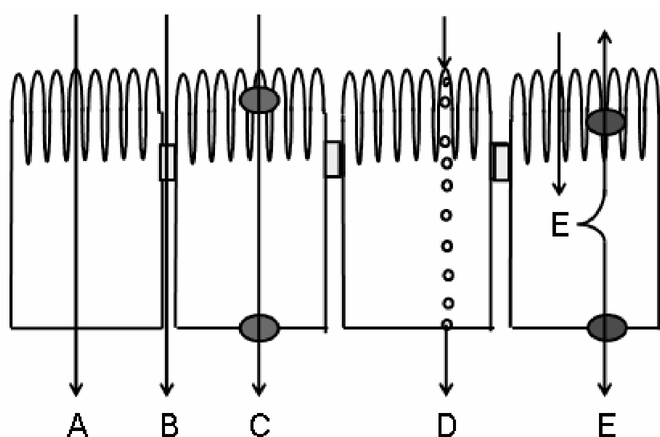


Figure 1.5

Transport mechanisms of the small intestine.
(A) Transcellular diffusion
(B) Paracellular diffusion
(C) Transcellular carrier-mediated transport
(D) Transcelullar endocytose
(E) Efflux transport with apical or basolateral located efflux pumps

In this thesis, human colon carcinoma (Caco-2) cells grown on semi-permeable inserts in a two-compartment transport system (Figure 1.6) were used to study the intestinal transport of folate. Caco-2 cells have been widely used as an *in vitro* model for human intestinal absorption as they display, after differentiation, both biochemical and morphological characteristics of small intestinal enterocytes (65-68). Also the permeability characteristics of compounds across Caco-2 monolayers were found to correlate well with *in vivo* absorption data in humans after an oral intake (69-71).

Figure 1.6.
Monolayers of Caco-2 cells grown on semi-permeable inserts in a two-compartment cell culture system.

Determinants of folate status

After absorption of folate from the intestinal lumen in the blood, folate is transported via the portal vein to the liver. The plasma concentrations of folate, which are found to be strongly dependent on the first-pass liver effect, are indicators of folate bioavailability from a certain food matrix. The absolute bioavailability of folate is difficult to establish in human studies. As a result, the bioavailability of folate from a certain food matrix is often determined in relation to a reference compound, mostly folic acid. In short-term intervention studies with human volunteers, folate levels in plasma or serum are used as indicator of the bioavailability of folate from the food source (72,73). Plasma or serum folate concentrations reflect recent dietary intake and is best evaluated when fasting measurements are taken repeatedly over time in the same individual. A steady-state plasma concentration is reached within 4 weeks of supplementation. In studies with an enlarged (a sufficiently long) intervention period (of at least 3 months), also the RBC folate level is indicative for the folate status of the volunteers (73). Thus, the RBC folate concentration is considered as an indicator of long-term status as it reflects the body stores. In addition, plasma homocysteine levels of the subjects are regularly used as a biomarker in a human intervention study. Changes in plasma total homocysteine concentrations, in response to a certain folate intake, can be used as a measure of functional bioefficacy (73).

In this thesis, serum folate levels after consumption of fortified milk products were measured *in vivo* in a human intervention study. Besides measurements of actual serum folate levels in a human study, serum folate levels were predicted with a computational kinetic model which integrates the results on folate bioaccessibility and intestinal transport obtained in *in vitro* studies with the gastrointestinal model and Caco-2 cells. The impact of different supplements and food products at various time points and concentrations can not be easily studied in a human study. The use of a kinetic model, in combination with *in vitro* studies, offers the possibility to test in a short time period many food products on their efficacy of enhancing the folate status of the population. In this thesis, a scientific strategy is presented in which many fortified milk products are tested in *in vitro* studies to predict *in vivo* plasma levels with kinetic modeling. Following this approach the most suitable milk matrix and supplement can be selected which might be incorporated in an alternative dietary strategy to enhance the folate status of the population. Based on *in vitro* studies and kinetic modeling, the optimal supplements and test conditions can be established for an efficient design for a human trail avoiding the need to perform multiple human intervention studies.

Aim and outline of the thesis

The objective of this thesis was to study the bioavailability of folate from fortified milk products, which will provide information about the suitability of the milk products for folate fortification. The bioavailability of folate from fortified milk products was investigated following five research questions:

1. What is the bioaccessibility of folic acid and 5-CH_3-H_4folate from fortified milk products and does it differ from the bioaccessibility of natural folate from unfortified food products?

The first step in the overall bioavailability process is the release of folate from the food matrix during gastrointestinal passage. The bioaccessibility of folic acid and 5-CH_3-H_4folate from fortified milk products was investigated using an *in vitro* model simulating human gastrointestinal conditions. Chapters 2, 3 and 4 describe studies in which the bioaccessibility of folic acid and 5-CH_3-H_4folate from fortified UHT milk, pasteurized milk, yoghurt and fermented milk (filmjölk) was studied. Chapter 4 describes studies in which unfortified food products (such as spinach, orange juice, beer and milk) varying in folate content, food matrix and processing were tested to get insight in the effect of the food matrix on the bioaccessibility of native folate. In addition, the bioaccessibility of supplemental folate (Chapters 2 to 4) was compared with that of native folate from unfortified foods (Chapter 4).

2. What is the effect of FBP on the bioaccessibility of folic acid and 5-CH_3-H_4folate from fortified milk products?

Folate occurs mainly bound to folate-binding proteins (FBP) in milk. In our *in vitro* studies, the stability of FBP, the extent of binding to FBP for folic acid and 5-CH_3-H_4folate and the bioaccessibility of folic acid and 5-CH_3-H_4folate from milk products with additional FBP was investigated during gastrointestinal passage. The effect of FBP on the bioaccessibility of folic acid and 5-CH_3-H_4-folate was studied by testing fortified milk products in absence and in presence of additional FBP (Chapters 2,3 and 4). FBP was added to the milk products to reach equimolar ratios between FBP and folic acid or 5-CH_3-H_4-folate similar to the ratio between FBP and folate in natural (unfortified) milk. The stability of FBP was measured in fortified pasteurized milk, UHT milk and yogurt during gastrointestinal passage (Chapters 2 and 3). Chapter 5 describes *in vitro* studies designed to study the binding characteristics of FBP for folic acid and 5-CH_3-H_4folate during gastric passage of fortified milk products.

3. Is there a difference in transport across human intestinal cells between folic acid and 5-CH_3-H_4folate?

This question was studied using human intestinal Caco-2 cells cultured as monolayers in a two-compartment system (Chapter 6). Next to this research question, Chapter 6 also evaluates the permeability of folic acid and 5-CH_3-H_4folate across the intestinal wall using reference compounds for low and high absorption. In general, the permeability characteristics of compounds across Caco-2 monolayers correlate well with human *in vivo* absorption data. The permeability rate of folic acid

and 5-CH_3-H_4folate across Caco-2 cells indicate whether the intestinal absorption is expected to be limiting for the overall bioavailability. Chapter 7 describes *in vitro* studies in which the mechanisms are investigated which are involved in the transport of folic acid and 5-CH_3-H_4folate across Caco-2 cells. Information about the mechanisms underlying the transport of folic acid and 5-CH_3-H_4folate may be helpful for the development of a dietary strategy with an optimal bioavailability of folate.

4. What is the effect of FBP on the intestinal absorption of folic acid and 5-CH_3-H_4folate?

The binding of folic acid and 5-CH_3-H_4folate to FBP in the intestinal lumen might affect the intestinal transport of both folate compounds. Whether FBP de- or increases the intestinal absorption of folic acid and 5-CH_3-H_4folate was studied using monolayers of Caco-2 cells as described in Chapter 6. Several molar ratios between folate and FBP were tested to measure whether FBP concentration-dependently affects the intestinal transport of folic acid and 5-CH_3-H_4folate. The binding to FBP of folic acid and 5-CH_3-H_4folate was measured at different molar ratios between FBP and folate to correlate the extent of binding to the effect of FBP on the intestinal absorption of both folate compounds.

5. Will the consumption of fortified milk products lead to an enhanced folate status in humans? And if so, which combination of milk product, FBP content and supplement is most effective in enhancing the plasma folate levels in humans?

In addition to the *in vitro* studies described in Chapters 2 to 7, a human intervention study was performed to investigate whether the consumption of folic acid-fortified milk leads to an enhanced folate status (Chapter 8). Plasma homocysteine levels and folate levels in serum and RBC were measured in subjects who during four weeks received UHT-treated milk or pasteurized milk with or without folic acid (400 µg/l). To answer the research question which combination of milk product, FBP content and supplement leads to the highest plasma response, the results from the *in vitro* and *in vivo* studies were integrated as discussed in Chapter 9. In the human intervention study only two types of fortified milk products could be tested, while a variety of fortified milk products were tested in the *in vitro* studies with the gastrointestinal model. To extrapolate the results from the *in vitro* studies on kinetics of folate release (Chapters 2 to 4) and absorption (Chapter 6) to the human situation *in vivo*, a kinetic model was used as described in Chapter 9.

References

1. Blakley, R.L. (1988) IUPAC-IUB Joint Commission on Biochemical Nomenclature (JCBN). Nomenclature and symbols for folic acid and related compounds. Recommendations 1986. J. Biol. Chem. 263: 605-607.
2. Wagner, C. (1995) Biochemical role of folate in cellular metabolism. In: Folate in health and disease, 23-42. Edited by Bailey, L., New York, Marcel Dekker, Inc.
3. Fishman, S.M., Christian, P., West, K.P. (2000) The role of vitamins in the prevention and control of anaemia. Public Health Nutr 3:125-150.
4. Wald, N., Sneddon, J., Frost, C., Stone, R. (1991) MRC Vitamin Study Group Prevention of neural tube defects. Results of the Medical Research Council Vitamin Study. Lancet 338: 131-137.
5. Daly, L.E., Kirke, P.N., Molloy, A., Weir, D.G., Scott, J.M. (1995) Folate levels and neural tube defects. J. Am. Med. Assoc. 247: 1698-1702.
6. Boushey, C.J., Beresford, A.A., Omen, G.S., Motulsky, A.G. (1996) A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. J. Am. Med. Assoc. 274: 1049-1057.
7. Graham, I.M., O'Callaghan, P. (2000) The role of folic acid in the prevention of cardiovascular disease. Curr. Opin. Lipidol. 11: 577-587.
8. Rampersaud, G.C., Bailey, L.B., Kauwell, G.P.A. (2002) Relationship of folate to colorectal and cervical cancer: review and recommendations for practitioners. J. Am. Diet Assoc. 102: 1273-1282.
9. Health council of the Netherlands. Dietary reference intakes: Vitamin B_6, folate and vitamin B_{12}. The Hague: Health Council of the Netherlands, 2003.
10. Van Oort, F.V.A., Melse-Boonstra, A., Brouwer, I.A., Clarke, R., West, C.E., Katan, M.B., Verhoef, P. (2003) Am. J. Clin. Nutr., 77, 1318-1323.
11. De Bree, A., Van Dusseldorp, M., Brouwer, I.A., Van het Hof, K.H., Steegers-Theunissen, R.P. (1997) Folate intake in Europe: recommended, actual and desired intake. Eur J Clin Nutr 51: 643-660.
12. Bailey, L.B. (1998) Dietary Reference Intakes for folate: the debut of dietary folate equivalents. Nutr Rev 56: 294-299.
13. Suitor, C.W., Bailey, L.B. (2000) Dietary folate equivalents: interpretation and application. J Am Diet Assoc 100: 88-94.
14. Konings, E.J.M., Roomans, H.H.S., Dorant, E., Goldbohm, R.A., Saris, W.H.M., van den Brandt, P.A. (2001) Folate intake of the Dutch population according to newly established liquid chromatography data for foods. Am. J. Clin. Nutr. 73: 765-776.
15. Bausch-Goldbohm, R.A., Hulshof, K.F.A., Brants, H.A., van den Berg, H., Bouman, M. The intake of folic acid by different population groups in the Netherlands before and after fortification of certain food. Zeist: 1995.
16. Gregory, J.F. (1996) Vitamins. In Food Chemistry. Ed. O.R. Fennema, Marcel Dekker New York, pp 531-616.
17. Gregory, J.F. (1989) Chemical and nutritional aspects of folate research: analytical procedures, methods of folate synthesis, stability and bioavailability of dietary folates. In advances in food and nutrition research, Academic press, vol.33, pp. 1-101.
18. Konings, E.J.M. (1999) A validated liquid chromatographic method for determining folates in vegetables, milk powder, liver and flour. J. AOAC Int. 82: 119-127.
19. Venn, B.J., Mann, J.I., Williams, S.M., Riddell, L.J., Chisholm, A., Harper, M.J., Aitken, W. (2002) Dietary counseling to increase natural folate intake: a randomized placebo controlled trial in free-living subjects to assess effects on serum folate and plasma total homocysteine. Am J Clin Nutr 76: 758-765

20. Brouwer, I.A., van Dusseldorp, M., West, C.E., Meyboom, S., Thomas, C.M.G., Duran, M., van het Hof, K.H., Eskes, T.K.A.B., Hautvast, J.G.A.J., Steegers-Theunissen, R.P.M. (1999) Dietary folate from vegetables and citrus fruit decreases plasma homocysteine concentrations in humans in a dietary controlled trial. J. Nutr. 129: 1135-1139.
21. Van der Pal de Bruin, K.M., de Walle, H.E.K., de Rover, C.M., Jeeninga, W., Cornel, M.C., de Jong van den Berg, L.T.W., Buitendijk, S.E., Paulussen, T.G.W.M. Influence of educational level on determinants of folic acid use. Paediatric and Perinatal Epidemiology, 17: 256-263.
22. De Walle, H.E.K., Cornel, M.C., de Jong van den Berg, L.T.W. Three years after de dutch folic acid campaign: Growing socioeconomic differences. Preventive Medicine. 35:65-69.
23. Honein, M.A., Paulozzi, L.J., Mathews, T.J., Erickson, J.D., Wong, L.Y. (2001) Impact of folic acid fortification of the US food supply on the occurrence of neural tube defects. J Am Med Assoc, 285, 2981-2986.
24. Ray, J.G., Meier, C., Vermeulen, M.J., Boss, S., Wyatt, P.R., Colem, D.E.C. (2002) Association of neural tube defect and folic acid food fortification in Canada. Lancet, 360, 2047-2048.
25. Bailey, L.B. Evaluation of a new recommended dietary allowance for folate (1992). J Am Diet Assoc, 92, 463-468, 471.
26. Venn, B.J., Green, T.J., Moser, R., McKenzie, J.E., Skeaff, C.M., Mann, J. (2002) Increases in blood folate indices are similar in women of childbearing age supplemented with [6S]-5-methyltetrahydrofolate and folic acid. J Nutr, 132, 3353-3355.
27. Venn, B.J., Green, T.J., Moser, R., Mann, J. (2003) Comparison of the effect of low-dose supplementation with L-5-methyltetrahydrofolate or folic acid on plasma homocysteine: a randomized placebo-controlled study. Am J Clin Nutr, 77, 658-662.
28. Lamers, Y., Prinz-Langenohl, R., Moser, R., Pietrzik, K. (2004) Supplementation with [6S]-5-methyltetrahydrofolate or folic acid equally reduces plasma total homocysteine concentrations in healthy women. Am J Clin Nutr, 79, 473-478.
29. Pentieva, K., McNulty, H., Reichert, R., Ward, M., Strain, J.J., McKillop, D.J., McPartlin, J.M., Connolly, E., Molloy, A., Krämer, K., Scott, J.M. (2004) The short-term bioavailabilities of [6S]-5-methyltetrahydrofolate and folic acid are equivalent in men. J Nutr, 134: 580-585.
30. Wigertz, K., Svensson, U.K., Jägerstad, M. (1997) Folate and folate-binding protein content in dairy products. J. Dairy Res. 64: 239-252.
31. Becker, W. (1994) Dietary habits and nutrient intake in Sweden 1989. Swedish National Food Administration, Livsmedelsverkets förlag, Uppsala, Sweden.
32. Ghitis, J. (1967) The folate binding in milk. Am. J. Clin. Nutr. 20: 1-4.
33. Salter, D.N., Scott, K.J., Slade, H., Andrews, P. (1981) The preparation and properties of folate-binding protein from cow's milk. Biochem. J. 193: 469-476.
34. Forssén, K.M., Jägerstad, M.I., Wigertz, K., Witthöft, C.M. (2000) Folates and dairy products: a critical update. J Am Coll Nutr 19: 100S-110S.
35. Wigertz, K., Hansen, S.I., Høier-Madsen, M., Holm, J., Jägerstad, M. (1996) Effect of milk processing on the concentration of folate binding protein (FBP), the folate binding capacity and the retention of 5-methyltetrahydrofolate. Int. J. Food Sci. & Nutr. 47: 315-322.
36. Ford, J.E. (1974) Some observations on the possible nutritional significance of vitamin B_{12}- and folate binding proteins in milk. Br. J. Nutr. 31: 243-257.
37. Tani, M., Iwai, K. (1984) Some nutritional effects of folate binding protein in bovine milk on the bioavailability of folate to rats. J. Nutr. 114: 778-785.

38. Colman, N., Hettiarachchy, N., Herbert, V. (1981) Detection of a milk factor that facilitates folate uptake by intestinal cells. Science 211: 1427-1429.
39. Salter, D., Blakeborough, P. (1988) Influence of goat's-milk folate-binding protein on transport of 5-methyltetrahydrofolate in neonatal-goat small intestinal brush-border-membrane vesicles. Br J Nutr 59: 497-507.
40. Said, H.M., Horne, D.W., Wagner, C. (1986) Effect of human folate binding protein on folate intestinal transport. Archiv Biochem Biophys 251: 114-120.
41. Tani, M., Fushiki, T., Iwai, K. (1983) Influence of folate binding protein from bovine milk on the absorption of folate in gastrointestinal tract of rat. Biochim Biophys Acta 757: 274-281.
42. Iwai, K., Tani, M., Fushiki, T. (1983) Electrophoretic and immunological properties of Folate-Binding Protein isolated from Bovine milk. Agric. Biol. Chem. 47: 1523-1530.
43. Salter, D.N., Mowlem, A. (1983) Neonatal role of milk folate-binding protein: studies on the course of digestion of goat's milk folate binder in the 6-days old kid. Br. J. Nutr. 50: 589-596.
44. Wigertz, K. (1997) Milk Folates. Characterisation and Availability. Doctoral Thesis, Lund University, Sweden.
45. Arkbåge K (2003) Vitamin B_{12} folate and folate-binding proteins in dairy products. Analysis, process retention and bioavailability. Doctoral Thesis, Swedish University of Agricultural Sciences, Uppsala, Sweden.
46. Minekus, M., Marteau, P., Havenaar, R., Huis in 't Veld, J.H.J. (1995) A multicompartimental dynamic computer-controlled model simulating the stomach and small intestine. ATLA 23: 197-209.
47. Minekus, M. (1998) Development and validation of a dynamic model of the gastrointestinal tract. PhD Thesis, University of Utrecht; Elinkwijk b.v., Utrecht, Netherlands.
48. Krul, C.A.M., Luiten-Schuite, A., Baan, R., Verhagen, H., Mohn, G., Feron, V., and Havenaar, R. (2000) Application of a dynamic *in vitro* gastrointestinal tract model to study the availability of food mutagens, using heterocyclic aromatic amines as model compounds. Food Chem. Toxicol. 38: 783-792.
49. Zeijdner, E.E. and Havenaar, R. (2000) The fate of orally administrated compounds during passage through the gastrointestinal tract simulated in a dynamic *in vitro* model (TIM). European Pharmaceutical Contractor Febr. issue: 76-81.
50. Krul, C.A.M (2001) Mutagenic and Antimutagenic activity of food compounds: Application of a dynamic in vitro gastrointestinal model. PhD Thesis, University of Utrecht. Febodruk BV, Enschede, The Netherlands.
51. Larsson, M., Minekus, M., and Havenaar, R. (1997) Estimation of the bio-availability of iron and phosphorus in cereals using a dynamic in-vitro gastrointestinal model. J. Sci. Food Agric. 73: 99-106.
52. Leichter, J., Landymore, A.F., Krumdieck, C.L., Klein, B.P., Kuo, C.H., Boyd, G. Folate conjugase activity in fresh vegetables and its effect on the determination of free folate content. Am J Clin Nutr 1979;32:92-95.
53. Halsted, C.H. The intestinal absorption of folates. Am J Clin Nutr 1979; 32:846-855.
54. Said, H.M., Grishan, F.K., Murrell, J.E. (1985) Ontogenesis of the intestinal transport of 5-methyltetrahydrofolate in the rat. Am. J. Physiol. 249, G567-571.
55. Said, H.M., Strum, W.B. (1983) A pH-dependent, carrier-mediated system for transport of 5-methyltetrahydrofolate in rat jejunum. J. Pharmacol. Exp. Ther. 226: 95-99.
56. Selhub, J., Powell, G.M., Rosenberg, I.H. (1984) Intestinal transport of 5-methyltetrahydrofolate. Am.J.Physiol. 246: G515-G520.

57. Said, H.M., Grishan, F.K., Redha, R. (1987) Folate transport by human intestinal brush-border membrane vesicles. Am.J.Physiol. 252: G229-G236.
58. Dudeja, P.K., Kode, A., Alnounou, M., Tyagi, S., Torania, S., Subramamian, V.S., Said, H.M. (2001) Mechanism of folate transport across the human colonic basolateral membrane. Am J Physiol Gastrointest Liver Physiol, 281: G54-G60.
59. Birn, H., Selhub, J., Christensen, E.I. (1993) Internalization and intracellular transport of folate binding protein in rat kidney proximal tubule. Am. J. Physiol. 264, C302-C310.
60. Verma R.S., Gullapalli, S., Antony, A.C. (1992) Evidence that the hydrophobicity of isolated, in situ, and de novo-synthesized native human placental folate receptors is a function of glycosyl-phosphatidylinositol anchoring to membranes. J. Biol. Chem., 267 (6), 4119-4127.
61. Van Hoozen, C.M, Ling, E., Halsted, C.H. (1996) Folate binding protein: molecular characterization and transcript distribution in pig liver, kidney and jejunum. Biochem. J., 319, 725-729.
62. Wagner C. Cellular Folate binding proteins; function and significance. Ann Rev Nutr, 1982, 229-248.
63. Assaraf, Y.G., Rothem, L., Hooijberg, J.H., Stark, M., Ifergan, I., Kathman I., Dijkmans, B.A.C., Peters, G.J., Jansen, G. (2003) Loss of Multidrug resistance protein 1 expression and folate efflux activity results in a highly concentrative folate transport in human leukaemia cells. J Biol. Chem. 278 (9), 6680-6686.
64. Zeng, H., Chen, Z.S., Belinsky, M.G., Rea, P.A., Kruh, G.D. (2001) Transport of methotrexate (MTX) and folate by multidrug resistance protein (MRP)3 and MRP1: effect of polyglutamation on MTX transport. Cancer Research, 61, 7225-7232.
65. Hidalgo, I.J., Raub, T.J., Borchardt, R.T. (1989) Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. Gastroenterology 96: 736-749.
66. Hillgren, K.M., Kato, A., Borchardt, R.T. (1995) In vitro systems for studying intestinal drug absorption. Med Res Rev 15: 82-109.
67. Vincent, M.L., Russell, R.M., Sasak, V (1985) Folic acid uptake characteristics of a human colon carcinoma cell line Caco-2. A newly described cellular model for small intestinal epithelium. Human Nutrition: Clinical Nutrition 39C: 355-360.
68. Duizer, E., Penninks, A.H., Stenhuis, W.H., Groten, J.P. (1997) Comparison of permeability characteristics of the human colonic Caco-2 and rat small intestinal IEC-18 cell lines. J. Contr. Rel. 49: 39-49.
69. Artusson, P., Karlsson, J. (1991) Correlation between oral drug absorption in humans and apparent drug permeability coefficients in human intestinal epithelial (Caco-2) cells. Biochem Biophys Res Com 175: 880-885.
70. Yazdanian, M., Glynn, S.L., Wright, J.L., Hawi, A. (1998) Correlating partitioning and Caco-2 cell permeability of structurally diverse small molecular weight compounds. Pharm. Res. 15: 1490-1494.
71. Yee, S. (1997) In vitro permeability across Caco-2 cells (colonic) can predict in vivo (small intestinal) absorption in man – fact or myth. Pharm. Res. 14: 763-766.
72. Gregory, J.F. Case study: folate bioavailability. J. Nutr. 131: 1376S-1382S (2001).
73. Brouwer, I.A., van Dusseldorp, M., West, C.E., Steegers-Theunissen, R.P.M. (2001). Bioavailability and bioefficay of folate and folic acid in man. Nutr.res.rev. 14, 267-293.

2

Folic acid and 5-methyltetrahydrofolate in fortified milk are bioaccessible as determined in a dynamic in vitro gastrointestinal model

Journal of Nutrition 133: 2377-2383, 2003

Miriam Verwei[1,2]
Karin Arkbåge[3]
Robert Havenaar[2]
Henk van den Berg[4]
Cornelia Witthöft[3]
Gertjan Schaafsma[1,2]

[1]TNO Nutrition and Food Research, Zeist, The Netherlands
[2]Department of Human Nutrition, Wageningen University, Wageningen, The Netherlands
[3]Department of Food Science, Swedish University of Agricultural Sciences, Uppsala, Sweden
[4]The Netherlands Nutrition Centre, Den Haag, The Netherlands

Abstract

Dairy products are a potential matrix for folate fortification to enhance the 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 (TIM). We used both Ultra High Temperature (UHT)-processed milk and pasteurized milk, differing in endogenous FBP content, fortified with folic acid or 5-CH_3-H_4folate. 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 quantifying their folate and FBP content. The folate bioaccessibility from folic acid-fortified milk products without additional FBP was 58-61%. This was significantly lower ($P<0.05$) as compared to that of the 5-CH_3-H_4folate-fortified milk products (71%). Addition of FBP significantly reduced ($P<0.05$) the 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 content in the folic acid and 5-CH_3-H_4folate-fortified milk products after gastrointestinal passage was 13-16% and 0-1%, respectively, of the starting amounts subjected to TIM. In conclusion, milk seems to be a suitable carrier for folate fortification as both folic acid and 5-CH_3-H_4folate 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 inhibits the folic acid bioaccessibility from milk in this model.

Introduction

Prevention of neural tube defects and lowering serum homocysteine, an emerging risk factor for vascular disease, can be achieved by an adequate folate intake (1-5). A limiting factor, however, is the bioavailability of folate from various food sources. Research on folate bioavailability has strongly focused on folate-rich vegetables and citric fruits and on deconjugation of polyglutamates as a limiting step in digestion and absorption (6-8). Dairy products should also be considered as an interesting food category for folate absorption studies and as a potential matrix for folate fortification, as milk might enhance the folate bioavailability from the diet (9,10). Although the natural folate content in milk is low compared to folate-rich food products, in some European countries with a high milk consumption, such as the Netherlands (11) and Sweden (12), milk is responsible for 10-15% of the daily folate intake.

The naturally occurring form of folate in unprocessed milk, 5-methyltetrahydrofolate (5-CH_3-H_4folate), is bound to Folate Binding Proteins (FBP) (13,14) and present as a mixture of mono- and polyglutamates (11,15). The role of FBP in human gastrointestinal folate transport and absorption is unclear. It has been speculated that FBP protects folate from bacterial uptake and degradation (16,17) and/or could enhance folate absorption by mucosal cells (18,19). However, this enhancement was not found in rat studies (20,21), which showed that FBP reduced the folate absorption. In this rat study (20) it was also found that under acidic conditions in the stomach folic acid is released from FBP, but recombines after reaching the intestine. This is in line with the observation that the dissociation of folate occurs at pH of approximately 5 and lower (22).

Folate bioavailability has in many studies been assessed on basis of the plasma folate response after oral doses (meal) or in rat bioassays. These studies, however, do not allow investigating the separate processes occurring during *in vivo* gastrointestinal passage. An alternative method is an *in vitro*, dynamic, computer-controlled gastrointestinal model (TIM, (23,24)). This model has been 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 bio-active compounds (25-28). The bioaccessible folate corresponds to the folate fraction, which is released from the food matrix and available for absorption in the small intestine.

In this report we want to give answers to 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-CH_3-H_4folate 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 Ultra High 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-CH_3-H_4folate, folic acid was also used as fortification as this synthetic compound is used in supplements and folate-fortified food products. Next 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% 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 down to 7°C. The 5-CH_3-H_4folate-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-CH_3-H_4folate (sodium salt) to fortify the milk products were a gift from Eprova (Schaffhausen, Switzerland). The stock solutions of folic acid and 5-CH_3-H_4folate for folate fortification were calibrated spectrophotometrically at wavelengths 283 nm (ε = 27.6 µmol/mL.cm) and 290 nm (ε = 31.7 µmol/mL.cm), respectively, to check their purity (30). The folate fortification was performed by the addition of 142 µL folic acid (1.02 mg/mL) or 178 µL 5-CH_3-H_4 folate (0.81 mg/mL) as stock solutions (in 0.1 mol/L 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 sodium ascorbate was added to the 360 g of milk (containing 7.2 mg natural ascorbic acid (31)). The FBP fortification was aimed for a molar ratio of folate:FBP of 1:1 as 1 mol of FBP binds 1 mol of folate at pH 7.2 (32,33). FBP was added as 0.432 g concentrated FBP-rich whey powder (735 µmol 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 (TIM)

The TIM system (Figure 2.1) 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 37ºC 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 pre-set curves based on literature information for human *in vivo* conditions (23,24). Artificial oral fluid and gastric juice with lipase (150.000 U/g, *Rhizopus* lipases F-AP 15, Amano Pharmaceuticals, Nagoya, Japan) and pepsin (2200 U/mg, Sigma, P7012) were gradually added into the gastric compartment (24,28). Bile (porcine-bile extract, Sigma, P8631), 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 semi-permeable hollow fiber membranes with a cut-off of 5 kDa (Cobe Hemophan Hemodialyzers, Hospal GmbH, Germany), which mimic the absorption of digested products and water (Figure 2.1, points N, O and P). The non-absorbed fractions were collected after passage through the ileo-coecal valve as 'ileal delivery' (Figure 2.1, point H).

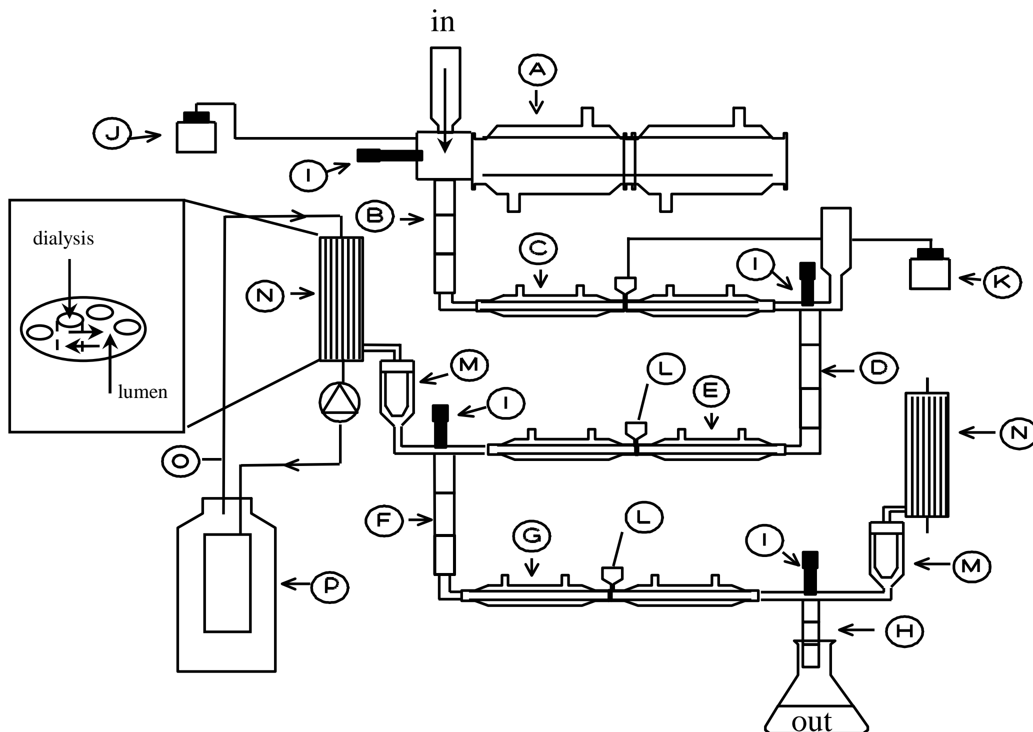


Figure 2.1. Schematic diagram of the dynamic *in vitro* gastrointestinal model (TIM): A. gastric compartment; B. pyloric sphincter; C. duodenal compartment; D. peristaltic valve; E. jejunal compartment; F. peristaltic valve; G. ileal compartment; H. ileo-caecal valve; I. pH electrodes; J. gastric secretion bottles with acid and enzymes; K. duodenal secretion bottles with bile, pancreatin, bicarbonate; L. secretion of bicarbonate to control the intestinal pH; M. pre-filter system; N. hollow fiber semi-permeable membrane system; O. water absorption system; P. closed dialyzing system.

TIM experiment with milk products

In preliminary experiments we established that folate recoveries of approximately 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 five-hour lasting 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. It was found that

both mono- and polyglutamates could pass the membranes. As the membranes do not discriminate between mono- and polyglutamates, the incorporation of conjugase 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 2.1) were tested in duplicate for folate bioaccessibility in TIM. Appropriate software and working protocol were used in order 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 semi-permeable hollow fiber membranes (Figure 2.1, point N) connected to the jejunal and ileal compartments. Total ileal delivery (Figure 2.1, point H) was collected over a period of 0-5 h. The dialysate contained the 'absorbable' (bioaccessible) fraction, while the ileal delivery material corresponds to the 'non-absorbable' (non-bioaccessible) fraction. After ending the experiment (after 5 h), the residues from the compartments of the stomach, duodenum, jejunum and ileum were analyzed to calculate the mass balance of folate. Directly after sampling, sodium-ascorbate (10 g/L) was added to all samples for preservation reasons. After mixing the samples were stored directly at minus 20˚C until folate analysis by HPLC.

Table 2.1. Characteristics of the milk products tested in the *in vitro* gastrointestinal model

Number	Milk product	Folate fortification	Folate (nmol/L)[1,2]	FBP fortification	FBP (nmol/L)[1,3]
1	UHT	Folic acid	834 ± 5	-	0
2	UHT	Folic acid	834 ± 5	+	919 ± 74
3	Pasteurized	Folic acid	816 ± 25	-	140 ± 2
4	Pasteurized	Folic acid	816 ± 25	+	1135 ± 32
5	Pasteurized	5-CH_3-H_4folate	901 ± 78	-	157 ± 11
6	Pasteurized	5-CH_3-H_4folate	812 ± 4	+	1223 ± 1

[1] Values are means ± range, n=2, [2] Quantified by HPLC. 1-4: folic acid concentration; 5,6: natural 5-CH_3-H_4folate concentration of ca. 65 nmol/L plus 5-CH_3-H_4folate fortification, [3] Quantified by ELISA.

Folate analysis

A HPLC method was set up to analyze the individual folate compounds, folic acid and 5-CH_3-H_4folate. Folates were determined by RP-HPLC (Waters 2690, Waters Corporation, Milford, USA) with UV (photodiode array detector, monitored at 290 nm) and fluorescence (excitation 290 nm, emission 360 nm) detection similar to the method of Konings (35). An inertsil 5 OD-3 column (2x10 cm, 3 mm id., Varian CP28308) was used in combination with a Chromsep guard column (10x2 mm, Varian CP28141). Gradient elution with 0.033 mol/L phosphate buffer (pH 2.1) and acetonitrile (HPLC-S grade) were used to separate folic acid and 5-CH_3-H_4folate. The start gradient was a mixture of 95% phosphate buffer and 5% acetonitrile as described in Konings (35). A flow of

0.4 mL/min was used and the time between 2 consecutive injections was 45 min. Calibration curves (five points) of folic acid and 5-CH_3-H_4folate were used for calculation of the concentrations.
All analytical steps including quantification were performed on one day under subdued light to reduce folate degradation. In each set of samples to be analyzed, two spiked samples were included to measure the recoveries of folic acid and 5-CH_3-H_4folate from the food, dialysate and ileal delivery samples.
Before quantification with HPLC the samples were extracted, deconjugated, cleaned and concentrated. The samples, analyzed as duplicates, were diluted 1:2 (v/v) with extracting buffer (0.1 mol/L phosphate buffer, pH 6.1, with 10 g/L ascorbic acid) for 12 min in a boiling water bath. After extraction the pH was adjusted to 4.5-5.0 with 1.5 mol/L H_3PO_4 and the samples were deconjugated with 200 µL human plasma conjugase (Sigma, P9523) for 3.5 h at 37°C in a waterbath with shaking device. Directly after centrifuged for 15 min at 3000 x g and 4ºC, 40-50 mL of the supernatants were cleaned and concentrated using FBP affinity columns. The FBP affinity columns were prepared according to Konings (35) with FBP from Scripps Laboratories (San Diego, USA) and Affi-Gel 10 Gel from Bio-Rad Laboratories (Herculus, USA). The folate compounds were consequently eluted in 5 mL elution solution (0.02 mol/L trifluoroacetic acid - 0.02 mol/L dithioerytritol) resulting in an increase of folate concentration before HPLC analysis. To check if 50 mL was not exceeding the maximum capacity volume of the FBP affinity columns, we added a mixture of folate compounds (1 µg of folic acid, 5-CH_3-H_4folate, 5-HCO-H_4folate and H_4folate) to different volumes of TIM jejunal and ileal dialysate (Table 2.2).

Table 2.2. The recovery of folate compounds (1 µg of folic acid, 5-CH_3-H_4folate, 5-HCO-H_4folate and H_4folate), which were added as a mixture to different volumes of TIM jejunal and ileal dialysate and eluted over the FBP affinity columns.

TIM dialysate (mL)	Recovery of folate standards (%)[1]			
	Folic acid	5-CH_3-H_4folate	5-HCO-H_4folate	H_4folate
0[2]	108 ± 2	102 ± 2	79 ± 4	94 ± 2
5	102 ± 2	100 ± 4	39 ± 1	90 ± 1
10	104 ± 1	100 ± 1	37 ± 0	78 ± 3
15	104 ± 0	103 ± 0	29 ± 2	70 ± 0
25	107 ± 1	102 ± 1	31 ± 1	61 ± 1
50	102 ± 1	102 ± 0	14 ± 0	44 ± 2

[1] Values are means ± range, n=2, [2] 5 mL 0.1 mol/L phosphate buffer instead of TIM dialysate

FBP analysis

FBP was quantified by a two-site enzyme-linked immunosorbent assay (ELISA) according to Høijer-Madsen et al. (36). The antibody against FBP from bovine milk (Rabbit anti-bovine FBP 24740) was obtained from the State Serum Institute (Copenhagen, Denmark) and the FBP calibrant from the Central Hospital (Hillerød, Denmark). Briefly, to 3 g of TIM sample 0.09 g of Triton X-100 was added. The sample was put on a shaking device, incubated for 45 min at room temperature

and thereafter diluted to a concentration of 0.4 nmol FBP/L and applied to a microtiter plate. An eight-point calibration curve from 0.002-1.1 nmol FBP/L was prepared and included in each ELISA run. The ELISA quantification procedure is described in detail elsewhere (37). The in-house reference material, wpc 65, was included into every analysis. The coefficient of variation between runs did not exceed 15%.

Calculations and statistics

The recovery (mass balance) of folate was calculated by the formula:

$Recovery\ (\%) = ((Folate_{dialysate}+Folate_{ileal\ delivery}+Folate_{residues})/(Folate_{food}+Folate_{endogenous})) * 100$

where, $Folate_{dialysate}$ is the folate content in jejunal plus ileal dialysate, $Folate_{ileal\ delivery}$ is the folate content in the total material collected behind the ileocaecal valve, $Folate_{residues}$ is the folate content in the residues collected from the TIM system after ending the experiment, $Folate_{food}$ is the folate content in the test portion of the milk product and $Folate_{endogeneous}$ is the folate content in the bile and pancreatic solutions secreted into the duodenal compartment (Figure 2.1, point K).

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

$Folate\ bioaccessibility\ (\%) = Folate_{dialysate}/(Folate_{food}+Folate_{endogenous})*100.$

The data were analyzed by ANOVA, after Barlett's test for testing the homogenicity of the variances. ANOVA was performed to test the overall difference between foods. When an overall significance was found, pairwise tests on individual means were performed using the least squares means method of SAS. P-value < 0.05 was considered as significant.

Results

The six different milk products contained 360-412 µg folate/L. This was close to the aimed concentration of 400 µg/L. In the FBP-fortified milk products, the aimed ratio of folate:FBP fortification of ca 1:1 on molar basis was reached (Table 2.1).

The TIM and food samples were analyzed by HPLC after cleaning/concentrating by FBP affinity columns. The column capacity was tested and recoveries equal or above 100% were obtained for folic acid and 5-CH_3-H_4folate with dialysate samples up to 50 mL (Table 2.2). For H_4folate and 5-HCO-H_4folate recoveries were incomplete and lower with increasing sample volume. As the milk products contained mainly folic acid and 5-CH_3-H_4folate, this lower recovery of the other folates was not considered a problem. The spiked samples, which were included in each set of samples analyzed on one day, gave analytical recoveries of approximately 100% for both folic acid and 5-CH_3-H_4folate.

In the HPLC chromatograms of the TIM samples only the parent (added) compounds were found, with no other peaks than folic acid and 5-CH_3-H_4folate. This indicates that no other metabolites were formed during the digestive process. In the folic acid-fortified milk a small 5-CH_3-H_4folate peak was observed corresponding to the natural 5-CH_3-H_4folate content of 25-35 µg/L. Also in some dialysate samples of the experiments with folic acid-fortified milk products a small 5-CH_3-H_4folate peak was visible. This peak was not exceeding the above mentioned natural 5-CH_3-H_4folate content in the milk, indicating no conversion of folic acid into 5-CH_3-H_4folate during

gastro-intestinal passage. For this reason, only the bioaccessibility of the parent compounds (folic acid or 5-CH_3-H_4folate from the folic acid- or 5-CH_3-H_4folate-fortified milk products, respectively) are given (Table 2.3). The bioaccessibility of folic acid in total dialysate (jejunal plus ileal) was 61 ± 2% from folic acid-fortified UHT milk and 58 ± 1% from folic acid-fortified pasteurized milk (no significant difference). However, the folate content was significantly lower ($P < 0.05$) in the jejunal dialysate and significantly higher ($P < 0.05$) in the ileal dialysate from pasteurized milk as compared with UHT milk. The FBP fortification of both milk products lowered significantly ($P < 0.05$) the bioaccessible fractions of folic acid from both UHT milk and pasteurized milk, to 51 ± 3% and 44 ± 2%, respectively. As a result the folic acid content in the ileal delivery was enhanced in both FBP-fortified milk products. The folate bioaccessibility from 5-CH_3-H_4folate-fortified pasteurized milk (71 ± 2%) was found to be significantly higher ($P < 0.05$) compared to the folic acid-fortified pasteurized milk (58 ± 1%). The addition of FBP to 5-CH_3-H_4folate-fortified pasteurized milk had no effect on the folate bioaccessibility.

Table 2.3. Folate content in jejunal dialysate, ileal dialysate, jejunal + ileal dialysate (=bioaccessible fraction) and ileal delivery (=non-bioaccessible fraction) samples collected between 0-5 h from the in vitro gastrointestinal model.

Milk product	Folate (as % of intake)			
	Jejunal dialysate	Ileal dialysate	Jejunal plus ileal dialysate	Ileal delivery
UHT milk +folic acid	48.4 ± 1.2^{a}	12.9 ± 1.0^{a}	61.2 ± 2.2^{a}	6.9 ± 0.8^{a}
UHT milk +folic acid+FBP	$38.6 \pm 2.1^{a,b}$	11.9 ± 0.6^{a}	50.5 ± 2.7^{b}	$22.0 \pm 2.9^{b,c}$
Past. milk+folic acid	37.1 ± 4.3^{b}	21.2 ± 3.7^{b}	58.3 ± 0.6^{a}	$16.5 \pm 5.2^{a,b}$
Past. milk+folic acid+FBP	34.1 ± 0.7^{b}	9.8 ± 1.2^{a}	43.9 ± 1.9^{b}	29.0 ± 4.9^{c}
Past. milk+5-CH_3-H_4folate	61.4 ± 3.1^{c}	9.3 ± 1.7^{a}	70.8 ± 1.5^{c}	4.3 ± 0.2^{a}
Past. milk+5-CH_3-H_4folate+FBP	60.3 ± 3.6^{c}	11.7 ± 0.6^{a}	72.0 ± 3.1^{c}	6.0 ± 4.2^{a}

[1] Values are means ± range, n = 2, [2] Means in column without at common letter differ significantly ($P<0.05$).

The endogenous folate content, $Folate_{endogenous}$, in bile and pancreatic juice was 1.3-1.4% of the folate in the food ($Folate_{food}$). Approximately 10% of the folate intake ($Folate_{food}$ + $Folate_{endogenous}$) was found as residue ($Folate_{residues}$) in the TIM system at the end of the experiments. Dividing the folate content in the TIM samples by the folate intake gave recoveries of 80-90% in the 12 experiments performed. No difference was found between folic acid and 5-CH_3-H_4folate concerning stability during passage through TIM (Table 2.3). The differences in folate bioaccessibility between the six milk products could therefore not be clarified by differences in total recoveries.

The kinetic profile of folate bioaccessibility was measured by collecting samples from jejunal and ileal dialysate during 0-1, 1-2, 2-3 and 3-5 h after starting the experiment. The maximum concentration of folic acid and 5-CH_3-H_4folate in jejunal dialysate was found in the samples collected between 1-2 h and in the ileal dialysate between 1-3 h (Figure 2.2).

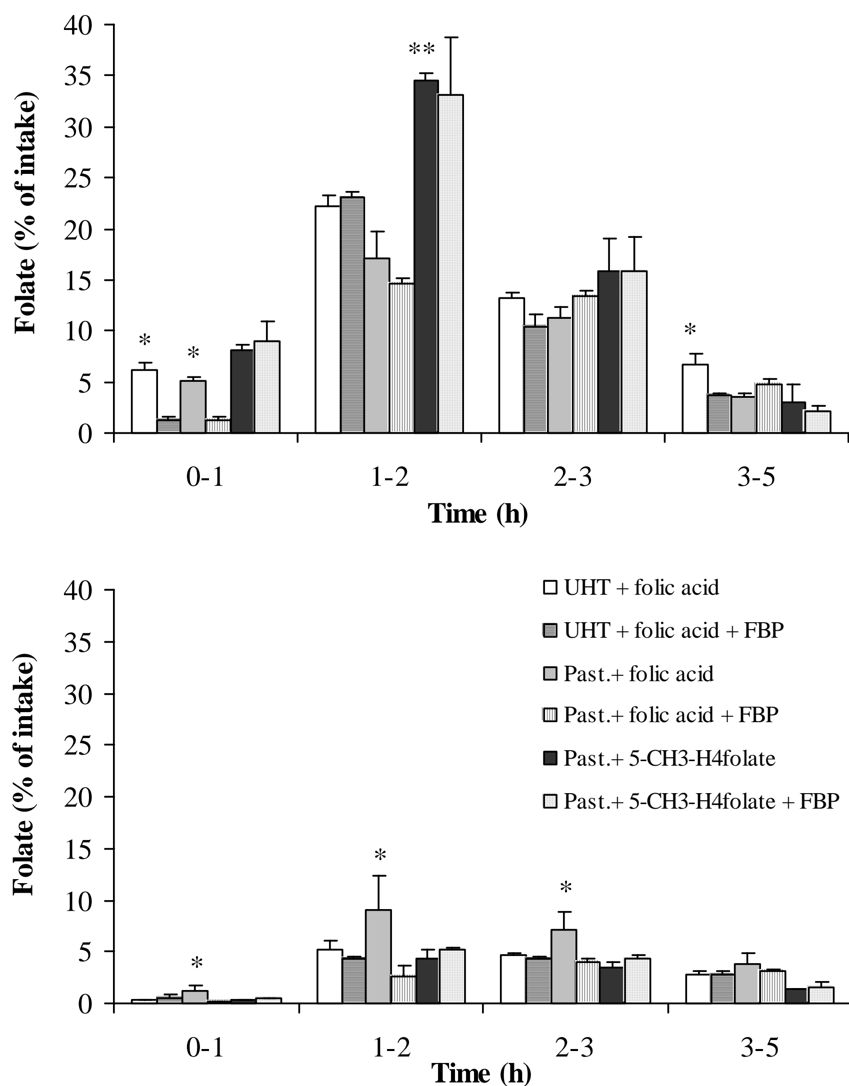


Figure 2.2. Folate content, as percentage of intake, in jejunal dialysate (upper panel) and ileal dialysate (lower panel) collected during 0-1, 1-2, 2-3, 3-5 h in UHT milk + folic acid, UHT milk + folic acid + FBP, pasteurized milk + folic acid, pasteurized milk + folic acid +FBP, pasteurized milk + 5-CH_3-H_4folate and pasteurized milk + 5-CH_3-H_4folate + FBP. The values are means ± range, n=2. * $P < 0.05$, UHT or pasteurized milk compared to FBP-fortified UHT or pasteurized milk, respectively, within one time interval. ** $P < 0.05$, 5-CH_3-H_4folate-fortified pasteurized milk compared to folic acid-fortified pasteurized and UHT milk within time interval 1-2h.

The analyzed FBP content decreased after passage through the TIM system. As expected, no FBP was found in the dialysate, as the molecular size of intact FBP is larger than the cut-off of the dialysate membrane. After folic acid fortification, 13-16% of the FBP naturally present in pasteurized milk and the FBP added to pasteurized and UHT milk was recovered in the ileal delivery. In the 5-CH_3-H_4folate-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 bioavailability of dietary folates varying between 40-70% 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_4folate leads to higher folate bioaccessibility (~70%) ($P < 0.05$) as compared to fortification with folic acid (~60%, Table 2.3). The higher bioaccessibility of 5-CH_3-H_4folate is mainly a result of the significant higher ($P < 0.05$) folate content found in the jejunal dialysate collected between 1-2 h (Figure 2.2). 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_4folate compared to folic acid at the pH range 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, no effect of FBP was found on the 5-CH_3-H_4folate bioaccessibility, while the inhibiting effect of added FBP on the bioaccessibility of folic acid was 11-14% (Table 2.3). In Figure 2.3, the level of FBP as measured in the folic acid-fortified milk products (Table 2.1, products 1-4), is plotted against the bioaccessibility of folic acid from the fortified UHT and pasteurized milk products. It appears that the FBP content of the milk products is inversely related with the bioaccessible folic acid fractions.

FBP did not affect 5-CH_3-H_4folate bioaccessibility; this could indicate that the 5-CH_3-H_4folate-FBP complex is less stable in the intestine than the folic acid-FBP complex. The results point out that after gastrointestinal passage the retention of FBP from the folic acid-fortified milk products was 13-16%. But, only 0-1% FBP retention was found after passage of 5-CH_3-H_4folate-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 non-fortified milk products, which could implicate the presence of a non-degradable 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 can not answer the question if the FBP-bound folate is absorbed or not, since there is no corresponding folate receptor system involved 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 the *in vivo* situation, especially during the neonatal period (9,16-21,42,43). However, some results are contradictory as 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), while Tani et al (20) and Said et al. (21) found a lower jejunal and equal ileal transport in rats of FBP-bound folate compared to 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 also more gradually and slowly. It is suggested that a slower absorption rate prevents the occurrence of high plasma folate levels, which could promote rapid excretion by the kidneys (17).

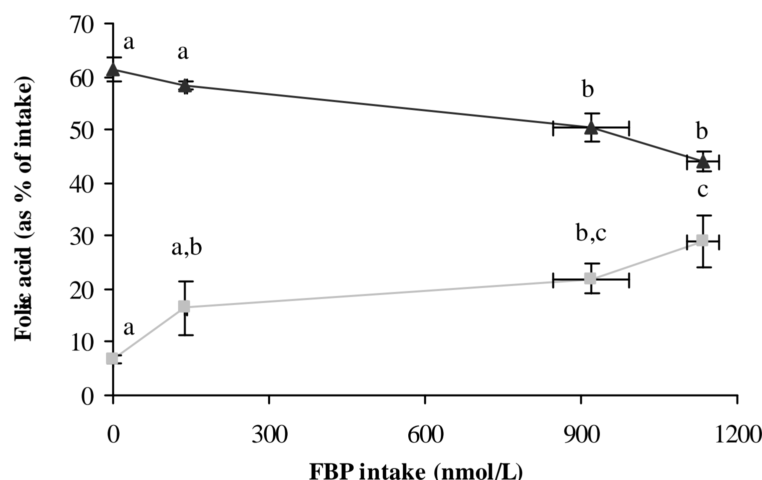


Figure 2.3. Folic acid content, as percentage of intake, in the bioaccessible fraction (jejunal plus ileal dialysate) (solid square) and non-bioaccessible fraction (ileal delivery) (solid triangle) collected during 0-5 h from the UHT milk + folic acid, UHT milk + folic acid + FBP, pasteurized milk + folic acid, and pasteurized milk + folic acid +FBP as tested in a gastrointestinal model (TIM), plotted against the FBP concentration in the folic acid-fortified milk products (intake). The values are means ± range, n = 2. Means without a common letter, within the bioaccessible and non-bioaccessible fractions, respectively, differ significantly ($P < 0.05$).

The kinetic pattern, i.e. the appearance of folate in the dialysate fractions, appeared to be different for the six milk products (Figure 2.2). This could be due to the amount of protein (such as FBP) in the food. Folate compounds could occur bound to proteins, which results in a slower release of folate from the food matrix. Overall, similar trends in folate bioaccessibility between the six milk products were found between 0-1 and 0-5 hour. These trends are a decrease in folic acid bioaccessibility in case of FBP enrichment, a higher folate bioaccessibility in case of 5-CH_3-H_4folate fortification and no difference in 5-CH_3-H_4folate bioaccessibility between FBP-fortified and non-fortified products. Within the first hour after milk consumption about half of the stomach content was delivered to the intestine. So, it seems that incubation of the food at 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 2.1, products 1 and 3), which showed in the TIM system a high bioaccessibility, were also studied in a human intervention study (de Jong *et al*, unpublished data). The results indicate also a high bioavailability, because an extra dose of 200 µg of folic acid added to milk, given daily for four weeks, increased significantly serum folate and red blood cell folate concentrations. No significant difference in folate level was found between the folic acid-fortified UHT and pasteurized milk, indicating that the small amount of endogenous FBP in the pasteurized milk has no significant effect on folate bioavailability. In a study with ileostomists (10), who consumed daily for three weeks, milk products either with or without endogenous FBP together with a standardized diet, no difference in total 5-CH_3-H_4folate excretion in the ileostomal effluent was found between the two groups, indicating similar absorption. These

results are in line with our *in vitro* study as we also found no effect of endogenous FBP on the folic acid content in dialysate and ileal delivery. No human studies are published about the effect of added FBP on the bioavailability of folic acid and 5-CH_3-H_4folate. However, the relation as we found in our study (Figure 2.3) between folic acid bioaccessibility and FBP was also found in a folate bioavailability study performed on rats (9). The relative folate bioavailability from diets containing human, bovine or goat milk were compared with a milk-free diet. Folic acid was added to the milk-free and milk-containing diets to obtain 0, 200, 400 or 600 µg folic acid/kg diet. The dose-response curves for plasma folate showed an enhancement of folate bioavailability in case human or bovine milk was incorporated, but it seemed that a milk factor other than FBP caused this enhancement, as goat milk, with the highest FBP content, 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 folate fortification as folate was easily released from the matrix and highly available for absorption (60-70%). A small but statistically significant difference was found in the bioaccessibility of folic acid (60%) and of 5-CH_3-H_4folate (70%). Concerning FBP fortification, our *in vitro* data suggest that additional FBP decrease the bioaccessibility of folic acid, in contrast to 5-CH_3-H_4folate, from milk products. Therefore, FBP seems not beneficial for enhancement of folate bioavailability. The TIM system can be considered as a good methodology for nutritional science for evaluating the bioaccessibility as the model simulates accurately and reproducibly the kinetic luminal conditions. As 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 which is complementary to the studies with the TIM system. Whether FBP could possibly influence folate transport, can also be answered by investigating the FBP binding activity during gastrointestinal passage for both folic acid and 5-CH_3-H_4folate, as the occurrence of folate-FBP complexes at the site of absorption is relevant. This will give more insight in the mechanisms underlying the effect of FBP on folate bioavailability.

Acknowledgments

The authors thank Jan Lelieveld and Hans Mocking for their technical assistance.

References

1 Wald, N., Sneddon, J., Frost, C., Stone, R. (1991) MRC Vitamin Study Group Prevention of neural tube defects. Results of the Medical Research Council Vitamin Study. Lancet 338: 131-137.

2 Daly, L.E., Kirke, P.N., Molloy, A., Weir, D.G., Scott, J.M. (1995) Folate levels and neural tube defects. J. Am. Med. Assoc. 247: 1698-1702.

3 Boushey, C.J., Beresford, A.A., Omen, G.S., Motulsky, A.G (1996) A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. J. Am. Med. Assoc. 274: 1049-1057.

4 Cuskelly, G.J., McNulty H., Scott, J.M. (1996) Effect of increasing dietary folate on red-cell folate: implications for prevention of neural tube defects. Lancet 347: 657-659.

5 Graham, I.A., O'Allaghan, P. (2000) The role of folic acid in the prevention of cardiovascular disease. Curr. Opin. Lipidol. 11: 577-587.

6 Brouwer, I.A., van Dusseldorp, M., West, C.E., Meyboom, S., Thomas, C.M.G., Duran, M., van het Hof, K.H., Eskes, T.K.A.B., Hautvast, J.G.A.J., Steegers-Theunissen, R.P.M. (1999) Dietary folate from vegetables and citrus fruit decreases plasma homocysteine concentrations in humans in a dietary controlled trial. J. Nutr. 129: 1135-1139.

7 Brouwer, I.A, van Dusseldorp, M., West, C.E., Steegers-Theunissen, R.P.M. (2001) Bioavailability and bioefficacy of folate and folic acid in humans. Nutr. Res. Rev. 14, 267-293.

8 Gregory III, J.F. (1995) The bioavailability of folate. In Bailey LB (ed): "Folate in Health and Disease", Marcel Dekker, New York: 195-235.

9 Swiatlo, N., O'Conner, D.L., Andrews, J., Picciano, M.F. (1990) Relative folate bioavailability from diets containing human, bovine and goat milk. J. Nutr. 120: 172-177.

10 Wigertz, K. (1997) Milk Folates. Characterisation and Availability. Doctoral Thesis, Lund University, Sweden.

11 Konings, E.J.M., Roomans, H.H.S., Dorant, E., Goldbohm, R.A., Saris, W.H.M., van den Brandt, P.A. (2001) Folate intake of the Dutch population according to newly established liquid chromatography data for foods. Am. J. Clin. Nutr. 73: 765-776.

12 Becker, W. (1994) Dietary habits and nutrient intake in Sweden 1989. Swedish National Food Administration, Livsmedelsverkets förlag, Uppsala, Sweden.

13 Ghitis, J. (1967) The folate binding in milk. Am. J. Clin. Nutr. 20: 1-4.

14 Wagner, C. (1985) Folate Binding Proteins. Nutr. Rev., 43: 293-299.

15 Karlin, R. (1969) Investigations about variations in cows'mixed milks. J. Int. Vitaminol. 39: 359-371.

16 Ford, J.E. (1974) Some observations on the possible nutritional significance of vitamin B_{12}- and folate binding proteins in milk. Br. J. Nutr. 31: 243-257.

17 Tani, M., Iwai, K. (1984) Some nutritional effects of folate binding protein in bovine milk on the bioavailability of folate to rats. J. Nutr. 114: 778-785.

18 Colman, N., Hettiarachchy, N., Herbert, V. (1981) Detection of a milk factor that facilitates folate uptake by intestinal cells. Science 211: 1427-1429.

19 Salter, D., Blakeborough, P. (1988) Influence of goat's-milk folate-binding protein on transport of 5-methyltetrahydrofolate in neonatal-goat small intestinal brush-border-membrane vesicles. Br. J. Nutr. 59: 497-507

20 Tani, M., Fushiki, T., Iwai, K. (1983) Influence of folate binding protein from bovine milk on the absorption of folate in gastrointestinal tract of rat. Biochim. Biophys. Acta 757: 274-281.

21 Said, H.M., Horne, D.W., Wagner, C. (1986) Effect of human folate binding protein on folate intestinal transport. Archiv. Biochem. Biophys. 251: 114-120.

22 Ghitis, J., Mandelbaum-Shavit, F., and Grossowicz, N. (1969) Binding of folic acid and derivatives by milk. Am. J. Clin. Nutr. 22: 156-162.

23 Minekus, M., Marteau, P., Havenaar, R., Huis in ‘t Veld, J.H.J. (1995) A multicompartimental dynamic computer-controlled model simulating the stomach and small intestine. ATLA 23: 197-209.

24 Minekus, M. (1998) Development and validation of a dynamic model of the gastrointestinal tract. PhD Thesis, University of Utrecht; Elinkwijk b.v., Utrecht, Netherlands.

25 Krul, C.A.M., Luiten-Schuite, A., Baan, R., Verhagen, H., Mohn, G., Feron, V., and Havenaar, R. (2000) Application of a dynamic *in vitro* gastrointestinal tract model to study the availability of food mutagens, using heterocyclic aromatic amines as model compounds. Food Chem. Toxicol. 38: 783-792.

26 Zeijdner, E.E. and Havenaar, R. (2000) The fate of orally administrated compounds during passage through the gastrointestinal tract simulated in a dynamic *in vitro* model (TIM). European Pharmaceutical Contractor Febr. issue: 76-81.

27 Krul, C.A.M (2001) Mutagenic and Antimutagenic activity of food compounds: Application of a dynamic in vitro gastrointestinal model. PhD Thesis, University of Utrecht. Febodruk BV, Enschede, The Netherlands.

28 Larsson, M., Minekus, M., and Havenaar, R. (1997) Estimation of the bio-availability of iron and phosphorus in cereals using a dynamic in-vitro gastrointestinal model. J. Sci. Food Agric. 73: 99-106.

29 Wigertz, K., Hansen, S.I., Høier-Madsen, M., Holm, J., Jägerstad, M. (1996) Effect of milk processing on the concentration of folate binding protein (FBP), the folate binding capacity and the retention of 5-methyltetrahydrofolate. Int. J. Food Sci. & Nutr. 47: 315-322.

30 Van den Berg, H., Finglas, P.M., Bates, C. (1994) FLAIR intercomparison on serum and red cell folate. Int. J. Vitam. Nutr. Res. 64: 288-293.

31 Dutch Food Composition Table 2001 (2001) The Netherlands Nutrition Centre, The Hague, The Netherlands.

32 Salter, D.N., Scott, K.J., Slade, H., Andrews, P. (1981) The preparation and properties of folate-binding protein from cow’s milk. Biochem. J. 193: 469-476.

33 Anthony, A.C., Utley, P.D, Marcell, P.D., Kolhouse, J.F (1982) Isolation, characterization, and comparison of the solubilized particulate and soluble folate binding proteins from human milk. J. Biol. Chem. 257: 10081-10089.

34 Halsted, C.H. (1979) The intestinal absorption of folates. Am. J. Clin. Nutr. 32, 846-855.

35 Konings, E.J.M. (1999) A validated liquid chromatographic method for determining folates in vegetables, milk powder, liver and flour. J. AOAC Int. 82: 119-127.

36 Høier-Madsen, M., Hansen, S.I., Holm, J. (1986) Rabbit antibodies against the folate binding protein from Cows’milk. Production, characterisation and use for development of an enzyme-linked immunosorbent assay (ELISA). Biosci. Rep. 6: 895-900.

37 Wigertz, K., Svensson, U.K., Jägerstad, M. (1997) Folate and folate-binding protein content in dairy products. J. Dairy Res. 64: 239-252.

38 Tamura, T., Stokstad, E.L.R. (1973) The availability of food folate in man. Br. J. Nutr. 25: 513-532.

39 Abad, A.R., Gregory III, J.F. (1987) Determination of folate bioavailability with a rat bioassay. J. Nutr. 117: 866-873.

40 Holm, J., Hansen, S.I. (2001) Binding of radiolabeled folate and 5Methyltetrahydrofolate to cow’s milk folate binding protein at pH 7.4 and 5.0. Relationship to concentration and polymerization equilibrium of the purified protein. Biosci. Rep. 21: 733-743.

41 Kaarsholm, N.C., Kolstrup, A., Danielsen, S.E., Holm, J., Hansen, S.I. (1993) Ligand-induced conformation change in folate-binding protein. Biochem. J. 292: 921-925.

42 Ford, J.E., Knaggs, G.S., Salter, D.N., Scott, K. (1972) Folate nutrition in the kid. Br.J. Nutr. 27: 571-583.

43 Mason, J.B., Selhub, J. (1988) Folate-binding protein and the absorption of folic acid in the small intestine of the suckling rat. Am. J. Clin. Nutr. 48: 620-625.

3

Bioaccessibility of folic acid and 5-methyltetrahydrofolate decreases after the addition of folate-binding protein to yogurt

Journal of Nutrition 133: 3678-3683, 2003

Karin Arkbåge[1]
Miriam Verwei[2,3]
Robert Havenaar[2]
Cornelia Witthöft[1]

[1]Department of Food Science, Swedish University of Agricultural Sciences, Uppsala, Sweden
[2]TNO Nutrition and Food Research, Zeist, The Netherlands
[3]Department of Human Nutrition, Wageningen University, Wageningen, The Netherlands

Abstract

Milk products are only moderate sources of folate. Nevertheless, they are of interest due to their content of folate-binding proteins (FBP), which in some studies have been reported to increase folate bioavailability. The effect of FBP on folate bioavailability is widely discussed. The aim of this study was to investigate the bioaccessibility of folic acid and (6S)-5-methyltetrahydrofolate (5-CH_3-H_4folate) from fortified yogurt using a dynamic *in vitro* gastrointestinal model (TIM). In addition, the impact of FBP on folate bioaccessibility and the stability of FBP added to yogurt during gastrointestinal passage were investigated. A folate bioaccessibility of 82% was found from yogurt fortified with folic acid and 5-CH_3-H_4folate. Addition of FBP to yogurt significantly decreased ($P < 0.05$) the folate bioaccessibility. The lowering effect of FBP was more pronounced in yogurt fortified with folic acid (34% folate bioaccessibility) than from yogurt fortified with 5-CH_3-H_4folate (57% folate bioaccessibility). After gastrointestinal passage, 17% of the FBP in yogurt fortified with 5-CH_3-H_4folate and 34% of the FBP in yogurt fortified with folic acid was recovered. No difference in folate bioaccessibility was found between folate-fortified yogurt and folate-fortified pasteurized milk ($P = 0.10$), whereas the lowering effect of FBP was significantly ($P < 0.05$) stronger in yogurt compared to pasteurized milk. In conclusion, based on the high bioaccessibility of folic acid and 5-CH_3-H_4folate, yogurt without active FBP can be considered as an appropriate food matrix for folate fortification.

Introduction

Over the last decade, the literature on health effects of folates has grown. It is mainly focussed on neural tube defects in the developing fetus, spontaneous abortions, mental fitness and certain forms of cancer (1-5). This resulted in increased recommended dietary intakes of folates, which revealed a gap between actual intake and recommendation in Western populations. To bridge this gap there is a need for more accurate information on bioavailability of dietary folates from both native and fortified foods. Dairy products, especially fermented milk such as yogurt, are moderate folate sources. Yet, there is a lack of knowledge on the bioavailability of folate and eventual matrix effects caused by different pH, starter cultures, and presence of folate-binding proteins (FBP). The dominant folate compound in dairy products is 5-methyltetrahydrofolate (5-CH_3-H_4folate) of which ca 60% is in monoglutamate form (6). In unprocessed bovine milk most of the folate is bound to FBP (7).

The possible physiological functions of the milk FBP remain controversial, especially whether or not it affects the absorption of folates. Some investigators observe an increase (8,9), some observe no difference (10) and some observe less overall absorption (11,12) of folates when bound to FBP.

Evidence for partial stability and effect of added FBP on folate bioaccessibility during transit through stomach and small intestine was recently obtained using TNO´s dynamic *in vitro* gastroIntestinal Model (TIM) (13). The term bioaccessibility describes the amount of folate released from the food matrix being able to pass the cell membranes with a molecular cut-off of 5 kDa, during transit through a simulated stomach and small intestine, thereby reflecting the availability for absorption *in vitro*. This study compares the folate bioaccessibility and FBP stability in milk fortified with folic acid or biologically active (6S)-5-CH_3-H_4folate in the absence or presence of additional FBP in equimolar amounts to folate. Without added FBP, the folate bioaccessibility from milk fortified with folic acid and 5-CH_3-H_4folate was 58% and 71%, respectively. The addition of FBP resulted in a significant decrease in bioaccessibility of folic acid but not of 5-CH_3-H_4folate. Approximately 15% of the FBP in the food were found to pass the gastrointestinal tract intact from folic acid-fortified products. In the pasteurized milk fortified with 5-CH_3-H_4folate, however, only 0-1% was recovered after gastrointestinal passage.

The present study aimed to study the stability of FBP and its impact on the bioaccessibility of added folic acid and (6S)-5-CH_3-H_4folate in yogurt. Furthermore, we compared the folate bioaccessibility between two dairy matrices, yogurt and pasteurized milk. The lower pH of yogurt (pH 4.2) compared to milk (pH 6.8) (14), might affect the FBP binding activity and its stability during gastrointestinal transit. At a pH below 5, FBP loses its folate-binding capacity allowing dissociation between FBP and folate in yogurt. Moreover, the starter culture might have proteolytic enzymes that hydrolyze FBP during the gastrointestinal transit. This could result in a different folate bioaccessibility from yogurt as compared with milk.

Materials and Methods

Dairy samples

Four different yogurt products (Arla Foods), containing 3% fat, were used to study folate bioaccessibility (Table 3.1). The yogurt products were fortified to reach a folate and FBP concentration of 900 nmol/L to achieve a 1:1 molar ratio. Folic acid was obtained from Schircks Laboratories (Jona, Switzerland) and (6S)-5-CH_3-H_4folate (sodium salt) from Merck-Eprova (Schauffhausen, Switzerland). The folate compounds were checked for purity according to Van den Berg *et al*. (15), using molar extinction coefficients as described by Blakley (16). Stock solutions of (6S)-5-CH_3-H_4folate (0.73 g/L) and folic acid (1.00 g/L) in 0.1 mol/L phosphate buffer, pH 6.1, containing 10 g ascorbic acid/L were stored at -20°C. Folate fortification was performed on the day of the TIM experiment by addition of 220 µL of the (6S)-5-CH_3-H_4folate stock solution or 160 µL of the folic acid stock solution to 400 g of yogurt. FBP was added as 16 g concentrated whey powder containing 75% protein and 15% fat (WPC 75; Arla Foods, Sweden) into 400 g of yogurt. The yogurt product was stirred and placed in the dark at 20°C for 1 h before the start of the TIM experiment. A portion of 300 g of yogurt was applied into the gastric compartment of the TIM system. The remaining 100 g was stored in subsamples at -20°C for folate and FBP analyses.
Samples deriving from previous TIM experiments (13) with fortified pasteurized milk products (Table 3.1) were also analyzed by RPBA for comparison of the folate bioaccessibility in yogurt and pasteurized milk, and for confirmation of HPLC results.

Table 3.1. Characteristics of dairy products tested in the in vitro gastrointestinal model[1]

Dairy product	Folate fortificant	Folate (nmol/L)	FBP fortification	FBP (nmol/L)
Yogurt	folic acid	1153±44	–	0
Yogurt	folic acid	1203±1	+	1233±30
Yogurt	5-CH_3-H_4folate	913±42	–	0
Yogurt	5-CH_3-H_4folate	1039±76	+	1172±80
Pasteurized milk	folic acid	936±1	–	140±2 [2]
Pasteurized milk	folic acid	911±3	+	1135±32 [2]
Pasteurized milk	5-CH_3-H_4folate	1169±225	–	157±11 [2]
Pasteurized milk	5-CH_3-H_4folate	1012±113	+	1223±1 [2]

[1] Folate and FBP concentrations measured with RPBA and ELISA, respectively. Values are means ± range, n= 2,[2] From Verwei *et al.* (13)

TIM experiment

The dynamic *in vitro* gastrointestinal model, TIM, has been described in detail by Minekus *et al.* (17) and applied in folate studies by Verwei *et al*. (13). The gastric small-intestinal model represents the stomach, duodenum, jejunum, and ileum. The pH curves, peristaltic movements,

gastric emptying, intestinal transit, and gradual additions of digestive juices are computer-controlled events and comparable with human conditions (17).
Each dairy product was tested in duplicate in TIM in experiments lasting 5 h. At the beginning of each experiment a test portion of 300 g of yogurt was put into the gastric compartment of TIM. During passage of the food through the TIM system, total jejunal and ileal dialysate were collected during 0-1, 1-2, 2-3 and 3-5 h. The dialysate fractions contained the absorbable (bioaccessible) fraction (membrane cut-off of 5 kDa). The ileal delivery was collected during 0-5 h representing the non-absorbable (non-bioaccessible) fraction. At the end of each experiment the residues in the compartments were collected. The same TIM protocol (including passage time, peristaltic movements, pH curves) was used for the yogurt products as used in a previous study with milk products (13) to enable optimal comparison between the two matrices.

FBP analysis

FBP concentrations in the food and in TIM samples were analyzed, in duplicates, using a two-site enzyme-linked immunosorbent assay (ELISA) (18) according to the procedure by Wigertz *et al.* (19). The antibody against FBP from bovine milk (Rabbit anti-bovine FBP 24739) was obtained from the State Serum Institute (Copenhagen, Denmark) and the FBP calibrant from the Central Hospital Hillerød (Hillerød, Denmark). Briefly, to 3 g of sample 0.09 g of Triton X-100 was added. The sample was put on a shaking device, incubated for 45 min at room temperature, diluted to approximately 0.4 nmol FBP/L and applied to a microtiter plate. A calibration curve from 0.002-1.1 nmol FBP/L was prepared and included in each run. A whey protein concentrate containing 65% protein, (WPC 65. Arla Foods, Götene, Sweden), was used as an in-house reference material and included in every analysis. The coefficient of variation between runs did not exceed 15%.

Folate analysis

The samples from the TIM experiments, milk and yogurt products and the solutions of bile and pancreatic juice “secreted” into TIM (endogenous fraction) were extracted, in duplicate, as described by Strålsjö *et al* (20). Briefly, 3 g of test food and TIM sample or 0.7 g of WPC 65 were mixed with 5-8 or 30 volumes, respectively, of extraction buffer (0.1 mol/L phosphate buffer pH 6.1, containing freshly added ascorbic acid 10 g/L (w/v) and 2-mercaptoethanol 1 mL/L (v/v)). Samples were heat extracted in a boiling water bath for 12 min, cooled down to 37°C, and subjected to conjugase treatment for 3 h, 1 mL chicken pancreas conjugase suspension, 10 g/L, (lyophilized chicken pancreas, Difco, Detroit, MI, USA). A chicken pancreas blank was included in every analysis to correct for folate from the enzyme suspension. After centrifugation (27,000 g for 15 min), the supernatants were collected and diluted to 25 mL or 50 mL. Aliquots of extracts were stored at -20°C until RPBA analysis. To prevent folate oxidation, samples were protected by nitrogen, subdued light and cooled on ice throughout sample preparation. WPC 65 was included in every analysis as an in-house material. The coefficient of variation between runs did not exceed 10%.

A commercial RPBA kit, SimulTrac SNB Radioassay kit, Vitamin B_{12} [^{57}Co]/Folate [^{125}I] (ICN Pharmaceuticals, Costa Mesa, CA, USA) was used for folate quantification. The use of external calibrants and dilution of samples and calibrants were according to Strålsjö *et al.* (20). Folic acid or (6S)-5-CH_3-H_4folate was used for preparation of calibration curves in the concentration range of 0.5 to 10 µg/L (five-point curve, duplicates for each concentration).
To check for losses of folate during sample preparation and RPBA quantification, known amounts of both folate standards were added to samples prior to extraction in concentrations of 100% of the native folate content of the samples (n=2). Recovery was calculated using the equation:

$$R(\%) = \left(\frac{c_s - c_u}{c_t}\right) * 100,$$

where R (%) = recovery, c_s = folate content in spiked sample, c_u = folate content in unspiked sample, and c_t = theoretical content of added spike (21). The recovery of folic acid and 5-CH_3-H_4folate ranged between 90 and 102% in jejunal dialysate, ileal dialysate, and ileal delivery samples (n=2).

Statistics and calculations

FBP stability during gastrointestinal passage of yogurt is presented as percentage of FBP found in the ileal delivery sample compared to the FBP content in the test food portion (intake). The bioaccessible and non-bioaccessible folate fractions are given as folate content in dialysate and ileal delivery, respectively, and expressed as percentage of folate content in the intake.
The data was analyzed by ANOVA, using Minitab Statistical Software release 13.3 for Windows (Minitab Inc., PA, USA), to evaluate the effect of FBP on folate bioaccessibility in yogurt, to compare folate bioaccessibility from yogurt to that from pasteurized milk, and to compare folate bioaccessibility data from milk obtained by the two methods of analysis. Tukey´s test was performed for pairwise comparison of data. A P-value < 0.05 was considered significant.

Results

Folate concentrations in the fortified dairy products (yogurt and pasteurized milk) ranged from 911 to 1203 nmol/L. The FBP concentrations amounted to 1150 to 1233 nmol/L in the FBP-fortified products (Table 3.1) resulting in the intended molar ratio of folate: FBP of 1:1. No FBP was detected in the yogurt products without FBP fortification. The folate concentrations in yogurt after fortification exceeded the natural folate concentration by about four times. Folate concentrations in the fortified pasteurized milk products were approximately nine times higher compared to their natural folate concentration. In the pasteurized milk products without added FBP a native FBP concentration of 140-157 nmol/L was found.
The amount of FBP was reduced during passage through the gastrointestinal tract. No FBP was found in the dialysate, as the molecular size of intact FBP is physically hindered to pass the dialysate membrane. From folic acid-fortified yogurt 34% of the initially added FBP was recovered in the ileal delivery fraction, whereas 17% FBP was recovered from the yogurt fortified with 5-CH_3-H_4folate (Table 3.2).

Table 3.2. Folate content, measured with RPBA, in food samples and subsequent jejunal dialysate, ileal dialysate and ileal delivery samples from TIM experiments with yogurt and pasteurized milk. FBP content, measured with ELISA, in food and in ileal delivery [1]

Dairy product	Total folate (nmol/fraction)				FBP (nmol/fraction)	
	Food	Jej. dial.	Ileal dial.	Ileal del.	Food	Ileal del.
Yogurt+folic acid	329±13	203±7[d]	65±11[a,b]	21±2[a]	0[a]	0[a]
Yogurt+folic acid+FBP	346±0	86±4[a]	33±5[a]	196±10[d]	354±9[b]	122±4[c]
Yogurt+5-CH_3-H_4folate	261±12	152±11[b,c,d]	64±11[a,b]	22±3[a]	0[a]	0[a]
Yogurt+5-CH_3-H_4folate +FBP	298±22	125±9[a,b]	44±0[a,b]	139±14[c]	335±23[b]	56±5[b]
Milk+folic acid	279±0	132±11[a,b,c]	63±5[a,b]	36±4[a,b]	42±1[a]	7±1[a]
Milk+folic acid+FBP	271±1	126±4[a,b]	40±1[a,b]	75±19[b]	338±10[b]	46±10[b]
Milk+5-CH_3-H_4folate	348±67	182±12[c,d]	87±20[b]	21±3[a]	47±3[a]	0[a]
Milk+5-CH_3-H_4folate+FBP	302±34	186±15[c,d]	84±11[b]	33±6[a,b]	365±0[b]	5±0[a]

[1] Values are means ± range, n= 2. Means in a column without a common letter differ, P<0.05.

A folate bioaccessibility of 82% was found from yogurt fortified with folic acid and 5-CH_3-H_4folate (Table 3.2). In addition, 8-9% was found as the non-absorbable fractions from both yogurt products. In the two yogurt products fortified with FBP, a different folate absorption pattern was found as compared to yogurt without FBP (Table 3.2, Figure 3.1). The bioaccessibility of 5-CH_3-H_4folate and folic acid from FBP-fortified yogurt was significantly lowered (P< 0.05) to 57% and 34%, respectively. This inhibiting effect of FBP on folate bioaccessibility was significantly more pronounced in yogurt fortified with folic acid than in yogurt fortified with 5-CH_3-H_4folate (P< 0.05). In addition, the non-absorbed fractions from yogurt fortified with folic acid and 5-CH_3-H_4folate were enhanced from 8-9% to 57% and 47%, respectively. In total, approximately 90% of the folic acid or 5-CH_3-H_4folate, originally present in the test food (= intake) was recovered in the jejunal dialysate, the ileal dialysate, and the ileal delivery together.

Folate bioaccessibility was found to be higher from the jejunum compared to the ileum compartment for all products (P< 0.05) (Figure 3.1). A maximum concentration of folic acid and 5-CH_3-H_4folate was found in jejunum during the second hour after starting the experiments for all products but the yogurt fortified with folic acid and FBP.

The samples from previous TIM experiments, in which pasteurized milk with folic acid or 5-CH_3-H_4folate were tested on folate by HPLC (13), were also analyzed by RPBA (Table 3.1 and 3.2). No statistical difference in folate bioaccessibility from folate-fortified yogurt and folate-fortified pasteurized milk was found (P= 0.10). However, the addition of FBP to yogurt led to a significantly lowered (P< 0.05) folate bioaccessibility as compared with addition of FBP to pasteurized milk.

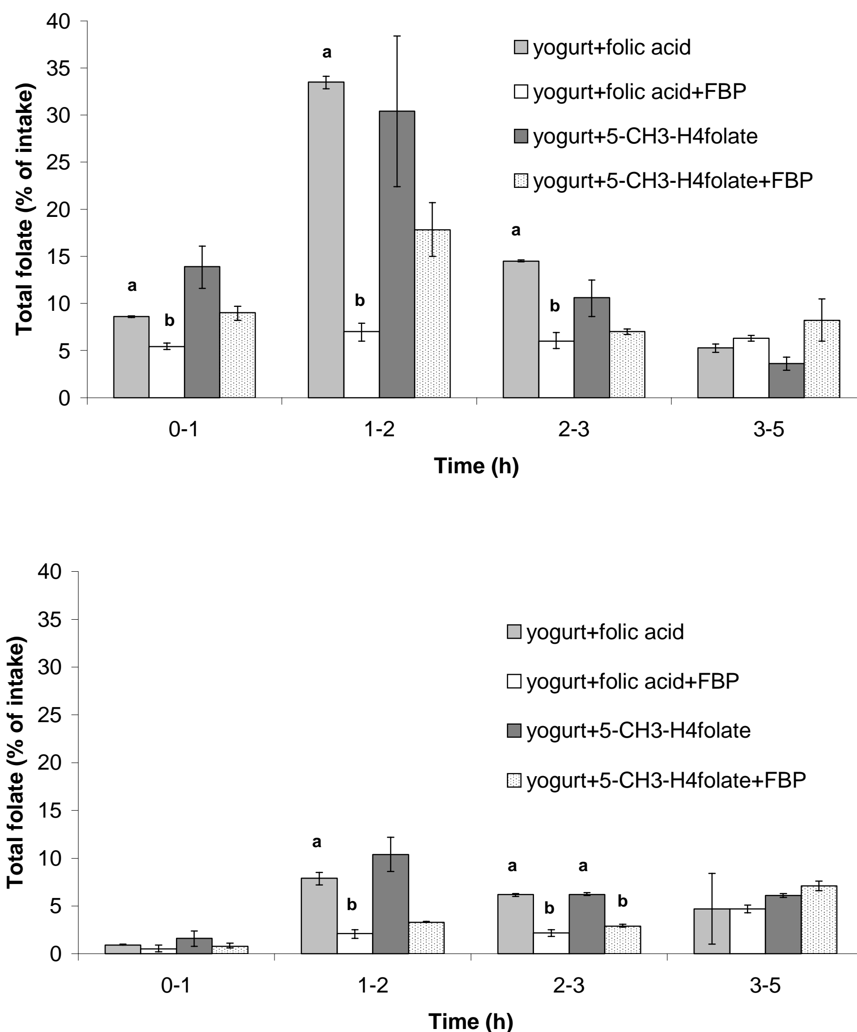


Figure 3.1. Folate content, as percentage of intake, in jejunal dialysate (upper panel) and ileal dialysate (lower panel) collected during 0-1, 1-2, 2-3, 3-5 h, in yogurt + folic acid, yogurt + folic acid + Folate-Binding Protein (FBP), yogurt + 5-methyltetrahydrofolate (5-CH_3-H_4folate) and yogurt + 5-CH_3-H_4folate + FBP. The values are means ± range (n=2) and based on RPBA quantification. Within each time interval, products with and without FBP fortification are compared, products with different letters differ significantly ($P < 0.05$).

Discussion

In this study, the bioaccessibility of folate from yogurt fortified with folic acid or (6S)-5-CH_3-H_4folate was studied using an *in vitro* gastrointestinal model. A bioaccessibility of 82% was found for both folic acid and 5-CH_3-H_4folate from yogurt without FBP. All native FBP is inactivated by thermal processing, 90ºC for 5 min, prior to inoculation with the starter culture. FBP fortification of yogurt, to equimolar ratio with folate, significantly decreased ($P< 0.05$) the bioaccessibility of folic acid (34%) and 5-CH_3-H_4folate (57%). Our results suggest that approximately half of the folate (47-

57%) was protein-bound during passage of the FBP-fortified yogurt through jejunum and ileum, since it did not pass the dialysis membrane and was found in the ileal delivery samples.

Our study design allows parallel quantification of folate and FBP during passage of fortified food through a simulated gastrointestinal tract. Therefore, we could demonstrate that in a folate-fortified matrix FBP was only partly resistant against the digestive enzymes in stomach and small intestine. We found that this resistance of FBP during TIM passage depended on the folate form present in yogurt. FBP stability in yogurt fortified with folic acid (34%) was twice as high as the stability in yogurt fortified with 5-CH_3-H_4folate (17%). A similar pattern was observed in pasteurized milk fortified with FBP and folate (13). A relation between inhibition effect of FBP on the bioaccessibility of folic acid and 5-CH_3-H_4folate, and FBP stability in folic acid and 5-CH_3-H_4folate fortified milk and yogurt appears to exist. Previous studies found that the ligand binding of FBP increases the folding stability of the protein (22,23). Moreover, as FBP has a lower affinity for 5-CH_3-H_4folate compared to folic acid at pH 5 and 7.4 (23), this could explain the differences found in FBP effect and FBP stability between dairy products fortified with folic acid and 5-CH_3-H_4folate. Based on these differences, more stable folic acid-FBP complexes seem to exist compared to 5-CH_3-H_4folate-FBP complexes in both yogurt and milk products. In addition, more FBP-folate complexes appear to exist in yogurt as compared to pasteurized milk. Next to the occurrence of more stable FBP-folate complexes as compared to free FBP molecules, another possible explanation could be the presence of the carbohydrate part in bovine FBP, which also is suggested to influence the stability of the molecules.

One molecule of FBP binds one molecule of folate at a pH of about 7 (24). In addition, the molar ratio between FBP and folates naturally present in milk appeared to be 1:1. Therefore, we aimed for a 1:1 molar ratio when fortifying the yogurt and pasteurized milk with FBP and folate in the present study and in the previous study (13). Yet, more folate than FBP was proportionally found in the ileal delivery fraction. This suggests that other constituents in the yogurt could bind folates and prevent them from being absorbed. Jones *et al*. (25) report that in a rat model the effect of bovine FBP on folate bioavailability is influenced by the presence of certain milk components. Whereas soluble casein and whey proteins increase the folate bioavailability, acid-precipitated casein and a lipid-enriched whey preparation decrease it. Another explanation can be that in our study the stability of FBP was analyzed by quantifying FBP by an ELISA method based on antibodies raised against FBP. Whether FBP still possesses complete or reduced folate-binding capacity after passage of TIM remains to be studied, although the enhancement of folate in ileal delivery after FBP fortification indirectly suggests that FBP could bind folate and prevent folate from being absorbed (pass the membranes in jejunum and ileum).

Our results seem to partly be in agreement with an *in vivo* study performed on 6-day-old goat kids (26). This study shows that gastric acidity and gastrointestinal digestive enzymes only slightly affect the folate-binding capacity of FBP in goat's milk. In addition, Tani *et al* (10) find in rats that the folate binding activity of FBP fully recovers in jejunum after being reversibly inactivated under the gastric acidic conditions. However, contradictory results were obtained in an *in vitro* study (27) which shows that half of the folic acid-binding capacity was lost during pepsin treatment and that all folic acid-binding capacity was lost after further digestion with trypsin.

Dietary folates are difficult to analyze due to low natural concentrations, the presence of non-stable native folate forms, and food matrix (33). By applying both HPLC and RPBA methods for analysis of all samples from the pasteurized milk products subjected to TIM, we could compare both analytical methods. No statistical difference was found between the methods of analysis neither in the bioaccessible (P= 0.21) or the non-bioaccessible (P= 0.77) folate fraction. The bioaccessibility pattern from the different milk products was similar with both methods of analysis. On average, the RPBA method gave 16% higher values in the jejunal dialysate samples and 3% higher values in the ileal delivery samples which in part could be due to the difference in quantifying the total folate content (RBPA method) instead of the individual folate forms (HPLC method). The residues denoted strong matrix effects resulting in a false high response with the RPBA method. Except for the residue samples, we found that the values deriving from HPLC were confirmed by RPBA (Figure 3.2). This implies that the results from this study can be compared with those of the previous milk study and with literature data.

No statistical difference in the folate bioaccessibility was seen between folate-fortified yogurt and folate-fortified pasteurized milk (P= 0.10). In contrast, addition of FBP to both dairy matrices resulted in a significantly lower (P< 0.05) folate bioaccessibility in yogurt compared to milk. This was accompanied by a two- to 16-fold higher ileal excretion of intact FBP from yogurt compared to the corresponding pasteurized milk. Thus, it seems that FBP is more stable in yogurt. The TIM protocols were identical for yogurt and milk excluding that pH might have had an impact. Interestingly, the viable starter culture in yogurt seemed to have no degradable effect on FBP. Neither seemed the microorganisms affect the folate content during the TIM experiment.

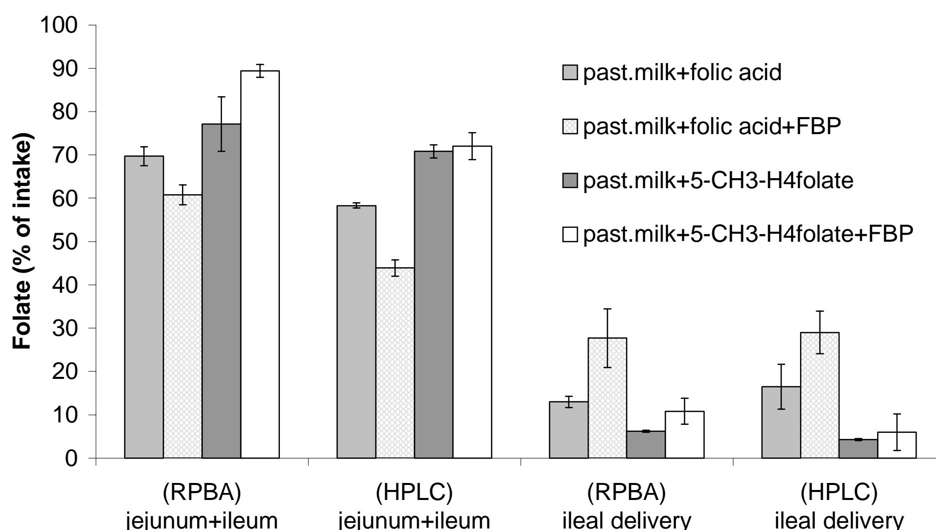


Figure 3.2. Folate content, as percentage of intake, in the bioaccessible fraction (jejunal plus ileal dialysate) and non-bioaccessible fraction (ileal delivery) collected during 0-5 h from the pasteurized milk + folic acid, pasteurized milk + folic acid + Folate-Binding Protein (FBP), pasteurized milk + 5-methyltetrahydrofolate (5-CH_3-H_4folate), and pasteurized milk + 5-CH_3-H_4folate + FBP. Quantified by RPBA (total folate) or HPLC (folic acid and 5-CH_3-H_4folate). The values are means ± range (n=2). HPLC results from Verwei *et al.* (13).

The TIM system provides an excellent opportunity for comparison of folate bioaccessibility from different (dairy) matrices. The model is strictly controlled for parameters such as pH curves, enzyme activities, peristaltic movements, and transit times, compared to the human system. Therefore, biases from e.g. pathologic or physiological conditions are avoided. The TIM system was found to kinetically mimic the *in vivo* situation as the absorption of free folate was found to be higher in jejunum than in ileum (12). Moreover, maximum folate absorption was seen 1-2 h after application of the test food into the TIM system, which is in accordance with data on folate absorption in humans (34). Folate bioaccessibility studies in TIM provide a good complement to more costly and time consuming folate absorption studies on humans, but need more validation against human experiments. Several *in vivo* and *in vitro* studies indicate that bovine FBP contribute to the absorption and/or retention of folates from milk, especially during the neonatal period (9,12,27-32). It has been reported that whereas free folates are mainly absorbed in jejunum (7), FBP-bound folates are absorbed more efficiently in ileum (11,29). However, as the TIM system has no intestinal receptor systems, it can not answer the question whether FBP-bound folates can be absorbed. For this purpose, *in vitro* studies with cultured intestinal cells or intestinal segments could be performed to study FBP-bound or free folate transport across the intestinal wall.

In conclusion, both folic acid and (6S)-5-CH_3-H_4folate in fortified yogurt are highly bioaccessible (82%). The addition of FBP to yogurt significantly ($P< 0.05$) decreased the folate bioaccessibility with a more pronounced effect in yogurt fortified with folic acid than in yogurt fortified with (6S)-5-CH_3-H_4folate. Besides, the inhibiting effect of FBP on folate bioaccessibility was significantly higher ($P< 0.05$) in yogurt as compared with milk. The stability of FBP during gastrointestinal transport of yogurt depended on the folate form used for fortification, ranging between 17 to 34%, and appeared to be higher than the FBP stability in pasteurized milk (0-15%).

Further studies are needed to elucidate the stability of FBP during gastrointestinal passage and its effect on folate bioavailability. Work is now in progress in our laboratory to validate the folate bioavailability from both fermented milk and pasteurized milk in adults in the presence of equimolar amounts of FBP and folate using a human ileostomy model.

Acknowledgments

The authors thank Jan Lelieveld and Hans Mocking at TNO, Zeist, for their technical assistance. Prof. Steen Ingemann Hansen at the Central Hospital Hillerød is gratefully acknowledged for discussions on the ELISA method and for kindly providing the FBP calibrant, and Prof. Margaretha Jägerstad at SLU, Uppsala, for critical evaluation of the manuscript.

References

1. Berry, R. J., Li, Z., Erickson, D., Li, S., Moore, C. A., Wang, H., Mulinare, J., Zhao, P., Wong, L. C., Gindler, J., Hong, S., & Correa, A. (1999) Prevention of neural-tube defects with folic acid in China. N. Engl. J. Med. 341: 1485-1490.
2. Honein, M. A., Paulozzi, L. J., Mathews, T. J., Erickson, D. J., & Wong, L.-Y. C. (2001) Impact of folic acid fortification of the US food supply on the occurrence of neural tube defects. J. Am. Med. Assoc. 285: 2981-2986.
3. Richter, B., Stegmann, K., Röper, B., Böddeker, I., Ngo, E. T. K. M., & Koch, M. C. (2001) Interaction of folate and homocysteine pathway genotypes evaluated in susceptibility to neural tube defects (NTD) in a German population. J. Human Gen. 46: 105-109.
4. Giovannucci, E., Rimm, E. B., Ascherio, A., Stampfer, M. J., Colditz, G. A., & Willett, W. C. (1995) Alcohol, low-methionine-low-folate diets, and risk of colon cancer in men. J. Natl.Cancer I. 87: 265-273.
5. Seshadri, S., Beiser, A., Selhub, J., Jacques, P. F., Rosenberg, I. H., D'Agostino, R. B., Wilson, P. W. F., & Wolf, P. A. (2002) Plasma homocysteine as a risk factor for dementia and Alzheimer's disease. N. Engl. J. Med. 346: 476-483.
6. Selhub, J. (1989) Determination of tissue folate composition by affinity chromatography followed by high pressure ion pair liquid chromatography. Anal. Biochem. 182: 84-93.
7. Gregory, J. F. (1995) The Bioavailability of Folate. In L.B. Bailey, ed. Folate in Health and Disease Marcel Dekker, New York.
8. Salter, D. N., & Blakeborough, P. (1988) Influence of goats-milk folate-binding protein on transport of 5-methyltetrahydrofolate in neonatal-goat small intestinal brush-border-membrane vesicles. Br. J. Nutr. 59: 497-507.
9. Colman, N., Hettiarachchy, N., & Herbert, V. (1981) Detection of a milk factor that facilitates folate uptake by intestinal cells. Science 211: 1427-1429.
10. Tani, M., Fushiki, T., & Iwai, K. (1983) Influence of folate-binding protein from bovine milk on the absorption of folate in gastrointestinal tract of rat. Biochim. Biophys. Acta 757: 274-281.
11. Izak, G., Galewski, K., Rachmilewitz, M., & Grossowicz, N. (1972) The absorption of milk-bound pteroylglutamic acid from small intestine segments. Proc. Soc. Expt. Biol. Med. 140: 248-250.
12. Said, H. M., Horne, D. W., & Wagner, C. (1986) Effect of human-milk folate- binding protein on folate intestinal transport. Arch. Biochem. Biophys. 251: 114-120.
13. Verwei, M., Arkbåge, K., Havenaar, R., Van den Berg, H., Witthöft, C., & Schaafsma, G. (2003) Folic acid and 5-methyltetrahydrofolate in fortified milk are bioaccessible as determined in a dynamic in vitro gastrointestinal model. J. Nutr. 133: 2377-2383.
14. Renner, E. (1989) Micronutrients in milk and milk-based food products. Elsevier Applied Science, London and New York.
15. Van den Berg, H., Finglas, P. M., & Bates, C. (1994) FLAIR Intercomparisons on serum and red cell folate. Intern. J. Vit. Nutr. Res. 64: 288-293.
16. Blakley, R. L. (1969) The biochemistry of folic acid and related pteridines. Frontiers of Biology North Holland Publishing Co, Amsterdam, The Netherlands.
17. Minekus, M., Marteau, P., Havenaar, R., & Huisintveld, J. H. J. (1995) A multicompartmental dynamic computer-controlled model simulating the stomach and small-intestine. Atla-Altern. Lab. Anim. 23: 197-209.

18. Høier-Madsen, M., Hansen, S. I., & Holm, J. (1986) Rabbit antibodies against the folate-binding protein from cows milk - production, characterization and use for development of an enzyme-linked-immunosorbent-assay (Elisa). Bioscience Reports 6: 895-900.
19. Wigertz, K., Svensson, U. K., & Jägerstad, M. (1997) Folate and folate-binding protein content in dairy products. J. Dairy Res. 64: 239-252.
20. Strålsjö, L., Arkbåge, K., Witthöft, C. M., & Jägerstad, M. (2002) Evaluation of a radioprotein-binding assay (RPBA) for folate analysis in berries and milk. Food Chem. 79: 525-534.
21. Rodriguez, L. C., Campana, A. M. G., Barrero, F. A., Linares, C. J., & Ceba, M. R. (1995) Validation of an analytical instrumental method by standard addition methodology. J. AOAC Int. 78: 471-476.
22. Kaarsholm, N. C., Kolatrup, A., Danielsen, S. E., Holm, J., & Hansen, S. I. (1993) Ligand-induced conformation change in folate-binding protein. Biochem. J. 292: 921-925.
23. Holm, J., & Hansen, S. I. (2001) Binding of radiolabeled folate and 5-methyltetrahydrofolate to cow's milk folate binding protein at pH 7.4 and 5.0. Relationship to concentration and polymerization equilibrium of the purified protein. Bioscience Reports 21: 733-743.
24. Salter, D. N., Scott, K. J., Slade, H., & Andrews, P. (1981) The preparation and properties of folate-binding protein from cow´s milk. Biochem. J. 193: 469-476.
25. Jones, M. L., Treloar, T., & Nixon, P. F. (2003) Dietary interactions influence the effects of bovine folate- binding protein on the bioavailability of tetrahydrofolates in rats. J. Nutr. 133: 489-495.
26. Salter, D. N., & Mowlem, A. (1983) Neonatal role of milk folate-binding protein: studies on the course of digestion of goat's milk folate binder in the 6-d-old kid. Br. J. Nutr. 50: 589-596.
27. Ford, J. E. (1974) Some observations on the possible nutritional significance of vitamin B_{12}- and folate-binding proteins in milk. Br. J. Nutr. 31: 243-257.
28. Ford, J. E., Knaggs, G. S., Salter, D. N., & Scott, K. J. (1972) Folate nutrition in the kid. Br. J. Nutr. 27: 571-583.
29. Tani, M., & Iwai, K. (1984) Some nutritional effects of folate-binding protein in bovine milk on the bioavailability of folates to rats. J Nutr 114: 778-785.
30. Mason, J. B., & Selhub, J. (1988) Folate-binding protein and the absorption of folic acid in the small intestine of the suckling rat. Am J Clin Nutr 48: 620-625.
31. Salter, D. N., & Blakeborough, P. (1988) Influence of goat's-milk folate-binding protein on transport of 5-methyltetrahydrofolate in neonatal-goat small intestinal brush-border-membrane vesicles. Br. J. Nutr. 59: 497-507.
32. Swiatlo, N., Oconnor, D. L., Andrews, J., & Picciano, M. F. (1990) Relative folate bioavailability from diets containing human, bovine and goat milk. J. Nutr. 120: 172-177.
33. Witthöft, C. M., Forssén, K., Johannesson, L., & Jägerstad, M. (1999) Folates-food sources, analysis, retention and bioavailability. Scand. J. Nutr. 43: 138-146.
34. Prinz-Langenohl, R., Brönstrup, A., Thorand, B., Hages, M., & Pietrzik, K. (1999) Availability of food folate in humans. J. Nutr. 129: 913-916.

4

Bioaccessibility of folate from several liquid and solid food products

Submitted for publication

Miriam Verwei[1,2]
Ana Belén Olivares[3]
Trinette van Vliet[1]
Henk van den Berg[4]
Robert Havenaar[1]

[1]TNO Nutrition and Food Research, Zeist, The Netherlands
[2]Department of Human Nutrition, Wageningen University, Wageningen, The Netherlands
[3]Food Science and Human Nutrition, University of Murcia, Murcia, Spain
[4]The Netherlands Nutrition Centre, Den Haag, The Netherlands

Abstract

Background In many countries average folate intake was found to be lower than recommended. The actual intake can be enhanced by a higher consumption of folate-rich food products or by the intake of supplements or fortified food products. The amounts of natural and fortified food products to be consumed to reach the recommended intake level are not only dependent on the folate content but also on the bioavailability of folate from the food products. *Aim of the study* To investigate the effect of the food matrix on the release of dietary folate during gastrointestinal passage. *Methods* The bioaccessible folate fractions, corresponding to the fractions which are released from the food matrix and available for absorption, from several food products were studied in an *in vitro* dynamic model simulating human gastrointestinal conditions. *Results* A large portion (68-94%) of the native or added folate was released from the food products during gastrointestinal passage. The bioaccessible folate fractions from peas and spinach leaves (78-80%) were significantly lower ($P<0.05$) than those from alcoholic beer (88%), orange juice (87-91%), gazpacho (94%), knäckebröd (87%) and fortified filmjölk (90%), but not from milk (86%), spinach soup (83%) and non-alcoholic beer (83%). Additional folate-binding protein led to a 22% lower bioaccessible folate fraction from fortified filmjölk. *Conclusions* Folate appeared to be highly bioaccessible from liquid foods and knäckebröd and ~10% less available from green vegetables indicating an impact, although limited, of the food matrix on the bioaccessibility of folate during small-intestinal transit.

Introduction

The folate intakes needed for risk reduction of neural tube defects (1,2), cardiovascular diseases (3,4) and colon cancer (5,6) appear to be higher than the actual intakes in many (European) countries (7,8). The gap between actual and recommended intake can be bridged by an increased consumption of natural foods rich in folate, such as spinach and orange juice. A high consumption of folate-rich food products has shown to improve the folate status, including a lower plasma homocysteine (9,10). However, the compliance to a diet with high amounts of fruits and vegetables appeared to be low in several European countries and, therefore, this dietary strategy seems difficult to apply for the whole population and for a long time period. An alternative dietary strategy to improve the folate status of the whole population might be the consumption of folate-rich products in combination with fortified food products or supplements. The amounts of natural and fortified food products to be consumed to reach the recommended intake level are not only dependent on the folate content but also on the bioavailability of folate from the food products.

Several processes might limit the bioavailability of folate, such as the release of folate from the food matrix, enzymatic conversion of dietary folate polyglutamates to monoglutamates in the intestine prior to absorption (11) and the absorption of folate monoglutamates across the intestinal cells (12). Current research is focussed on the first step in the overall bioavailability process, which is the release of folate from food products during gastrointestinal passage. The impairment of the food matrix on the bioavailability of folate was shown in a controlled three-week dietary intervention study (13) in which the subjects received either a control diet or the control diet plus whole-leaf spinach, minced spinach or liquefied spinach. The consumption of minced or liquefied spinach led to a higher plasma folate response than consumption of whole-leaf spinach, which suggests that the food matrix of spinach has an effect on the bioavailability of folate (13). Also in a study of Van het Hof et al. (14) it was found that disruption of the whole leaf spinach matrix enhanced the bioavailability of folate as the consumption of chopped spinach induced a larger plasma folate response in humans.

The aim of this study was to investigate the influence of the food matrix on the release of folate from food products during gastrointestinal passage as a potential limiting step in folate bioavailability. The fraction of folate which is released during gastrointestinal transit and, as a result, available for absorption is also called the bioaccessible folate fraction. The bioaccessible fraction of native and supplemental folate from several foods varying in matrix, folate content and processing was investigated in an *in vitro* dynamic gastrointestinal model (TIM) simulating human gastrointestinal conditions (15-19). Information about the effect of the food matrix on the bioavailability of folate can contribute to the development of a dietary strategy with the aim of optimizing the folate status of the population.

Materials and methods

Chemicals

Protease (P-5147), α-amylase (A-6211), chloramphenicol (C-0378), pepsin (P-7012), bile (porcine-bile extract, P-8631) and folic acid (F-7876) were obtained from Sigma (St. Louis, MO, USA). Pancreatic juice (Pancrex V powder), lipase (*Rhizopus* lipases F-AP 15) and conjugase (chicken pancreas, 0459-12) were obtained from Paines&Byne (Greenford, UK), Amano Pharmaceuticals (Nagoya, Japan), and Difco (Sparks, USA), respectively. (6-S-)5-methyltetrahydrofolate was obtained from Eprova (Schaffausen, Switzerland). All other chemicals were derived from Merck (Darmstadt, Germany).

Food products

As part of the EU sponsored multicentre study "Folate: From Food to Functionality and Optimal Health" several products were investigated with respect to the bioaccessibility of folate in an *in vitro* gastrointestinal model. These food products were obtained from the various centres that participated in this study and which performed further (*in vivo*) studies with these products. The characteristics of the foods are described in Table 4.1. The liquids (beer, orange juice, milk and a tomato-vegetable soup 'gazpacho') did not need further processing prior to consumption and were tested directly from the package. The green vegetables were prepared prior to testing in the gastrointestinal model according to household procedures. Before addition to the gastric compartment, the knäckebröd and vegetables were ground or cut to smaller particles to simulate chewing of the food. Oral fluid with electrolytes and α-amylase was added to simulate saliva excretion accompanying the chewing process.
In addition to the food products with native folate, a fermented milk product (filmjölk) with supplemental 5-methyltetrahydrofolate (5-CH_3H_4folate) (500 µg/L) was investigated for its folate bioaccessibility. Filmjölk (fortified with folate as previously described for other dairy products (18,19)) was investigated in the absence and presence of additional folate-binding protein (FBP) similar as in our earlier studies (18,19). FBP was added as 14.4 g whey powder (20 nmol FBP/g whey) to 360 g of filmjölk to reach an equimolar ratio between folate and FBP in the milk products.

The dynamic in vitro gastrointestinal model

The dynamic *in vitro* gastrointestinal model (TIM, Figure 4.1) has been described in detail in previous studies (15-19). The gastric small-intestinal model represents the stomach, duodenum, jejunum, and ileum. The pH curves, peristaltic movements, gastric emptying, intestinal transit, and gradual additions of digestive juices are computer-controlled events and comparable with human conditions. For simulation of absorption of low-molecular digested compounds and water, semi-permeable hollow fibre membranes with a cut-off of 5 kDa are connected with the jejunal and ileal compartments to measure the bioaccessible folate fraction.

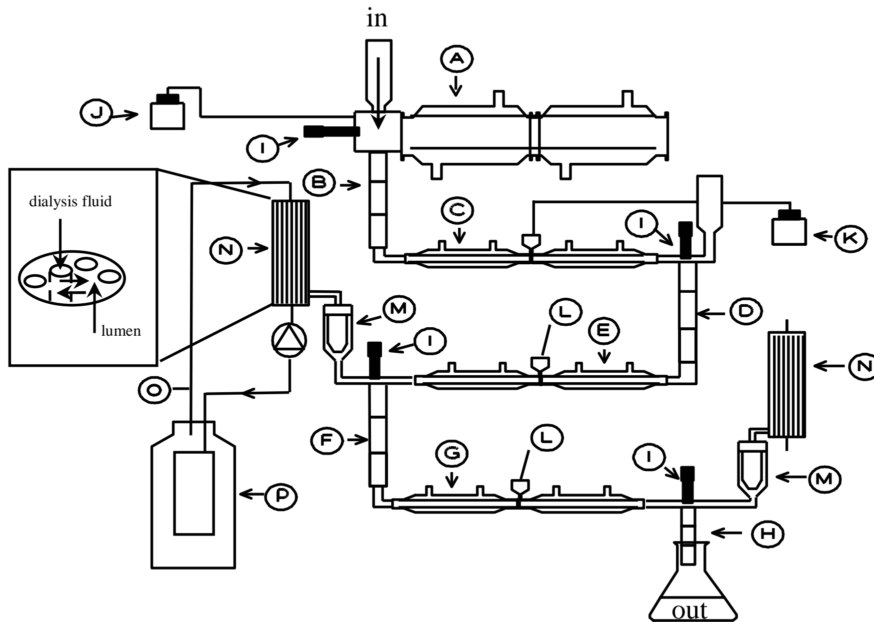


Figure 4.1. Schematic diagram of the dynamic *in vitro* gastrointestinal model (TIM): A. gastric compartment; B. pyloric sphincter; C. duodenal compartment; D. peristaltic valve; E. jejunal compartment; F. peristaltic valve; G. ileal compartment; H. ileo-caecal valve; I. pH electrodes; J. gastric secretion bottles with acid and enzymes; K. duodenal secretion bottles with bile, pancreatin, bicarbonate; L. secretion of bicarbonate to control the intestinal pH; M. pre-filter system; N. hollow fibre semi-permeable membrane system; O. water absorption system; P. closed dialysing system.

Experiments with the in vitro gastrointestinal model

In preliminary 5 h experiments in the *in vitro* gastrointestinal model we established that folate recoveries were approximately 100% if precautions were taken against folate oxidation and degradation by nitrogen flushing, protection against (UV-)light and addition of sodium-ascorbate after sampling. Dietary folate occurs as a mixture of mono- and polyglutamates. The polyglutamates are deconjugated *in vivo* prior to absorption to monoglutamates by jejunal brush-border conjugase. Since these brush-border enzymes are not present in the gastrointestinal model, we checked the dialysis membranes used in the model on complete passage of mono- and polyglutamates with liver homogenate as a source of polyglutamates. We found that all mono- and polyglutamates could pass through the membrane. Therefore, the addition of conjugase into the small-intestinal compartments was not necessary for measuring the bioaccessible folate fraction during transit through the jejunal and ileal compartments. But, external deconjugation of the samples collected from the gastrointestinal model was performed before folate analysis.

At the beginning of each experiment a test portion of 300 g of the (prepared) food products (Table 4.1), the so-called gastric intake, was put into the gastric compartment of the model. Jejunal and ileal dialysates (Figure 4.1, point N) were collected over 5 h to measure the bioaccessible fraction of folate. The non-bioaccessible folate fraction, which was transported to the large intestine, was collected as ileal delivery (Figure 4.1, point H) over a period of 5 h. At the end of the experiments,

the residues from all compartments were collected and analysed to calculate the recovery of folate. Directly after sampling, 1% (w/v) sodium-ascorbate was added to all samples for preservation reasons. After mixing, the samples were stored at minus 20˚C until folate analysis.

Folate analysis

The food (gastric intake), jejunal and ileal dialysate, ileal delivery and residual samples were analysed, in duplicate, for their folate content using a microbiological assay based upon the growth of the folate-requiring chloroamphenical-resistent *Lactobacillus casei* (NCBI 10463, ATCC, Manassas, USA) (20). Yeast extract and water were included as samples in each analysis to check the analytical performance and to correct for the background activity, respectively. From each sample, 1-20 g of was extracted using a tri-enzyme-method. Successively, 1 ml protease (2 g/L), 1 mL amylase (20 g/L) and 4 mL of chicken pancreas conjugase (5 g/L) was added to the samples and incubated for at least 3, 2 and 4h, respectively. Afterwards, the solutions were filtrated and the filtrates were diluted with phosphate buffer (pH 6.2) to a folate concentration of approximately 0.4 µg/L, corresponding with the highest concentration of folic acid included as standard on the micro titre plates. Subsequently, the wells were filled with the diluted samples and incubation medium (folic acid casei medium, 0822-15-9, Difco), containing chloramphenicol, and inoculated with the *Lactobacillus casei* strain. After an incubation period of 18-22h at 37˚C, growth was measured by fluorescence spectrometry (at 600 nm) relative to the standard concentration curve of folic acid on each micro titre plate to measure the folate content of the samples.

Calculations and statistics

The recovery (mass balance) of folate and the folate bioaccessibility were calculated as previously described (18). A high variation in folate recovery, i.e. 112 ± 24%, in the experiments with the *in vitro* model were found which could be a result of the higher variation (>20%) between duplicate analyses of the foods gazpacho, knäckebröd and canned peas as compared with the variation in duplicate analyses (<4%) for all other food products. As the high variation in folate recovery limits the comparison between tested foods with respect to their folate bioaccessibility if calculated as percentage of intake, we corrected the folate content in jejunal and ileal dialysate and in ileal delivery found in the corresponding experiments.

The data were statistically analysed by ANOVA, after Barlett's test for homogeneity of the variances. ANOVA was performed to test the overall difference between foods. When an overall significance was found, pair-wise tests on individual means were performed using the least squares means method of SAS. A P-value < 0.05 was considered as significant.

Table 4.1. Characteristics of the food products tested in the *in vitro* gastrointestinal model

Food product	Obtained from partner EU project (QLK1-1999-00576)	Preparation of food products prior to addition to gastric compartment of the gastrointestinal model[1]	Folate content of gastric intake (µg/100g)
Beer (alcoholic)	Brewing Research International (Lyttel Hall, UK)	No preparation, added directly from package	11 ± 0
Beer (non-alcoholic)	Brewing Research International (Lyttel Hall, UK)	No preparation, added directly from package	11 ± 0
Orange juice (pasteurised)	Istituto Nazionale della Nutrizione (Rome, Italy)	No preparation, added directly from package	49 ± 9
Orange juice (high presure)	Istituto Nazionale della Nutrizione (Rome, Italy)	No preparation, added directly from package	37 ± 2
Milk (pasteurised)	TNO Nutrition and Food Research (Zeist, The Netherlands)	No preparation, added directly from package	8 ± 0
Gazpacho	University of Murcia (Murcia, Spain)	No preparation, added directly from package	44 ± 26
Filmjölk + 5-CH_3H_4folate	Swedish Univ. of Agricultural Sciences (Uppsala, Sweden)	180 µg 5-CH_3H_4folate + 360 g filmjölk, stirred, incubated for 1 h at 20ºC in dark	52 ± 4
Filmjölk + 5-CH_3H_4folate +FBP	Swedish Univ. of Agricultural Sciences (Uppsala, Sweden)	180 µg 5-CH_3H_4folate + 14.4 g whey powder + 360 g filmjölk, stirred, incubated for 1 h at 20ºC in dark	68 ± 3
Spinach (soup)	Institute of Food Research (Norwich, UK)	57.3 g mashed spinach oral fluid leaves + 173.2 g water, heated in magnetron (700 W) for 3 min, mixed with 144.6 g oral fluid	12 ± 0
Spinach (leaves)	TNO Nutrition and Food Research (Zeist, The Netherlands)	80 g un-mashed spinach leaves, heated in magnetron (700 W) for 3 min, mixed with 160 g water and 160 g oral fluid	22 ± 3
Peas (canned)	University of Murcia (Murcia, Spain)	80 g peas (excluded from fluid) + 160 g water, heated in magnetron (700 W) for 3 min, mixed with 160 g oral fluid	8 ± 2
Peas (frozen)	University of Murcia (Murcia, Spain)	80 g peas (thawed) + 160 g water, heated in magnetron (700 W) for 3 min, mixed with 160 g oral fluid	13 ± 2
Knäckebröd	University of Helsinki (Helsinki, Finland)	50 g ground knäckebröd + 162.5 g water + 162.5 g oral fluid	10 ± 3

[1] 300 g of the prepared or unprepared food products was added to the gastric compartment of the gastrointestinal model (gastric intake).

Results

The folate content of the liquid and solid foods (after preparation) as added to the gastric compartment, the gastric intake, was measured and gave values between 8 and 68 µg/100g (Table 4.1). As the liquids were added directly from the package, the folate content in the (original) food products equalled the folate content in the gastric intake. The folate concentration in the mashed spinach, spinach leaves, canned peas, frozen peas and knäckebröd before preparation was calculated based upon their measured folate content in the gastric intake, and taking into account the dilution factors as a result of preparation (Table 4.1), and gave values of respectively, 80 ± 1, 111 ± 17, 39 ± 10, 67 ± 9 and 78 ± 25 µg folate/100g food.

Table 4.2. Mean ± range (n=2) folate content, given as percentage of intake corrected for recovery, in jejunal dialysate, ileal dialysate, jejunal plus ileal dialysate and ileal delivery collected between 0-5 h from the *in vitro* gastrointestinal model. Means in column with same letter are not significantly different (P < 0.05).

Food product	Jejunal dialysate	Ileal dialysate	Jejunal plus ileal dialysate	Ileal delivery
Beer (alcoholic)	67.0 ± 1.1[c]	20.4 ± 1.3[b]	87.5 ± 0.2[bc]	9.1 ± 1.1[cd]
Beer (non-alcoholic)	70.2 ± 0.6[bc]	12.7 ± 1.9[d]	82.9 ± 2.5[cde]	8.8 ± 1.9[cd]
Orange juice (pasteurised)	71.8 ± 1.3[bc]	18.9 ± 0.5[bc]	90.7 ± 1.8[ab]	8.0 ± 1.4[cde]
Orange juice (high pressure)	67.1 ± 1.4[c]	19.7 ± 1.4[b]	86.8 ± 0.1[bc]	7.9 ± 0.3[cde]
Milk (pasteurised)	55.5 ± 2.4[de]	30.4 ± 2.3[a]	85.9 ± 0.2[bcd]	6.3 ± 0.6[cde]
Filmjölk+5-CH_3H_4folate	68.2 ± 0.8[bc]	21.3 ± 3.2[b]	89.6 ± 2.4[ab]	9.1 ± 2.5[cd]
Filmjölk+5-CH_3H_4folate +FBP	50.7 ± 2.3[e]	17.1 ± 0.4[bcd]	67.8 ± 1.9[f]	30.5 ± 2.2[a]
Gazpacho	88.9 ± 1.5[a]	5.4 ± 1.1[e]	94.3 ± 2.6[a]	3.5 ± 1.4[e]
Spinach (soup)	67.9 ± 2.1[bc]	15.2 ± 0.0[cd]	83.0 ± 2.2[cde]	11.9 ± 1.9[bc]
Spinach (leaves)	59.7 ± 4.0[d]	20.6 ± 0.9[b]	80.3 ± 3.2[de]	14.8 ± 2.3[b]
Peas (canned)	58.7 ± 1.2[d]	20.2 ± 0.8[b]	78.9 ± 2.3[e]	14.6 ± 2.4[b]
Peas (frozen)	57.0 ± 0.5[d]	20.7 ± 1.5[b]	77.7 ± 0.9[e]	16.0 ± 0.4[b]
Knäckebröd	72.7 ± 1.2[b]	13.9 ± 0.5[d]	86.6 ± 1.8[bc]	9.0 ± 2.2[cd]

The kinetic profile of folate release during gastrointestinal passage varied between the food products (Table 4.2). The biggest difference in site of folate release was found between gazpacho and milk. Almost all dietary folate was released from the gazpacho matrix in the jejunal compartment while milk showed a more gradual release of folate during passage through the jejunal and ileal compartment. Disruption of the spinach matrix (spinach soup) was found to result in a higher jejunal bioaccessible folate fraction as compared with the jejunal fraction from spinach leaves (Table 4.2).

Discussion

In the present study, the effect of the food matrix on the bioaccessibility of folate was investigated by testing a variety of liquid and solid food products for their folate release during passage through an *in vitro* gastrointestinal model. Folate was found to be highly (68-94%) bioaccessible from the tested food products, which indicates that the release of folate during gastrointestinal passage will only minimally affect the bioavailability of folate. However, as a significantly lower ($P<0.05$) bioaccessible folate fraction from vegetables (78-80%) was found as compared to alcoholic beer (88%), orange juice (87-91%), knäckebröd (87%) and gazpacho (94%), the matrix of vegetables such as spinach and peas limits the release of folate, which could result in a lower folate bioavailability.

The bioavailability of dietary folate in humans is not precisely known due to the variability of data derived from several *in vivo* studies. The variability can probably be attributed to a difference in experimental approach and in response criteria. Besides, *in vivo* bioavailability studies are usually focussed only on folate-rich products such as liver, spinach, and orange juice. In our study we determined the bioaccessibility of folate from several food products varying from a low to high folate content (8 to 110 µg/100g product). For the folate-rich products spinach and orange juice, a folate release of 80 and 90%, respectively, was found which is in line with reported values for bioavailability of folate in *in vivo* studies. In a study with healthy ileostomists between 73 and 91% of the dietary folate was absorbed from two spinach meals based on 24 h postdose stomal folate excretion (21). In a controlled dietary human intervention study a bioavailability of 60 to 98% of dietary folate from vegetables and citrus fruit, relative to folic acid, was found (10). The lower bioaccessible fraction of folate from green vegetables as we found *in vitro* seem to agree with results from a rat bioassay, which showed a lower bioavailability of dietary folate from peas and spinach as compared with that from orange juice based upon serum and liver folate concentrations (22).

In human studies was found that the disruption of the whole leaf matrix induced a larger plasma folate response than whole leaf spinach (13-14). In our *in vitro* studies, we also tested mashed spinach, prepared as a soup, and whole leaf spinach to measure the bioaccessible fractions of both spinach matrices. A significantly higher ($P<0.05$) bioaccessible fraction of folate from spinach soup (68%) was found in the jejunal compartment as compared with that from whole leaf spinach (60%). Present study indicates that disruption of the vegetable matrix can lead to an earlier (faster) release of folate in the gastrointestinal tract. As folate is absorbed mainly in the proximal part of the small intestine, an early release of folate from the food matrix in the gastrointestinal tract can result in a higher bioavailability of folate. A difference in kinetic profile of folate release during transit through the gastrointestinal tract was not only found for the spinach soup and spinach leaves but also between several other products tested in the *in vitro* model. For example, folate from gazpacho was released almost exclusively in the jejunal compartment, while folate from milk was released more gradually (Table 4.2). The impact of a difference in kinetic profile of folate release in the human gastrointestinal tract on the bioavailability of folate is not investigated so far.

The bioaccessibility of native folate appeared not to be different from added folate as no significant difference was found between the bioaccessibility of folate from beer (88%), orange juice (87-91%), gazpacho (94%), milk (86%), knäckebröd (87%) and that from filmjölk fortified with 5-CH_3H_4folate (90%) (Table 4.2). In previous studies we evaluated the bioaccessibility of 5-CH_3H_4folate from fortified pasteurised milk (18) and from yoghurt (19). Calculated in the same way as in the present study, the bioaccessibility of 5-CH_3H_4folate from fortified filmjölk (90%) was found to be in the same range as that from 5-CH_3H_4folate-fortified milk and yoghurt (82-83%). These earlier studies demonstrated that FBP significantly lowered the folate bioaccessibility from 5-CH_3H_4folate-fortified yoghurt but had no significant effect on the folate bioaccessibility from 5-CH_3H_4folate-fortified milk (18,19). Current study showed that the addition of FBP led to a 22% lower bioaccessible 5-CH_3H_4folate fraction from filmjölk, which is in line with the 25% decrease in bioaccessibility of 5-CH_3H_4folate from yoghurt (19). Fortified filmjölk, with or without additional FBP, was recently tested in a study with nine healthy ileostomy volunteers as part of the EU sponsored multicentre project. This *in vivo* study demonstrated that FBP reduced the bioavailability of 5-CH_3H_4folate from filmjölk (23) which confirms our *in vitro* findings.

In this study, we focussed on the release of folate from the food matrix as this is one of the processes which determine the bioavailability of folate in humans. These studies showed that folate is easily released from a variety of food matrices, including fortified products. Only a limited effect of the (vegetable) matrix was found on the bioaccessibility of folate which will probably result in a lower bioavailability of folate from green vegetables as compared with other food matrices (such as orange juice, milk and knäckebröd). After folate is released from the food matrix, it can be absorbed from the intestinal lumen to reach the blood stream. This second step in the bioavailability of folate can also be influenced by the food matrix as certain ingredients might interact with the absorption of folate. Information about these individual processes (gastrointestinal release and absorption) in the bioavailability of folate should be integrated to be able to recommend a combination of food products in a dietary strategy leading to an optimal bioavailability of folate and, as a result, an enhanced folate status of the population.

In conclusion, this study showed that folate is highly bioaccessible from liquid foods and knäckebröd and ~10% less available from vegetables. This indicates that the food matrix has a limited impact on the release of folate from food products during gastrointestinal passage.

References

1. MRC Vitamin Study Research Group (1991) Prevention of neural tube defects: results of the Medical Research Council Vitamin Study. Lancet 338: 131-137
2. Cuskelly GJ, McNulty H, Scott JM (1996) Effect of increasing dietary folate on red-cell folate: implications for prevention of neural tube defects. Lancet 347: 657-659
3. Boushey CJ, Beresford AA, Omen GS, Motulsky AG (1995) A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. JAMA 274: 1049-1057
4. Graham IM, O'Callaghan P (2000) The role of folic acid in the prevention of cardiovascular disease. Curr Opin Lipidol 11:577-587
5. Giovannucci E, Stampfer MJ, Colditz GA, Hunter DJ, Fuchs C, Rosner BA, Speizer FE, Willet WC (1998) Multivitamin use, folate, and colon cancer in women in the Nurses' Health Study. Ann Intern Med 129: 517-524
6. Rampersaud GC, Bailey LB, Kauwell GPA (2002) Relationship of folate to colorectal and cervical cancer: review and recommendations for practitioners. J Am Diet Assoc 102: 1273-1282
7. De Bree A, Van Dusseldorp M, Brouwer IA, Van het Hof KH, Steegers-Theunissen RP (1997) Folate intake in Europe: recommended, actual and desired intake. Eur J Clin Nutr 51: 643-660
8. De Bree A, Verschuren WMM, Blom HJ, Kromhout D (2001) Association between B vitamin intake and plasma homocysteine concentration in the general Dutch population aged 20-65y. Am J Clin Nutr 73: 1027-1033
9. Venn BJ, Mann JI, Williams SM, Riddell LJ, Chisholm A, Harper MJ, Aitken W (2002) Dietary counseling to increase natural folate intake: a randomized placebo controlled trial in free-living subjects to assess effects on serum folate and plasma total homocysteine. Am J Clin Nutr 76: 758-765
10. Brouwer IA, van Dusseldorp M, West CE, Meyboom S, Thomas CMG, Duran M, van het Hof KH, Eskes TKAB, Hautvast JGAJ, Steegers-Theunissen RPM (1999) Dietary folate from vegetables and citrus fruit decreases plasma homocysteine concentrations in humans in a dietary controlled trial. J Nutr 129: 1135-1139
11. Brouwer IA, Dusseldorp Mv, West CE, Steegers-Theunissen RPM (2001) Bioavailability and bioefficacy of folate and folic acid in humans. Nutr Res Rev 14:267-293
12. Verwei M, van den Berg H, Havenaar R, Groten JP (2004) Effect of folate-binding protein on intestinal transport of folic acid and 5-methyltetrahydrofolate across Caco-2 cells. Eur J Nutr (in press)
13. Castenmiller JJ, van der Poll CJ, West CE, Brouwer IA, Thomas CM, van Dusseldorp M (2000) Bioavailability of folate from processed spinach in humans. Effect of food matrix and interaction with carotenoids. Ann Nutr Metals 44: 163-169
14. Van het Hof KH, Tijburg LB, Pietzrik K, Westrate JA (1999) Influence of feeding different vegetables on plasma levels of carotenoids, folate and vitamin C. Effect of disruption of the vegetable matrix. Br J Nutr 82: 203-212
15. Minekus M, Marteau P, Havenaar R, Huis in 't Veld JHJ (1995) A multicompartimental dynamic computer-controlled model simulating the stomach and small intestine. ATLA 23: 197-209
16. Krul CAM, Luiten-Schuite A, Baan R, Verhagen H, Mohn G, Feron V, and Havenaar R (2000) Application of a dynamic *in vitro* gastrointestinal tract model to study the availability of food mutagens, using heterocyclic aromatic amines as model compounds. Food Chem Toxicol 38: 783-792
17. Krul CAM, Luiten-Schuite A, Tenfelde A, van Ommen B, Verhagen H, Havenaar R (2001) Antimutagenic activity of green tea and black tea extracts studied in a dynamic in vitro gastrointestinal model. Mut Res 474: 71-85

18. Verwei M, Arkbåge K, Havenaar R, van den Berg H, Witthöft C, Schaafsma G (2003) Folic acid and 5-Methyltetrahydrofolate in fortified milk are bioaccessible as determined in a dynamic in vitro gastrointestinal model. J Nutr 133: 2377-2383
19. Arkbåge K, Verwei M, Havenaar R, Witthöft C (2003) Bioaccessibility of folic acid and (6S)-5-methyltetrahydrofolate decreases after the addition of folate-binding protein to yogurt as studied in a dynamic in vitro gastrointestinal model. J Nutr 133: 3678-3683
20. Williams S (ed) (1984) Microbiological assays. In: Official Methods of Analysis of the Association of Official Analytical Chemist, fourteenth edition. AOAC Inc., Arlington, Virginia, USA, pp 862-873
21. Konings EJ, Troost FJ, Castenmiller JJ, Roomans HH, Van den Brandt PA, Saris WH (2002) Intestinal absorption of different types of folate in healthy subjects with an ileostomy. Br J Nutr 88: 235-242
22. Clifford AJ, Heid MK, Peerson JM, Bills ND (1991) Bioavailability of food folates and evaluation of food matrix effects with a rat bioassay. J Nutr 121: 445-453
23. Arkbåge K (2003) Vitamin B_{12} folate and folate-binding proteins in dairy products. Analysis, process retention and bioavailability. Doctoral Thesis, Swedish University of Agricultural Sciences, Uppsala, Sweden.

5

The binding of folic acid and 5-methyltetrahydrofolate to folate-binding proteins during gastric passage differs in a dynamic in vitro gastrointestinal model

Journal of Nutrition 134: 31-37, 2004

Miriam Verwei[1,2]
Karin Arkbåge[3]
Hans Mocking[1]
Robert Havenaar[1]
John Groten[1]

[1]TNO Nutrition and Food Research, Zeist, The Netherlands
[2]Department of Human Nutrition, Wageningen University, Wageningen, The Netherlands
[3]Department of Food Science, Swedish University of Agricultural Sciences, Uppsala, Sweden

Abstract

Despite its low natural folate content, milk is responsible for 10-15% of the daily folate intake in countries with a high dairy consumption. Milk products can be considered as a potential matrix for folate fortification, e.g. with synthetic folic acid, to enhance the daily intake of folate. In untreated milk, the natural folate, 5-methyltetrahydrofolate (5-CH_3-H_4folate), occurs bound to folate-binding proteins (FBP). In this study the extent of binding to FBP for folic acid and 5-CH_3-H_4folate was investigated in a dynamic in vitro model simulating human gastric passage. Protein binding of folic acid and 5-CH_3-H_4folate was characterized using gel-exclusion chromatography. Before gastric passage, folic acid and 5-CH_3-H_4folate were mainly bound to FBP (76-79%) while 7% was free. Folic acid remained bound to FBP to a similar extent after gastric passage. For 5-CH_3-H_4folate the FBP-bound fraction gradually decreased from 79% to 5% and the free fraction increased from 7% to 93%. So, while folic acid enters the proximal part of the intestine bound to FBP, 5-CH_3-H_4folate appears mainly to be present as free folate in the duodenal lumen. The stability of FBP was found to be similar in both folate/FBP mixtures, i.e. 70% of the initial FBP content could be retained after gastric passage. This study indicated that FBP are partly stable during gastric passage but show different binding characteristics for folic acid and 5-CH_3-H_4folate in the duodenal lumen. This could result in a different bioavailability from folic acid- and 5-CH_3-H_4folate-fortified milk products.

Introduction

Prevention of neural tube defects (1-2) and lowering risk for cardiovascular disease (3-4) and colon cancer (5-6) can be achieved by an adequate folate intake. This can be reached by a high consumption of folate-rich food products or by the intake of supplements or fortified food products (7-9). The dominant folate compound in natural food products is 5-methyltetrahydrofolate ($5\text{-}CH_3\text{-}H_4$folate), while in supplements and fortified products mostly folic acid is used.
Although the natural folate content in milk is low compared to folate-rich food products such as vegetables and citrus fruit, milk is responsible for 10-15% of the daily folate intake in European countries with a high milk consumption, such as The Netherlands (10) and Sweden (11). Milk can be considered as a potential matrix for folate (folic acid or $5\text{-}CH_3\text{-}H_4$folate) fortification as it is highly consumed and might enhance the folate bioavailability from the diet (12) and, in addition, folic acid and $5\text{-}CH_3\text{-}H_4$folate appeared to be highly bioaccessible from the milk matrix (13).
In untreated milk, $5\text{-}CH_3\text{-}H_4$folate occurs bound to Folate-Binding Proteins (FBP) (14-16). The role of FBP in folate bioavailability is unclear. It has been suggested that FBP protects folate from bacterial uptake and degradation (17,18) or may play a role in sequestering folate from the blood plasma into the mammary glands and thereby supplying folate to the newborn (19). FBP could also affect mucosal folate transport, although both inhibition and enhancement have been reported (20-23). The influence of FBP on folate absorption might depend on its binding to folate after gastric passage. In a previous study, the effect of FBP on the absorption of folic acid was investigated in rats (22) in which they received free folic acid or folic acid bound to bovine milk FBP. It was found that under acidic gastric conditions ($pH < 4.5$) folic acid was released from FBP and recombines in the small intestine (pH 6-7). Also in a study (24) with 6-d-old goat kids, who received by bottle FBP-bound folic acid in goat's milk, it was shown that gastric acidity and gastrointestinal digestive enzymes had little effect on the binding characteristics of FBP for folic acid. In our previous studies (13,25) using an *in vitro* dynamic gastrointestinal model it was found that FBP partly survives gastrointestinal passage and lowers the folate bioaccessibility and these effects appeared to be higher in folic acid-fortified milk products as compared with $5\text{-}CH_3\text{-}H_4$folate-fortified milk products. This suggests a different extent of binding to FBP for folic acid and $5\text{-}CH_3\text{-}H_4$folate.
The present study was performed to investigate the binding characteristics of FBP for folic acid and $5\text{-}CH_3\text{-}H_4$folate and to establish the impact of FBP stability on folate binding during gastric passage in a controlled *in vitro* model simulating human gastric conditions. The binding of folic acid and $5\text{-}CH_3\text{-}H_4$folate to an equimolar amount of FBP during gastric passage were compared to gain more insight in the effect of FBP on the bioavailability of folic acid and $5\text{-}CH_3\text{-}H_4$folate from milk products.

Materials and methods

Chemicals

5-CH_3-H_4folate and folic acid were studied as a mixture of radiolabeled and non-radiolabeled compounds. Radiolabeled folate compounds, [^{3}H]-folic acid (888 GBq/mmol; 37 GBq/L) and [^{14}C]-(6-RS)-5-CH_3-H_4folate (2.11 GBq/mmol; 3.7 GBq/L) were obtained from Amersham Pharmacia (Buckinghamshire, England) and checked for their purity. The non-radiolabeled standard solutions of folic acid and (6-RS)-5-CH_3-H_4folate were obtained from Schircks' Laboratories (Jona, Switzerland) and were controlled on purity according to Van den Berg (26). The whey powder with a FBP content of 20 nmol/g was provided by Arla Foods (Stockholm, Sweden). The antibody against FBP from bovine milk (Rabbit anti-bovine FBP 24739) was obtained from the State Serum Institute (Copenhagen, Denmark). Sephadex G75 Superfine powder, scintillation liquid (Superphase 2) and the low molecular weight gel filtration calibration kit were obtained from Amersham Pharmacia. The gastric enzymes pepsin (2200 U/mg, P7012) and lipase (150.000 U/g; Rhizopus lipases F-AP 15) were obtained from Sigma (St.Louis, MO) and Amano Pharmaceuticals (Nagoya, Japan), respectively. The 0.1 mol/L phosphate buffer (pH 7.2) used for gel filtration contained 13.4 g/L Na_2HPO_4, 3.5 g/L NaH_2PO_4, 8.3 g/L NaCl, and 0.02 g/L Na-azide (all from Sigma). For SDS-PAGE with immunoblotting Tris-Glycine gel (Pager™ Gold Precast Gels, 8 x10 cm, 12%, Sanvertech, Heerhugowaard, The Netherlands), nitrocellulose membranes (Protran, Schleicher&Schuell, Dassel, Germany) and goat-anti-rabbit IgG alkaline phosphatase conjugate (Dako, Glostrup, Denmark) were used. All other chemicals were obtained from Sigma.

Folate binding to FBP during gastric passage under static experimental conditions

First, the binding characteristics of FBP were studied under static experimental conditions, i.e. in test tubes for 1-2 h. Whey powder was used as the source of FBP. The FBP suspensions were made by mixing 50 mg whey powder (~ 1 nmol FBP) with 6 mL 0.1 mol/L phosphate buffer (pH 7.2). An equimolar FBP/folate suspension was made by adding [^{3}H]-folic acid or [^{14}C]-5-CH_3-H_4folate combined with non-radiolabeled folic acid or 5-CH_3-H_4folate to reach 1 nmol folate/6 mL suspension (167 nmol/L). The FBP and folate concentrations used in this study were comparable to the natural FBP and folate concentrations in milk, i.e. 110-220 nmol folate/L and 160-210 nmol FBP/L (27). The mixtures of folic acid, 5-CH_3-H_4folate or a combination of both folate compounds with the FBP suspensions were incubated for 1 h at pH 7.2 at ca 20 ºC to allow association. Various test conditions were simulated in the incubation experiments (n=2) (Table 5.1). Besides the incubation at pH 7, also an additional incubation period of 1 h at pH 3 (gastric pH conditions) with or without pepsin was tested. The pH of all mixtures was adjusted to pH 7 prior to elution over the Sephadex column to study the binding profile of the whey proteins.

Folate binding to and fate of FBP during gastric passage under dynamic experimental conditions

After the static experiments, FBP's binding characteristics for 5-CH_3-H_4folate and folic acid were studied in a dynamic *in vitro* gastrointestinal model (TIM) as described previously (13,25,28-30). This model has compartments for the stomach, duodenum, jejunum and ileum. Each compartment has a flexible inner wall surrounded by water at 37ºC to squeeze the walls in order to mix the food with the 'secreted' electrolytes and enzymes and to transport the chyme by peristaltic movements. The pH values as well as the gastric emptying and small intestinal passage of the food are controlled according to pre-set curves. Conditions were simulated according to the *in vivo* situation in adult humans.

In this model we studied the fate of the FBP-folate complex during gastric passage and the possible recombination or stability of the FBP-folate complex in the duodenum. The folic acid-fortified and the 5-CH_3-H_4folate-fortified FBP suspensions were tested separately in duplicate experiments. Artificial oral and gastric juices with lipase and pepsin were gradually added into the gastric compartment. The pH was continuously measured and regulated by the addition of HCl to follow the pre-set pH curve. This curve corresponded to the *in vivo* pH drop in the stomach of adults after consumption of milk products. In the duodenal compartment the pH was controlled at 6.5 by the addition of sodium bicarbonate.

The FBP suspension was made by dissolving 2.75 g whey powder in 330 mL 0.01 mol/L phosphate buffer. This resulted in a final FBP content of approximately 55 nmol. An equimolar folate/FBP mixture was obtained by adding 55 nmol folic acid or 5-CH_3-H_4folate, as a mixture of [^{3}H]-folic acid or [^{14}C]-5-CH_3-H_4folate with non-radiolabeled folic acid and 5-CH_3-H_4folate, to the FBP suspension. After one-hour incubation at ca 20 ºC to induce association between folate and FBP, a test portion of 300 g was put into the gastric compartment of the gastrointestinal model. The remaining 30 g of the FBP suspension was used for the determination of the folic acid and 5-CH_3-H_4folate binding to FBP before gastric passage. Intestinal material from the duodenal compartment was collected during 0-30, 30-60, 60-90 and 90-120 min after starting the experiments. All collected samples were stored at 2-8ºC and analyzed by gel filtration within five days. After 120 min the stomach was almost completely emptied, according to the pre-set curve for gastric emptying in humans, and the residual contents in the stomach and duodenum were collected for calculation of the mass balance of folate. The folate content of the collected samples was determined by radioactivity measurements with a scintillation counter (Wallac 1409, PerkinElmer).

Characterization of FBP in bovine whey

Gel filtration

The distribution of [^{3}H]-folic acid or [^{14}C]-5-CH_3-H_4folate over the whey proteins was studied by gel filtration on a Sephadex G75-column (2.6 cm x 30 cm). The column was equilibrated with 0.1 mol/L phosphate buffer (pH 7.2). The [^{3}H]-folic acid or [^{14}C]-5-CH_3-H_4folate-fortified FBP suspensions (3 mL), whether or not digested, were eluted with the phosphate buffer at a flow rate of 25 mL/h. The eluent was measured spectrophotometrically with an UV detector at 280 nm, as

indicator of protein content, and subsequently collected as fractions at 8 min interval during a total run time of 800 min. Portions (200-500 µL) of these fractions were quantified for their [^{3}H]-folic acid or [^{14}C]-5-CH_3-H_4folate content by measuring radioactivity with a scintillation counter after addition of 4 ml of scintillation suspension.
The column was calibrated with the elution volumes of proteins within a low molecular weight gel filtration calibration kit, including Blue Dextran 2000 (200 kDa), Albumin (67 kDa), Ovalbumin (43 kDa), Chymotrypsinogen A (25 kDa) and Ribonuclease A (14 kDa). The exact elution volume of FBP was determined by quantification of the FBP content in the collected fractions over time.

ELISA

The FBP content of the samples from the experiments with the gastrointestinal model and the gel filtration samples was analyzed by a two-site enzyme-linked immunosorbent assay (ELISA) according to Høier-Madsen et al. (31) to determine, respectively, the FBP stability after gastric passage and the elution volume of FBP. To 1 g of sample 0.09 g of Triton X-100 was added. The sample was put on a shaking device and incubated for 45 min at ca 20ºC. After this extraction the sample was applied to a microtiter plate and followed by ELISA procedure as described previously (32).

SDS-PAGE and Immunoblotting

The whey suspensions, before and after incubation with pepsin at pH 3, were subjected to a 12% gradient Tris-Glycine gel and, subsequently, blotted onto a nitrocellulose membrane. Immunodetection was carried out by incubating the blots with polyclonal rabbit antibodies against bovine FBP followed by goat-anti-rabbit IgG alkaline phosphatase conjugate, tetrazoliumchloride and 5-bromo-4-chloro-3-indolyl phosphate (33-34).

Results

Characterization of FBP in bovine whey

The whey suspension was analyzed via molecular size exclusion (Sephadex G75) at UV absorbance at 280 nm (Figure 5.1a). The proteins were separated into two visible peaks. Based on calibration proteins, the first peak (elution volume ~ 50 mL) corresponded to proteins larger than approximately 60 kDa and the second peak (elution volume ~ 75 mL) to proteins between 30-40 kDa. The ELISA analysis showed a maximum FBP content at an elution volume of approximately 75 ml (Figure 5.1b), corresponding with the elution volume of the second peak, i.e. proteins between 30-40 kDa (Figure 5.1a). No FBP was detected at the elution volume of the first peak of the UV-chromatogram indicating that the proteins larger than 60 kDa did not contain ELISA detectable FBP.

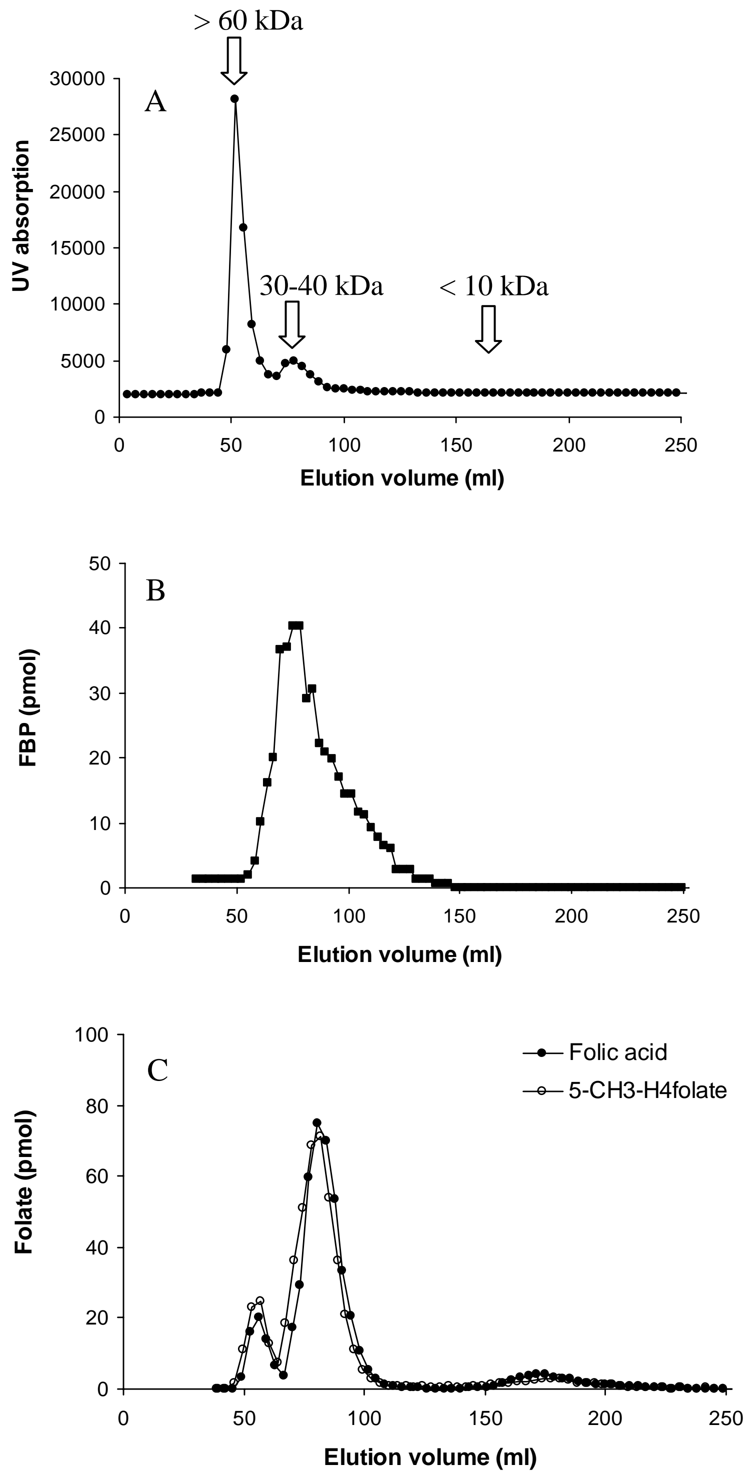


Figure 5.1. Sephadex gel filtration chromatography of a whey suspension. A) UV spectrum (280 nm) and B) FBP content (ELISA) of elution profile of the whey suspension and C) [^{3}H]-folic acid or [^{14}C]-5-CH_3-H_4folate binding characteristics, measured with a scintillation counter, in the elution profile of [^{3}H]-folic acid or [^{14}C]-5-CH_3-H_4folate fortified whey suspensions.

The extent of protein-bound folate was studied after incubation of the FBP suspension with radiolabeled 5-CH_3-H_4folate or folic acid (Figure 5.1c). Based on the folate content, three folate peaks were visible, successively corresponding to compounds with a molecular weight larger than 60 kDa, compounds between 30-40 kDa (i.e. FBP-bound folate), and compounds smaller than 10 kDa (i.e. free folate). The distribution of [^{3}H]-folic acid or [^{14}C]-5-CH_3-H_4folate over the 3 fractions was 11%, 79%, 10% and 14%, 79%, 7%, respectively (Table 5.1).

Table 5.1 Distribution of [^{3}H]-folic acid and [^{14}C]-5-CH_3-H_4folate over the collected whey protein fractions after gel filtration of the [^{3}H]-folic acid and/or [^{14}C]-5-CH_3-H_4folate fortified whey mixtures tested in static in vitro experiments.

Protein fraction (kDa)	Folate fortificant	Distribution of folic acid and 5-CH_3-H_4folate over protein fractions (%)			
		Folic acid or 5-CH_3-H_4folate fortified whey suspension[1,2]			Folic acid and 5-CH_3-H_4folate fortified whey suspension[1,3]
		pH 7	pH 3	pH 3 + pepsin	pH 7
> 60	Folic acid	11 ± 1	13 ± 0	10 ± 4	17 ± 1
30-40		79 ± 0	78 ± 0	78 ± 6	65 ± 1
< 10		10 ± 1	9 ± 0	12 ± 2	18 ± 1
> 60	5-CH_3-H_4folate	14 ± 0	16 ± 0	10 ±8	6 ± 0
30-40		79 ± 0	79 ± 0	27 ± 1	38 ± 2
< 10		7 ± 0	5 ± 0	63 ± 7	56 ± 2

[1] Values are means ± range, n=2, [2] The pH was adjusted to pH 7 prior to elution over the Sephadex column, [3] Folic acid and 5-CH_3-H_4folate were added as an equimolar mixture to the whey suspension.

Folate binding to FBP during gastric passage under static experimental conditions

The extent of binding to FBP for folic acid and 5-CH_3-H_4folate was studied under static experimental conditions simulating gastric passage. Incubation of the FBP suspension with folic acid or 5-CH_3-H_4folate at pH 7, showed that the major part (79%) of both folate compounds was initially bound to FBP prior to the incubation period at pH 3 (Figure 5.1c, Table 5.1). No difference in the amount of bound folic acid (78%) was found after incubation at pH 3 with or without pepsin (Table 5.1). Incubation at pH 3 without pepsin neither had an effect on the extent of binding to FBP for 5-CH_3-H_4folate (79%). However, the FBP-bound fraction of 5-CH_3-H_4folate decreased from 79% to 27% after incubation at pH 3 with pepsin for 1 h. At the same time the fraction of free 5-CH_3-H_4folate increased from 7% to 63%. This indicated that a major portion of 5-CH_3-H_4folate could occur free in the duodenal lumen. The whey proteins were also incubated with a mixture of folic acid and 5-CH_3-H_4folate (both in a 1:1 molar ratio with FBP) and the FBP-bound fractions were compared with those after the incubation with the single folate compounds. In this mixture of folic acid and 5-CH_3-H_4folate a small decrease in FBP-bound folic acid (from 79% to 65%) and a pronounced decrease in FBP-bound 5-CH_3-H_4folate (from 79% to 38%) were observed.

FBP in the whey suspension showed two clear bands between 30 and 40 kDa with SDS-PAGE combined with immunoblotting. After pepsin incubation at pH 3, the intensity of the bands was lowered.

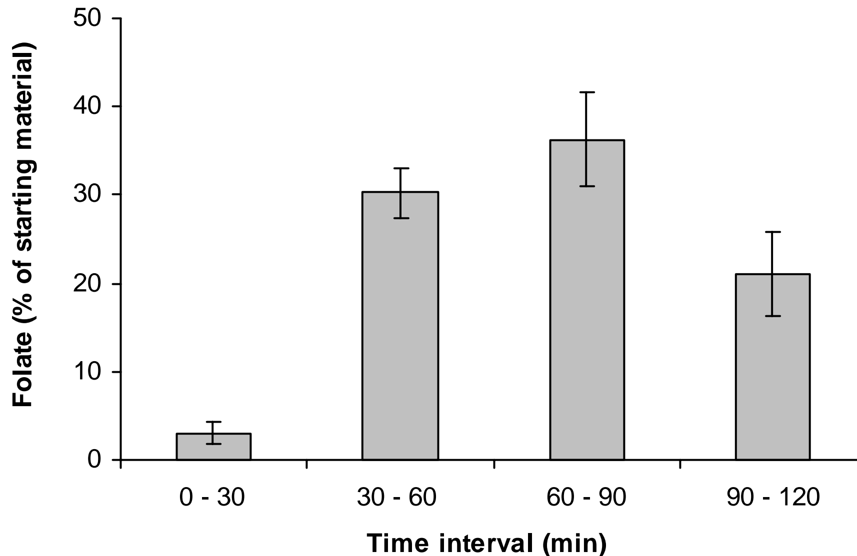


Figure 5.2. Folate content in the duodenal compartment, given as percentage of the starting material, collected during time intervals 0-30, 30-60, 60-90 and 90-120 min after gastric passage of folate-fortified whey suspensions in the *in vitro* gastrointestinal model. Results are mean ± stdev, n=4.

Folate binding to and fate of FBP during gastric passage under dynamic experimental conditions

The extent of binding of folic acid and 5-CH_3-H_4folate to FBP was investigated in duplicate experiments in the gastrointestinal model. The mass balance of folate in these experiments was 102 ± 1% (n=4). The gel filtration analyses of the whey suspension (gastric intake) and the samples of the duodenal lumen gave an analytical recovery of 98 ± 2% (n=20). The gastric passage of folic acid and 5-CH_3-H_4folate over time as measured in the duodenal compartment showed that most of the folate entered the proximal part of the intestine within 30-90 min after the start of the experiment (Figure 5.2). The distribution of folic acid and 5-CH_3-H_4folate over the protein fractions was determined in the gastric intake (0 min) and in the duodenal samples collected during 0-30, 30-60, 60-90, 90-120 min (Figure 5.3). At initial test conditions the major part of folic acid was bound to FBP and this fraction (76-81%) remained constant in the five successive samples over time during gastric passage (Table 5.2). Also the binding of folic acid to proteins larger than 60kDa and the free folic acid fraction remained unchanged over time. A similar initial FBP-bound fraction (79%) was observed for 5-CH_3-H_4folate before digestion. However, during gastric passage the FBP-bound 5-CH_3-H_4folate fraction was decreased from 79% to 5% in 2 h. Consequently, the fraction of free 5-CH_3-H_4folate increased from 7% in the initial whey suspension before gastric passage to 93%

in the duodenal sample collected between 90-120 min. The data from the duplicate experiments show a very low variation, which allows evaluation of the difference in the binding of folic acid and 5-CH_3-H_4folate to FBP during gastric passage.

The initial FBP concentration in the whey suspensions added to the gastric compartment was 158 ± 44 nmol/L (n=4). During gastric passage (0-120 min), 70% of the initial amounts of FBP in both folic acid- and 5-CH_3-H_4folate-fortified whey suspensions were recovered in the duodenal lumen (Table 5.3).

Table 5.2. Distribution of [^{3}H]-folic acid and [^{14}C]-5-CH_3-H_4folate over the collected whey protein fractions after gel filtration of the [^{3}H]-folic acid or [^{14}C]-5-CH_3-H_4folate fortified whey suspensions prior to (gastric intake) and after gastric passage (duodenal samples) in the dynamic in vitro gastrointestinal model.

		Distribution of folic acid or 5-CH_3-H_4folate over protein fractions (%)[1]				
Protein fraction (kDa)	Folate fortificant	Gastric intake	Duodenal samples collected between			
		0 min	0-30 min	30-60 min	60-90 min	90-120 min
> 60	Folic acid	17 ± 2	12 ± 2	12 ± 1	12 ± 2	10 ± 3
30-40		76 ± 2	80 ± 1	80 ± 1	80 ± 1	81 ± 2
< 10		7 ± 1	8 ± 1	8 ± 1	8 ± 1	9 ± 1
> 60	5-CH_3-H_4folate	14 ± 0	8 ± 2	5 ± 0	2 ± 0	2 ± 0
30-40		79 ± 0	57 ± 5	42 ± 3	9 ± 1	5 ± 0
< 10		7 ± 0	35 ± 7	53 ± 3	89 ± 1	93 ± 0

[1] Values are means ± range, n=2.

Table 5.3. The FBP content given as percentage of the initial amount in the whey suspension in the duodenal samples collected from the dynamic in vitro gastrointestinal model[1,2].

Folate fortificant	FBP (% of initial amount) in duodenal samples collected between				Sum
	0-30 min	30-60 min	60-90 min	90-120 min	0-120 min
Folic acid	2 ± 1	24 ± 2	28 ± 2	16 ± 1	70 ± 1
5-CH_3-H_4folate	3 ± 1	25 ± 2	33 ± 3	9 ± 1	70 ± 7

[1] The values are means ± range, n=2, [2] Quantified by ELISA.

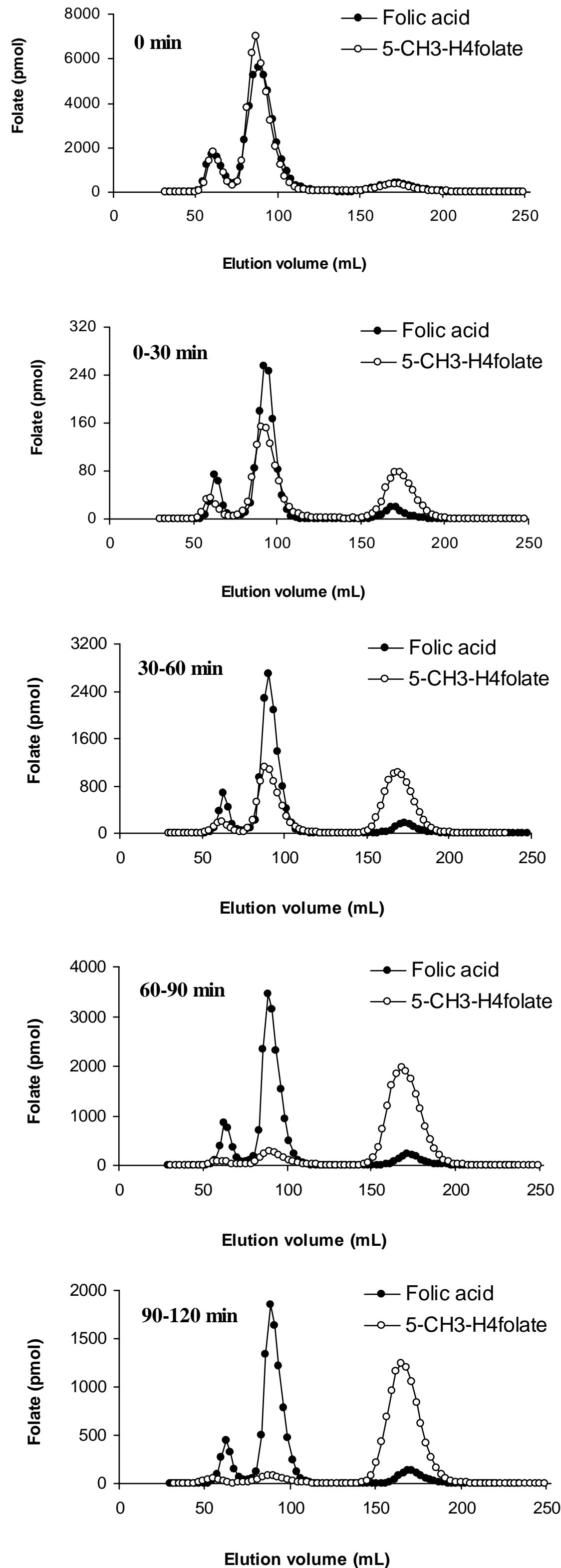


Figure 5.3. Sephadex gel filtration chromatography of [^{3}H]-folic acid-fortified or [^{14}C]-5-CH_3-H_4folate-fortified whey suspensions prior to and after gastric passage in the *in vitro* gastrointestinal model. The folate content (pmol), based on radioactivity, is plotted against the elution volume in the gastric intake (0 min) and in the duodenal samples collected between 0-30 min, 30-60 min, 60-90 min and 90-120 min.

Discussion

The present study was performed to investigate the stability and binding characteristics of FBP for folic acid and 5-CH_3-H_4folate of FBP during gastric passage. Before digestion of the folate-fortified FBP suspensions from whey, the major part of both labeled folate compounds (76-79%) appeared to be bound to proteins with a molecular weight of 30-40 kDa (Tables 5.1 and 5.2). ELISA analysis of the collected fractions (Figure 5.1b) showed that the fractions between 30-40 kDa contained FBP. This was confirmed by SDS-PAGE electrophoresis combined with immunoblotting. A molecular weight of FBP between 30-40 kDa is in line with the previous reported studies (35-37). It appeared that folic acid and 5-CH_3-H_4folate were initially bound to FBP to a similar extent. Only a minor part of folic acid and 5-CH_3-H_4folate was present as free folate (7-10%) or was bound to proteins larger than 60 kDa (11-17%). Interestingly, no FBP was detected in the protein fraction larger than 60kDa with the immuno-assays, which indicated that dimers/polymers of FBP were not present in the whey suspensions.

Exposing FBP, in a whey suspension, to an equimolar mixture of folic acid and 5-CH_3-H_4folate resulted in a low binding of 5-CH_3-H_4folate (38%) and a relatively high binding of folic acid (65%) to FBP (Table 5.1), indicating that FBP has a higher affinity for folic acid than for 5-CH_3-H_4folate. This is in agreement with the results found in previous studies in which FBP binding characteristics were investigated in *in vitro* experiments at pH 5.0 and 7.4 (38,39). This difference in affinity for FBP between folic acid and 5-CH_3-H_4folate was found to vary within the pH range of 7.4 to 10.1 (40).

The present study also showed that incubation at pH 3 had no effect on the extent of binding of folic acid and 5-CH_3-H_4folate to FBP once the pH of the incubation medium was returned to 7, reflecting the actual pH changes occurring during gastric and duodenum passage. An explanation may be that at low pH dissociation of folate takes place which is followed by a reassociation of folate to FBP at neutral pH. This is in line with other studies (22,35,36) which show that the dissociation of folic acid from FBP is completely reversible, even after pepsin treatment (22). We also found that incubation of the folic acid-FBP suspension at pH 3 with pepsin had no effect on the binding of folic acid to FBP (remained 78%). However, the FBP binding characteristics for folic acid are apparently different from the binding to 5-CH_3-H_4folate as we demonstrated a marked decrease in FBP-bound fraction (27%) after pepsin incubation of the 5-CH_3-H_4folate-FBP suspension. This different effect of pepsin on binding of folic acid and 5-CH_3-H_4folate to FBP suggests a difference in FBP binding characteristics for the folate vitamers.

Besides experiments under static conditions, experiments in a gastrointestinal model were performed as this model simulates the kinetic digestion and passage of the whey suspension from the stomach into the duodenum. These studies show that the amount of FBP-bound folic acid remained constant during the gastric passage from 0 to 120 min, indicating no change in the extent of folic acid binding to FBP (Table 5.2, Figure 5.3). In contrast, the FBP-bound 5-CH_3-H_4folate fraction gradually decreased during gastric passage from 79% to 5% within 120 min. The results obtained with these static and dynamic *in vitro* experiments simulating gastric conditions give the first evidence that the extent of binding to FBP is higher for folic acid than for 5-CH_3-H_4folate after

gastric passage. It should be noted that these FBP binding characteristics for folic acid and 5-CH_3-H_4folate have been established for FBP in whey powder. To draw conclusions about the FBP binding characteristics in milk products, results obtained in the present study should be extrapolated with caution. Direct extrapolation of the binding characteristics of FBP in whey protein concentrate to those in milk products might not as such be possible as a previous study (41) showed different binding properties of FBP in raw milk, pasteurized milk and whey protein concentrate. Nevertheless, this difference between folic acid and 5-CH_3-H_4folate in extent of binding to FBP is also supported by our previous studies (13, 25) in which the effect of FBP on the bioaccessibility of folic acid and 5-CH_3-H_4folate from fortified dairy products was investigated in the *in vitro* gastrointestinal model (bioaccessibility is, in these studies, defined as the free folate fractions which are available for absorption during gastrointestinal passage). The bioaccessibility of folic acid from folic acid-fortified milk and yogurt was significantly lower ($P < 0.05$), i.e. 11-14% and 47%, respectively, after the addition of FBP to the fortified milk (13) and yogurt (25). However, FBP did not lower the bioaccessibility of 5-CH_3-H_4folate from fortified milk (13) and lowered the bioaccessibility of 5-CH_3-H_4folate from fortified yogurt with 26% (25). These findings indicate that FBP in whey powder, milk and yoghurt have different binding characteristics for folic acid and 5-CH_3-H_4folate.

In this regard, one point to consider is the presence of endogenous folate in the whey protein concentrate (~ 4 µg 5-CH_3-H_4folate/g whey powder). This endogenous folate could compete with the added (exogenous) folic acid and 5-CH_3-H_4folate and as a result influence the extent of binding to FBP. However, this does not alter our general conclusions on extent of binding to FBP since we measured the relative binding of folic acid and 5-CH_3-H_4folate to FBP before and after gastric passage rather than focusing on the absolute quantification of the binding activity of FBP. As both folate compounds could be used for the fortification of dairy products, already containing endogenous FBP and 5-CH_3-H_4folate, this study provides information about the extent of binding of folic acid and 5-CH_3-H_4folate to FBP in the duodenal lumen after consumption of fortified dairy products.

An *in vivo* study (24) with 6-day old goat kids supports our *in vitro* studies as it showed that folic acid remained bound to FBP throughout the stomach and small intestine. Analysis of the goat's jejunal and ileal contents with gel filtration showed that a major part of the labeled folic acid (85-90%) was eluted in fractions corresponding to a molecular weight of 39 kDa (i.e. FBP-bound folic acid). Based on these results, the authors suggest that goat's milk FBP is resistant to digestion by gastric and intestinal enzymes. However, the fact that folic acid was bound to FBP in the goat's intestine, does not necessarily mean that FBP was completely resistant to degradation. The stability of FBP can only be investigated by quantitative determination of FBP before and after exposure to gastric and/or intestinal enzymes. Therefore, we studied the extent of binding to FBP in parallel with the quantitative determination of FBP. In contrast to the observed difference in FBP's binding characteristics for folic acid and 5-CH_3-H_4folate, we found no difference in FBP stability in the 5-CH_3-H_4folate/FBP and folic acid/FBP mixtures after gastric passage based on the ELISA measurements. From both mixtures 70% of the initial amount of FBP was recovered in the duodenum after gastric passage for 120 min. In our previous studies, in which folic acid- and 5-

CH_3-H_4folate-fortified dairy products were tested in the gastrointestinal model (13,25), the FBP content was quantified by ELISA in the samples collected after passage through the stomach and small intestine. It appeared that bovine FBP in a dairy matrix was less stable in combination with 5-CH_3-H_4folate (0-17%) than with folic acid (13-34%). Thus, a major portion of FBP passed the stomach intact and was largely digested by pancreatic enzymes along the passage through the small intestine. Apparently, this further digestion of FBP in the small intestine was dependent on the folate compound, folic acid or 5-CH_3-H_4folate, present in the dairy matrix.
It can be concluded that a major part of folic acid is still bound to FBP after gastric passage whereas a large portion of 5-CH_3-H_4folate is released from FBP. This difference in extent of binding to FBP for the two folate compounds can influence the folate bioavailability (i.e. release from the food matrix and intestinal transport) from milk products. To get insight in this overall picture, studies are underway in our laboratory concerning the effect of FBP on intestinal transport of folic acid and 5-CH_3-H_4folate.

Acknowledgments

The authors thank Corjan van den Berg for technical assistance. Henk van den Berg (The Netherlands Nutrition Centre, Den Haag, The Netherlands) and Margaretha Jägerstad (Swedish University of Agricultural Sciences, Uppsala, Sweden) are gratefully acknowledged for critical evaluation of the manuscript.

References

1. MRC Vitamin Study Research Group (1991) Prevention of neural tube defects: results of the Medical Research Council Vitamin Study. Lancet 338: 131-137.
2. Cuskelly, G.J., McNulty H., Scott, J.M. (1996) Effect of increasing dietary folate on red-cell folate: implications for prevention of neural tube defects. Lancet 347: 657-659.
3. Boushey, C.J., Beresford, A.A., Omen, G.S., Motulsky, A.G (1996) A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. J. Am. Med. Assoc. 274: 1049-1057.
4. Graham, I.A., O'Allaghan, P. (2000) The role of folic acid in the prevention of cardiovascular disease. Curr. Opin. Lipidol. 11: 577-587.
5. Giovannucci, E., Stampfer, M.J., Colditz, G.A., Hunter, D.J., Fuchs, C., Rosner, B.A., Speizer, F.E., Willet, W.C. (1998) Multivitamin use, folate, and colon cancer in women in the Nurses' Health Study. Ann. Intern. Med. 129: 517-524.
6. Rampersaud, G.C., Bailey, L.B., Kauwell, G.P.A. (2002) Relationship of folate to colorectal and cervical cancer: review and recommendations for practitioners. J. Am. Diet. Assoc. 102: 1273-1282.
7. Bailey, L.B., Rampsaud, G.C., Kauwell, G.P.A. (2003) Folic acid supplements and fortification affect the risk for neural tube defects, vascular disease and cancer: evolving science. J. Nutr. 133: 1961S-1968S.
8. Tamura, T. (1997) Bioavailability of folic acid in fortified foods. Am. J. Clin. Nutr. 66: 1299-1300.
9. Brouwer, I.A., van Dusseldorp, M., West, C.E., Meyboom, S., Thomas, C.M.G., Duran, M., van het Hof, K.H., Eskes, T.K.A.B., Hautvast, J.G.A.J., Steegers-Theunissen, R.P.M. (1999) Dietary folate from vegetables and citrus fruit decreases plasma homocysteine concentrations in humans in a dietary controlled trial. J. Nutr. 129: 1135-1139.
10. Konings, E.J.M., Roomans, H.H.S., Dorant, E., Goldbohm, R.A., Saris, W.H.M., van den Brandt, P.A. (2001) Folate intake of the Dutch population according to newly established liquid chromatography data for foods. Am. J. Clin. Nutr. 73: 765-776.
11. Becker, W. (1994) Dietary habits and nutrient intake in Sweden 1989. Swedish National Food Administration, Livsmedelsverkets förlag, Uppsala, Sweden.
12. Swiatlo, N., O'Conner, D.L., Andrews, J., Picciano, M.F. (1990) Relative folate bioavailability from diets containing human, bovine and goat milk. J. Nutr. 120: 172-177.
13. Verwei, M., Arkbåge, K., Havenaar, R., van den Berg, H., Witthöft, C., Schaafsma, G. (2003) Folic acid and 5-Methyltetrahydrofolate in fortified milk are bioaccessible as determined in a dynamic in vitro gastrointestinal model. J. Nutr. 133: 2377-2383.
14. Wigertz, K., Hansen, S.I., Høier-Madsen, M., Holm, J., Jägerstad, M. (1996) Effect of milk processing on the concentration of folate binding protein (FBP), the folate binding capacity and the retention of 5-methyltetrahydrofolate. Int. J. Food Sci. & Nutr. 47: 315-322.
15. Ghitis, J. (1967) The folate binding in milk. Am. J. Clin. Nutr. 20: 1-4.
16. Wagner, C. (1985) Folate Binding Proteins. Nutr. Rev. 43: 293-299.
17. Ford, J.E. (1974) Some observations on the possible nutritional significance of vitamin B_{12}- and folate binding proteins in milk. Br. J. Nutr. 31: 243-257.
18. Tani, M., Iwai, K. (1984) Some nutritional effects of folate binding protein in bovine milk on the bioavailability of folate to rats. J. Nutr. 114: 778-785.
19. Selhub, J., Arnold, R., Smith, A., Picciano, M.F. (1984) Milk folate binding protein (FBP): a secretory protein for folate? Nutr. Res. 4: 181-187.

20. Colman, N., Hettiarachchy, N., Herbert, V. (1981) Detection of a milk factor that facilitates folate uptake by intestinal cells. Science 211: 1427-1429.
21. Salter, D., Blakeborough, P. (1988) Influence of goat's-milk folate-binding protein on transport of 5-methyltetrahydrofolate in neonatal-goat small intestinal brush-border-membrane vesicles. Br. J. Nutr. 59: 497-507
22. Tani, M., Fushiki, T., Iwai, K. (1983) Influence of folate binding protein from bovine milk on the absorption of folate in gastrointestinal tract of rat. Biochim. Biophys. Acta 757: 274-281.
23. Said, H.M., Horne, D.W., Wagner, C. (1986) Effect of human folate binding protein on folate intestinal transport. Archiv. Biochem. Biophys. 251: 114-120.
24. Salter, D.N., Mowlem, A. (1983) Neonatal role of milk folate-binding protein: studies on the course of digestion of goat's milk folate binder in the 6-days old kid. Br. J. Nutr. 50: 589-596.
25. Arkbåge, K., Verwei, M., Havenaar, R., Witthöft, C. (2003) Folic acid and (6S)-5-methyltetrahydrofolate bioaccessibility decreases after addition of folate-binding protein to yogurt as studied in a dynamic in vitro gastrointestinal model. J. Nutr. 133: in press.
26. Van den Berg, H., Finglas, P.M., Bates, C. (1994) FLAIR intercomparison on serum and red cell folate. Int. J. Vitam. Nutr. Res. 64: 288-293.
27. Forssén, K.M., Jägerstad, M.I., Wigertz, K., Witthöft, C.M. (2000) Folates and dairy products: a critical update. J. Am. Coll. Nutr. 19: 100S-110S.
28. Minekus, M., Marteau, P., Havenaar, R., Huis in 't Veld, J.H.J. (1995) A multicompartimental dynamic computer-controlled model simulating the stomach and small intestine. ATLA 23: 197-209.
29. Minekus, M. (1998) Development and validation of a dynamic model of the gastrointestinal tract. PhD Thesis, University of Utrecht; Elinkwijk b.v., Utrecht, Netherlands.
30. Zeijdner, E.E. and Havenaar, R. (2000) The fate of orally administrated compounds during passage through the gastrointestinal tract simulated in a dynamic *in vitro* model (TIM). European Pharmaceutical Contractor Febr. issue: 76-81.
31. Høier-Madsen, M., Hansen, S.I., Holm, J. (1986) Rabbit antibodies against the folate binding protein from Cows'milk. Production, characterisation and use for development of an enzyme-linked immunosorbent assay (ELISA). Biosci. Rep. 6: 895-900
32. Wigertz, K., Svensson, U.K., Jägerstad, M. (1997) Folate and folate-binding protein content in dairy products. J. Dairy Res. 64: 239-252.
33. Blake, M.S., Johnston, K.H., Russell-Jones, G.J., Gotschlich, E.C. (1984) A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on Western blots. Anal. Biochem. 136: 175-179.
34. Nygren, L., Sternesjö, Å., Björk, L. (2003) Determination of folate-binding proteins from milk by optical biosensor analysis. Int. Dairy J. 13: 283-290.
35. Salter, D.N., Scott, K.J., Slade, H., Andrews, P. (1981) The preparation and properties of folate-binding protein from cow's milk. Biochem. J. 193: 469-476.
36. Iwai, K., Tani, M., Fushiki, T. (1983) Electrophoretic and immunological properties of Folate-Binding Protein isolated from Bovine milk. Agric. Biol. Chem. 47: 1523-1530.
37. Anthony, A.C., Utley, P.D., Marcell, P.D., Kolhouse, J.F. (1982) Isolation, characterization, and comparison of the solubilized particulate and soluble folate binding proteins from human milk. J. Biol. Chem. 257: 10081-10089.
38. Holm, J., Hansen, S.I. (2001) Binding of radiolabeled folate and 5-Methyltetrahydrofolate to cow's milk folate binding protein at pH 7.4 and 5.0. Relationship to concentration and polymerization equilibrium of the purified protein. Biosci. Rep. 21: 733-743.

39. Holm, J., Hansen, S.I. (2002) Ligand Binding Characteristics of two molecular forms, one equipped with a hydrophobic glycosyl phosphatidylinositol tail, of the folate binding protein purified from human milk. Biosci. Rep. 22: 455-463.
40. Givas, J.K., Gutcho, S. (1975) pH dependence of the binding of folates to milk binder in radioassay of folates. Clin. Chem. 21: 427-428
41. Gregory III, J.F. (1982) Denaturation of the folacin-binding protein in pasteurized milk products. J. Nutr. 112: 1329-1338.

6

Effect of folate-binding protein on intestinal transport of folic acid and 5-methyltetrahydrofolate across Caco-2 cells

European Journal of Nutrition (in press)

Miriam Verwei[1,2]
Henk van den Berg[3]
Robert Havenaar[1]
John P. Groten[1]

[1]TNO Nutrition and Food Research, Zeist, The Netherlands
[2]Department of Human Nutrition, Wageningen University, Wageningen, The Netherlands
[3]The Netherlands Nutrition Centre, Den Haag, The Netherlands

Abstract

Background Milk products are a potential matrix for fortification with synthetic folic acid or natural 5-methyltetrahydrofolate (5-CH_3-H_4folate) to enhance the daily folate intake. In milk, folate occurs bound to folate-binding proteins (FBP). Our previous studies with an *in vitro* gastrointestinal model showed that 70% of the initial FBP content of the milk product was retained in the duodenal lumen. While folic acid remained bound to FBP after gastric passage, 5-CH_3-H_4folate was mainly present as free folate in the duodenal lumen. *Aim of the study* To investigate the effect of FBP on the absorption of folic acid and 5-CH_3-H_4folate from the intestinal lumen. *Methods* The transport of [^{3}H]-folic acid and [^{14}C]-5-CH_3-H_4folate across enterocytes was studied in the presence or absence of bovine FBP using monolayers of Caco-2 cells grown on semi-permeable inserts in a two-compartment model. The apparent permeability coefficients (P_{app}) of folic acid and 5-CH_3-H_4folate were determined and compared with the permeability of reference compounds for low (mannitol) and high (caffeine) permeability. *Results* The transport from the apical to the basolateral side of the Caco-2 cells was higher ($P<0.05$) for folic acid ($P_{app} = 1.7*10^{-6}$ cm/sec) than for 5-CH_3-H_4folate ($P_{app} = 1.4*10^{-6}$ cm/sec) after 2h incubation to 1 μM folic acid or 5-CH_3-H_4folate test solutions (pH 7). The permeability of folic acid and 5-CH_3-H_4folate across Caco-2 monolayers appeared to be higher ($P<0.05$) than that of mannitol ($P_{app} = 0.5*10^{-6}$ cm/sec) but lower ($P<0.05$) than that of caffeine ($P_{app} = 34*10^{-6}$ cm/sec). The addition of FBP to the medium led to a lower ($P<0.05$) intestinal transport and cellular accumulation of folic acid and 5-CH_3-H_4folate. *Conclusions* Compared to the reference compounds, folic acid and 5-CH_3-H_4folate showed a moderate permeability across Caco-2 cells, which indicates that folate absorption from the intestinal lumen is not likely to be complete. The intestinal transport of folic acid and 5-CH_3-H_4folate was found to be dependent on the extent of binding to FBP at the luminal side of the cells.

Introduction

An adequate folate intake is preventive against megaloblastic anaemia (1) and reduces the risk for neural tube defects (2,3), colon cancer (4) and cardiovascular diseases (5,6). In many countries mean folate intake was found to be lower than recommended or desired (7,8) and supplementation or food fortification could be used to complement the folate intake from the natural diet. The main folate compound in non-fortified food products is 5-methyltetrahydrofolate (5-CH_3-H_4folate), while in supplements and fortified products mostly folic acid is used. Milk can be considered as a potential matrix for folate fortification because it is widely consumed and might enhance the folate bioavailability from the diet (9). In unprocessed milk, folate is essentially bound to folate-binding proteins (FBP) (10,11). At saturation, FBP bind approximately 1 mol folate/mol protein at pH 7.2 (12) with a somewhat higher affinity for folic acid than for 5-CH_3-H_4folate (13). The physiological role of FBP is unclear.

In recent studies, the bioaccessibility of folate from folic acid- or 5-CH_3-H_4folate-fortified milk products was investigated using a dynamic *in vitro* gastrointestinal model (14,15). Approximately 60-80% of the supplemental folate was released from the milk matrix during gastrointestinal passage and, therefore, available for absorption (bioaccessible). Addition of FBP reduced the bioaccessible fraction of folic acid to a higher extent than that of 5-CH_3-H_4folate from the fortified milk products (14,15). In additional studies with the gastrointestinal model it was found that approximately 80% of folic acid and 5-CH_3-H_4folate occurred bound to FBP in fortified whey suspensions with equimolar amounts of folate and FBP (16). FBP appeared to be highly stable during gastric passage in the gastrointestinal model as 70% of the initial FBP content could be retrieved in the duodenal lumen (16). While folic acid remained bound to FBP, the FBP-bound fraction of 5-CH_3-H_4folate gradually decreased from 79% to 5% during gastric passage. These studies show that the FBP binding characteristics are different for folic acid and 5-CH_3-H_4folate after gastric passage which could affect the absorption of both folate compounds from the intestinal lumen.

The intestinal absorption of folate has been characterized based upon *in vitro* and *in vivo* studies (mainly rat). The transport of folate across the intestinal cell membrane within physiological concentrations (<10 μM) was found, at least partly, to occur by a pH-dependent, active, carrier-mediated system (17-20). However, contradictory results have been reported about the influence of FBP on folate uptake (21,22) and transport (23,24) both *in vivo* in rats (24) as well as in *in vitro* studies using isolated rat mucosal cells (21), goat brush-border membrane vesicles (22) and everted sacs of rat intestine (23).

The present study was performed to investigate the effect of luminal FBP binding to folic acid and 5-CH_3-H_4folate on the transport of both folate compounds across human epithelial cells. For this purpose, monolayers of polarized human Caco-2 cells grown on semi-permeable inserts were used. Caco-2 cells cultured in a two-compartment system are widely used as an *in vitro* model for human intestinal absorption as they display after differentiation both biochemical and morphological characteristics of small intestinal enterocytes (25-27). Also the permeability characteristics of compounds across Caco-2 monolayers were found to correlate well with human oral absorption *in*

vivo (28-32). In the present study the permeability of folic acid and 5-CH_3-H_4folate across Caco-2 cells were compared with the permeability of reference compounds.

Materials and methods

Chemicals

Radiolabelled folate compounds, [^{3}H]-folic acid (888 GBq/mmol; 37 MBq/ml) and [^{14}C]-(RS)- 5-CH_3-H_4folate (2.11 GBq/mmol; 3.7 MBq/ml), were obtained from Amersham Pharmacia (Buckinghamshire, UK). Folic acid was studied as a mixture of radiolabelled and non-radiolabelled compounds. The non-radiolabelled standard solution of folic acid (Schirck's Laboratories, Jona, Switzerland) was controlled on purity according to Van den Berg et al. (33). The reference compounds, [^{3}H]-mannitol and [^{14}C]-caffeine, were obtained from ICN Biomedicals (Irvine, CA, USA) and Perkin-Elmer life sciences (Boston, MA, USA), respectively. Sephadex G75 Superfine powder, scintillation liquid (High Ionic Fluor and Ultima Gold) and the low molecular weight gel filtration calibration kit (17-0442-01) were obtained from Amersham Pharmacia. The FBP-rich whey fraction (821 nmol FBP/g) was kindly provided from DMV International (Veghel, The Netherlands). The elution solution used for gel filtration was 0.1 mol/L phosphate buffer with 0.15 mol/L NaCl (pH 7.2) containing 13.4 g/L Na_2HPO_4, 3.5 g/L NaH_2PO_4, 8.3 g/L NaCl, 0.02 g/L Na-azide (all from Sigma, St.Louis, MO, USA).

Cell culture

The human colon carcinoma cell line, Caco-2, was obtained from the American Type Culture Collection (Rockville, MD, USA). Cells grown in 75 cm^2 flasks (Corning-Costar, Cambridge, MA, USA) were passaged weekly at a split ratio of 1:10 using 0.05% trypsin in PBS with 0.022% EDTA. Caco-2 cells were used at passages 35-42. The Caco-2 cells were maintained at 37°C in an atmosphere of 5% CO_2 in culture medium, Hepes-buffered Dulbecco's Modified Eagle Medium (DMEM) containing 4.5 g/L glucose, supplemented with 1% (v/v) MEM non-essential amino acids, 6 mM L-glutamine, 50 µg/ml gentamycin and 10% (v/v) FCS (all from Gibco, Paisley, Scotland). For transport experiments approximately $1*10^5$ cells/cm^2 were seeded on Transwell polycarbonate cell culture inserts (12 well) with a mean pore size of 0.4 µm (Corning-Costar) (Figure 6.1). The integrity of the monolayers was tested by measuring the transepithelial electrical resistance (TEER) using the Millicell-ERS epithelial voltohmmeter (Millipore Co., Bedford, USA) before the transport experiments were started (34,35). The TEER values of the filter-grown cells used in the transport experiments were at least 400 $\Omega.cm^2$.

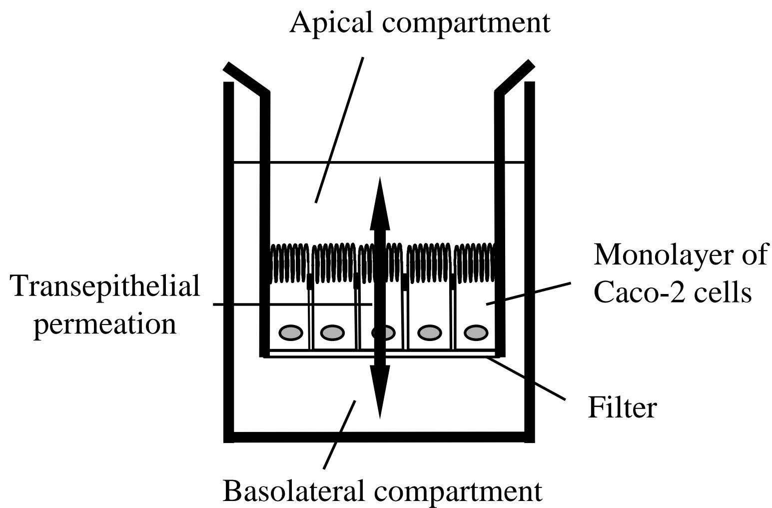


Figure 6.1. The two-compartment cell culture system. The system consists of a permeable cell culture (filter) insert which is placed in a well of a cell culture plate. After reaching confluence the Caco-2 cell layer forms a barrier between the apical and basolateral compartment (0.5 and 1.8 mL, respectively).

Intestinal transport and cellular accumulation

The transport experiments with folate compounds were performed after culturing the cells for 18-22 days at 37ºC in transwell inserts with 0.5 mL and 1.8 mL culture medium (DMEM) in the apical and basolateral compartments, respectively, corresponding to the luminal and serosal side of the enterocytes. For apical (Ap) exposure, culture medium was removed from the filter insert before moving them to a new 12-well plate containing 1.8 mL transport medium (Hanks Balanced Salt Solution (HBBS), Hepes-buffered, pH 7.0). The transport study started by filling the apical chambers with 0.5 mL of 1 µM folic acid or 5-CH_3-H_4folate test solutions (dissolved folic acid or 5-CH_3-H_4folate in transport medium). For basolateral (Bl) exposure, culture medium at the apical side was replaced by 0.5 mL of transport medium (HBBS, pH 7.0) and the transport study started by transferring the filter inserts to new 12-well plates containing 1.8 ml of 1 µM folic acid or 5-CH_3-H_4folate test solutions (dissolved folic acid or 5-CH_3-H_4folate in transport medium).

A concentration of 1 µM for both folic acid and 5-CH_3-H_4folate was studied as comparable folate concentrations (0.8-0.9 µM) were used in the folate-fortified milk in our previous *in vitro* (14,15) and *in vivo* (de Jong *et al*, publ. in prep.) studies. The effect of FBP on the absorption of folic acid and 5-CH_3-H_4folate from the intestinal lumen was studied by the addition of FBP (0.25 - 2 µM) to folic acid or 5-CH_3-H_4folate test solutions (1 µM) to reach molar ratios of FBP:folate of 0.25:1, 0.5:1, 0.75:1, 1:1, 1.5:1, 2:1. In milk, the natural ratio of FBP and 5-CH_3-H_4folate is approximately 1:1 as the natural FBP and folate concentrations in milk are 160-210 nmol FBP/L and 110-220 nmol folate/L (36). The FBP/folate mixtures were incubated in dark for 1 h at ca 20ºC before addition to the apical chambers.

All Caco-2 cultures were incubated on a rotating platform device (approximately 60 rpm) in a humidified incubator containing 5% CO_2 in air at 37°C. Transepithelial folate transport from the apical to the basolateral compartment (Ap>Bl) and from the basolateral to the apical compartment

(Bl>Ap) was measured by taking 500 µL or 200 µL samples from the basolateral or apical compartment, respectively, after 15, 30, 60, 90 and 120 min. After sampling, 500 µL or 200 µL HBBS was added to the corresponding compartment to restore the original volume. In each transport experiment mannitol (10 µM) and caffeine (10 µM) were included as reference compounds for low and high permeability, respectively. Their bidirectional transport rates were established similar to folic acid and 5-CH_3-H_4folate transport. Directly after sampling 4 mL scintillation liquid (Ultima Gold) was added to all collected samples and measured in a scintillation counter (Wallac 1409, Perkin-Elmer life sciences).
The apparent permeability coefficients (P_{app}, cm/sec) were determined on basis of appearance of the test compound (folic acid, 5-CH_3-H_4folate, caffeine, mannitol) in the receiver compartment before 10% of the compound was transported (i.e. under sink conditions) according to the following equation (34,35):
$P_{app} = (dQ/dt)/A*C_0$ (cm/sec), where dQ/dt = permeability rate (mol/sec), A = surface area of the filter insert (1.1 cm^2), C_0 = initial concentration (mol/mL).
The cellular accumulation was measured after 120 min of incubation. For this purpose, the medium was removed and the monolayers were washed rapidly with 1 and 2 mL of PBS in the apical and basolateral compartments, respectively. Subsequently, the filters with cells were detached from the inserts and incubated overnight in 1 mL 1.5 mol/L 20% KOH/EtOH solution. The cellular accumulation was measured, after the addition of 10 mL scintillation liquid (High Ionic Fluor), in a scintillation counter.

Folate-binding to FBP

The folate-binding to FBP was studied in the folic acid and 5-CH_3-H_4folate test solutions (1 µM) in the presence of FBP (0.5, 1 and 2 µM). For this purpose, the binding characteristics were determined with molecular size exclusion chromatography as described previously (16). One mL of these test solutions were applied to a Sephadex G75-column (2.6 cm x 30 cm), which had been equilibrated with 0.1 mol/L phosphate buffer containing 0.15 mol/L NaCl (pH 7.2), and eluted with the same buffer at a flow rate of 25 mL/h. The eluent was measured spectrophotometrically with an UV detector at 280 nm, as indicator of protein content, and subsequently collected as fractions at 8 min interval during a total run time of 800 min. Portions (200-500 µL) of these fractions were measured on their [^{3}H]-folic acid or [^{14}C]-5-CH_3-H_4folate content by measuring radioactivity with a scintillation counter after the addition of 4 ml of scintillation suspension (Ultima Gold).
The column was calibrated with the elution volumes of proteins within the gel filtration calibration kit, including Blue Dextran 2000 (200 kDa), Albumin (67 kDa), Ovalbumin (43 kDa), Chymotripsinogen A (25 kDa), and Ribonuclease A (14 kDa).

Statistical analysis

Data in the figures and text are expressed as mean ± SD of at least 3 experiments each performed in triplicate. Comparisons between group means were performed by a Student's two-tailed t-test. A significant difference between means was considered to be present when $P < 0.05$.

Results

Intestinal transport and cellular accumulation of folate

The time course of the intestinal transport of folic acid and 5-CH_3-H_4folate was examined after addition of 1 μM folic acid and 5-CH_3-H_4folate solutions to the apical compartment (0.5 mL) or basolateral compartment (1.8 mL) of the Caco-2 monolayers (Figure 6.2). After 2 h, the Ap>Bl transport of folic acid (13.2 ± 1.2 pmol/monolayer) was significantly ($P<0.05$) higher than that of 5-CH_3-H_4folate (9.4 ± 1.5 pmol/monolayer), corresponding with 2.6 ± 0.2% and 1.9 ± 0.3% of the dose, respectively. The Bl>Ap transport was found to be similar for folic acid (9.1 ± 1.1 pmol/monolayer) and 5-CH_3-H_4folate (8.7 ± 0.1 pmol/monolayer), corresponding with 0.5 ± 0.1% of the dose.

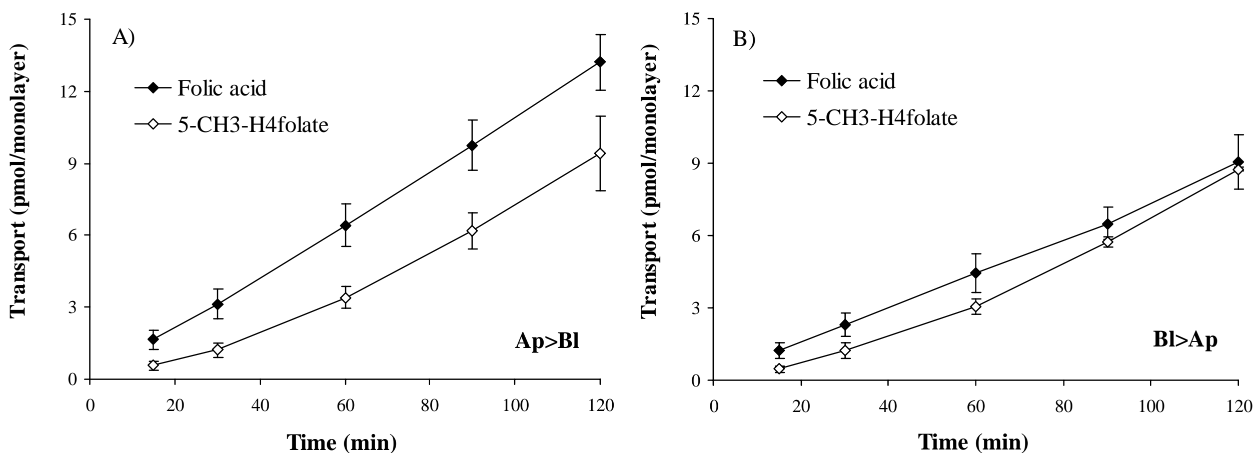


Figure 6.2. Transport of folic acid and 5-CH_3-H_4folate from A) the apical to the basolateral side (Ap>Bl) and B) the basolateral to the apical side (Bl>Ap) of a monolayer of Caco-2 cells (1.13 cm^2). Samples were taken from the receiver compartment at 15, 30, 60, 90 and 120 min after the addition of 1 μM folic acid or 5-CH_3-H_4folate to the apical or basolateral compartment. Values are expressed as means ± SD of at least three experiments each performed in triplicate.

The P_{app} values for the Ap>Bl and Bl>Ap transport of folic acid and 5-CH_3-H_4folate were determined between 60 and 120 minutes (linear range, Figure 6.2) after incubation with 1 μM folic acid or 5-CH_3-H_4folate at pH 7. Under these test conditions, the P_{app} value for the Ap>Bl transport of folic acid ($1.7 \pm 0.2*10^{-6}$ cm/sec) was significantly higher ($P<0.05$) than the P_{app} values for the Bl>Ap transport of folic acid ($1.1 \pm 0.1*10^{-6}$ cm/sec), the Ap>Bl transport of 5-CH_3-H_4folate ($1.4 \pm 0.2*10^{-6}$ cm/sec) and the Bl>Ap transport of 5-CH_3-H_4folate ($1.4 \pm 0.1*10^{-6}$ cm/sec). The transport rates of folic acid and 5-CH_3-H_4folate were significantly ($P<0.05$) higher than that of mannitol ($0.5 \pm 0.1*10^{-6}$ cm/sec), which is a transport marker for a low absorption in humans. On the other hand, the transport of both folate compounds was significantly ($P<0.05$) lower than that of caffeine ($34 \pm 4*10^{-6}$ cm/sec), which is a transport marker that is known to be highly absorbed (100%) from the human intestinal tract.

After 2 h of apical incubation, 5-CH_3-H_4folate showed a significantly ($P<0.05$) higher cellular accumulation than folic acid, i.e. 1.1% vs. 0.7% of the dose (Table 6.1). However, as the intestinal transport of folic acid was higher than that of 5-CH_3-H_4folate, the sum of transported and accumulated folate was not significantly different ($P<0.05$) between folic acid and 5-CH_3-H_4folate, i.e., 3.3% and 3.0% respectively. For both folic acid and 5-CH_3-H_4folate, a significant ($P<0.05$) lower cellular content, i.e., 0.3% and 0.5%, respectively, was found after basolateral incubation compared to apical incubation.

Table 6.1. Transport and cellular accumulation of folic acid and 5-CH_3-H_4folate, given as % of dose, after 2h apical incubation to folate/FBP test solutions (pH 7) containing 1μM folic acid or 5-CH_3-H_4folate with 0 to 2 μM FBP[1,2].

FBP	Transport (% of dose)		Cell. accumulation (% of dose)	
(μM)	Folic acid	5-CH_3-H_4folate	Folic acid	5-CH_3-H_4folate
0	2.6 ± 0.2[a]	1.9 ± 0.3[a]	0.7 ± 0.2[a]	1.1 ± 0.2[a]
0.25	2.2 ± 0[b]	1.9 ± 0[a]	0.4 ± 0[b]	0.9 ± 0.1[b]
0.5	1.9 ± 0.1[b]	1.4 ± 0.2[b]	0.4 ± 0.1[b,c]	0.8 ± 0.2[b]
0.75	1.8 ± 0.1[b,c]	1.0 ± 0.1[c]	0.3 ± 0.1[b,c]	0.5 ± 0.1[b,c]
1	1.5 ± 0.1[c]	0.5 ± 0.1[d]	0.3 ± 0.1[b,c]	0.4 ± 0.2[c]
1.5	1.5 ± 0.2[c]	0.5 ± 0.1[d]	0.3 ± 0[b,c]	0.3 ± 0[c]
2	1.5 ± 0.1[c]	0.4 ± 0[d]	0.2 ± 0.1[c]	0.3 ± 0.1[c]

[1] Values are expressed as means ± SD of at least three experiments each performed in triplicate, [2] Means in column without at common letter differ significantly ($P<0.05$).

Folate-binding to FBP

The binding characteristics of FBP for folic acid and 5-CH_3-H_4folate in the test solutions with 0.5, 1 or 2 μM FBP was studied with gel filtration analysis. For both folic acid and 5-CH_3-H_4folate test solutions, three folate peaks were visible after gel filtration (Figure 6.3). Based on calibration proteins, the first peak (elution volume ~ 60 mL) corresponded to compounds with a molecular weight larger than 60 kDa, the second peak (elution volume ~ 90 mL) to compounds between 30-40 kDa (i.e. FBP-bound folate), and the third peak (elution volume ~180 mL) to compounds smaller than 10 kDa (i.e. free folate). A few percent of the 5-CH_3-H_4folate and folic acid in the whey solutions appeared to be bound to the proteins larger than 60 kDa. The FBP-bound [^{3}H]-folic acid fractions in the 0.5, 1 and 2 μM FBP suspensions were 45, 86 and 87%, respectively, and the corresponding free [^{3}H]-folic acid fractions were 53, 9 and 8%, respectively. This indicates that at 1 and 2 μM FBP most of the folic acid was bound to FBP. For 5-CH_3-H_4folate, the binding characteristics were slightly different. The FBP-bound 5-CH_3-H_4folate fractions were 39, 78 and 86%, respectively, in the 0.5, 1 and 2 μM FBP suspensions with free 5-CH_3-H_4folate fractions of 59, 17 and 8%, respectively (Figure 6.3). Thus, most of the 5-CH_3-H_4folate was bound to FBP in the 1 and 2 μM FBP test solutions. However, compared to folic acid, a substantial amount of free 5-CH_3-H_4folate (17%) was still present in the 1 μM FBP solution.

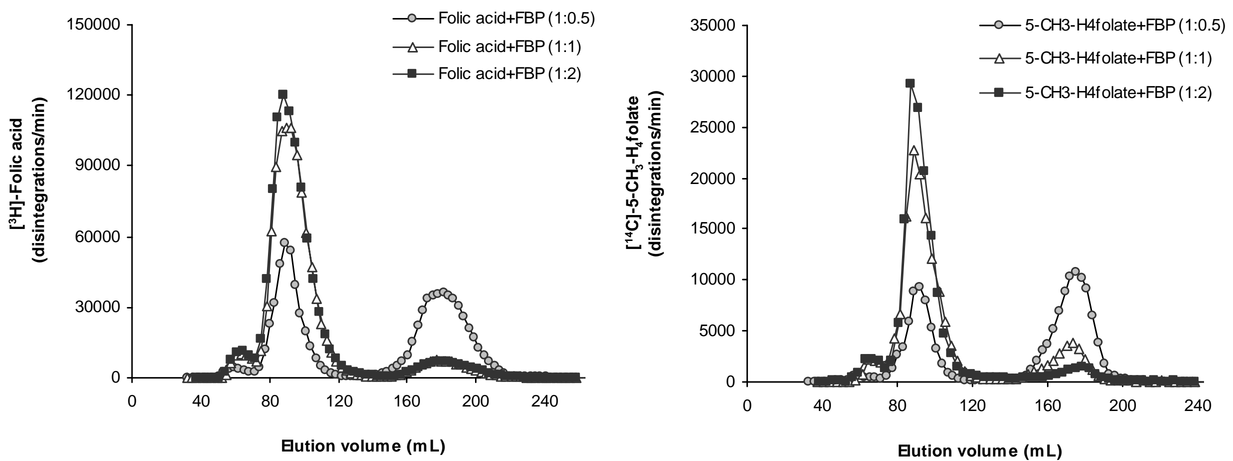


Figure 6.3. Sephadex gel filtration chromatography of [^{3}H]-folic acid or [^{14}C]-5-CH_3-H_4folate in 1 µM folic acid or 5-CH_3-H_4folate test solutions with 0.5, 1 or 2 µM FBP. The folate content in the fractions is plotted against the elution volume.

Discussion

In the present study we investigated the effect of luminal FBP binding on the transport of 5-CH_3-H_4folate and folic acid across monolayers of Caco-2 cells. In the absence of FBP in the 1 µM folic acid or 5-CH_3-H_4folate test solutions (pH 7), the Ap>Bl transport of folic acid was higher than that of 5-CH_3-H_4folate, while Bl>Ap transport was found to be similar for both folate compounds (Figure 6.2). This indicates that the absorption of folic acid from the intestinal lumen will be higher than that of a similar dose of 5-CH_3-H_4folate in the first 2h of passage. Two-directional transport in epithelial cells is usually investigated to demonstrate either a specific carrier-mediated influx or efflux of the test compound. The present study shows that the ratios between basolateral- and apical-directed transport (P_{app}Ap>Bl/P_{app}Bl>Ap) were 1.47 and 1.05 for folic acid and 5-CH_3-H_4folate, respectively. These results point towards a slightly polarized transport of folic acid with a higher transport to the basolateral side of the Caco-2 cells, while the transport of 5-CH_3-H_4folate was found not to be polarized.

Although the transport of folic acid appeared to be significantly ($P<0.05$) higher than that of 5-CH_3-H_4folate, its cellular accumulation was significantly ($P<0.05$) lower. Folate monoglutamates do not accumulate unless they are converted to folate polyglutamates in the cytoplasm or mitochondria, a reaction catalyzed by the enzyme folylpolyglutamate synthetase (FPGS) (37). Previous *in vitro* studies, in which the activity of hog liver FPGS for several folate derivatives was investigated, showed that FPGS had a higher affinity for (6RS)-5-CH_3-H_4folate than for folic acid (38). Thus, the higher degree of cellular accumulation of 5-CH_3-H_4folate might be attributed to its higher affinity for FPGS in Caco-2 cells. As the sum of transported and accumulated folate is similar for folic acid and 5-CH_3-H_4folate, apparently equal amounts of folic acid and 5-CH_3-H_4folate are absorbed into the Caco-2 cells, but a higher amount of folic acid also passes the basolateral membrane within 2 h luminal exposure.

To evaluate the permeability of folate, the reference compounds mannitol and caffeine were included in each study which are considered as markers for, respectively, low and high permeability across the human intestinal wall. The permeability of folate across Caco-2 monolayers was found to be somewhat higher than that of mannitol but much lower than the permeability of caffeine. Previous studies (28-32) have reported a correlation between the permeability characteristics of compounds across Caco-2 monolayers and the human oral absorption determined *in vivo*. This correlation shows that mannitol with its very poor absorption has a low oral bioavailability (16%), while caffeine with its excellent epithelial permeability is completely (100%) absorbed. An exact prediction of the human absorption is not possible with the Caco-2 model, but based on the moderate permeability of folic acid and 5-CH_3-H_4folate we anticipated that the folate compounds are not completely absorbed from the intestinal tract.
Gel filtration analysis showed that most of the folic acid and 5-CH_3-H_4folate (78-86%) in the 1 and 2 µM FBP solutions was bound to FBP, while lower FBP-bound fractions of folic acid (45%) and 5-CH_3-H_4folate (39%) were found in the 0.5 µM FBP solutions. These results are in line with previous *in vitro* binding studies (12) which showed that at saturation 1 mol FBP binds approximately 1 mol folate at pH 7.2. The transport and cellular accumulation of folic acid and 5-CH_3-H_4folate was found to be lower in presence of 1 µM FBP as compared with 0.5 µM FBP. Thus, these results indicate that the effect of FBP on the absorption of folate was dependent on the extent of binding to FBP at the luminal side of the cells. In addition, present study demonstrates that FBP-bound folate can not be taken up by the Caco-2 cells or at least that the FBP-bound folate transport is very slow and does not significantly contribute to the overall intestinal absorption.
In this study, the barrier function was maintained in a Caco-2 cell culture system and for the first time the absorption of 5-CH_3-H_4folate and folic acid were simultaneously investigated under identical test conditions, which allow a comparison in effect of FBP on the intestinal transport of both folate compounds. A decrease in folate transport by FBP as observed in our *in vitro* study was also found in an *in vitro* study with everted sacs of rat jejunum (23) and an *in vivo* rat study (24) on the absorption of 5-CH_3-H_4folate (23) and folic acid (24). In contrast, in *in vitro* uptake studies with isolated mucosal cells (21) and brush-border membrane vesicles (22) it was found that FBP increased the uptake of folic acid (21) and 5-CH_3-H_4folate (22). The difference in effect of FBP might be related to the presence of an intact barrier of intestinal cells in the *in vitro* study with everted sacs (23) and the *in vivo* (24) study indicating that the active, polarized transport is dependent on the free folate fraction in the intestinal lumen. Apparently this is not the case in the studies using membrane vesicles (22) or isolated single cells (21).
The present work was undertaken as a follow-up to our earlier studies with an *in vitro* gastrointestinal model (14-16) to get more in depth insight in the bioavailability of folic acid and 5-CH_3-H_4folate from (fortified) milk products. These studies demonstrated that the release of 5-CH_3-H_4folate (72%) was higher than that of folic acid (58%) from fortified pasteurised milk during gastrointestinal passage (14). Addition of FBP to the fortified milk led to a reduction of the free folic acid fraction (44%) in the gastrointestinal tract but had no effect on the release of 5-CH_3-H_4folate (71%) from the milk matrix. This difference in luminal binding between folic acid and 5-CH_3-H_4folate was also found in a study in which the FBP binding characteristics were investigated

before and after gastric passage of fortified whey suspensions (16). Before gastric passage, folic acid and 5-CH_3-H_4folate were equally bound to FBP. However, after gastric passage the extent of binding to FBP appeared to be higher for folic acid (80%) than for 5-CH_3-H_4folate (5-57%). The difference in extent of luminal binding should be taken into account when discussing the effect of FBP on the intestinal transport of folic acid and 5-CH_3-H_4folate as present study showed that the transport of folic acid and 5-CH_3-H_4folate was dependent on the extent of binding to FBP at the luminal side of the cells. As a consequence, we might speculate that the absorption of folic acid from fortified milk products will be hampered due to its binding to FBP whereas the absorption of 5-CH_3-H_4folate will be affected to a smaller extent. The separate processes occurring during gastrointestinal passage can be described in a physiologically-based kinetic model. Currently, studies are underway to integrate the data derived from the *in vitro* studies about the release of folate from the food matrix in the intestinal tract and the transport of free folate across the intestinal wall in a kinetic model for folate compounds to get more insight in the systemic distribution of folate and the overall effect of FBP on these processes.

References

1. Fishman SM, Christian P, West KP (2000) The role of vitamins in the prevention and control of anaemia. Public Health Nutr 3:125-150
2. Wald N, Sneddon J, Frost C, Stone R (1991) MRC Vitamin Study Group Prevention of neural tube defects. Results of the Medical Research Council Vitamin Study. Lancet 338: 131-137
3. Daly LE, Kirke PN, Molloy A, Weir DG, Scott JM (1995) Folate levels and neural tube defects. J Am Med Assoc 247: 1698-1702
4. Rampersaud GC, Bailey LB, Kauwell GPA (2002) Relationship of folate to colorectal and cervical cancer: review and recommendations for practitioners. J Am Diet Assoc 102: 1273-1282
5. Boushey CJ, Beresford AA, Omen GS, Motulsky AG (1996) A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. J Am Med Assoc 274: 1049-1057
6. Graham IA, O'Allaghan P (2000) The role of folic acid in the prevention of cardiovascular disease. Curr Opin Lipidol 11: 577-587
7. De Bree A, Van Dusseldorp M, Brouwer IA, Van het Hof KH, Steegers-Theunissen RP (1997) Folate intake in Europe: recommended, actual and desired intake. Eur J Clin Nutr 51: 643-660.
8. Konings EJM, Roomans H, Dorant E, Goldbohm R, Saris W, van den Brandt P (2001) Folate intake of the Dutch population based newly established liquid chromatography data for foods. Am J Clin Nutr 73: 765-776.
9. Swiatlo N, O’Conner DL, Andrews J, Picciano MF (1990) Relative folate bioavailability from diets containing human, bovine and goat milk. J Nutr 120: 172-177
10. Ghitis J (1967) The folate binding in milk. Am J Clin Nutr 20: 1-4
11. Wagner C (1985) Folate Binding Proteins. Nutr Rev 43: 293-299
12. Salter DN, Scott KJ, Slade H, Andrews P (1981) The preparation and properties of folate-binding protein from cow’s milk. Biochem J 193: 469-476
13. Holm J, Hansen SI (2001) Binding of radiolabeled folate and 5-methyltetrahydrofolate to cow’s milk folate binding protein at pH 7.4 and 5.0. Relationship to concentration and polymerization equilibrium of the purified protein. Biosci Rep 21: 733-743
14. Verwei M, Arkbåge K, Havenaar R, van den Berg H, Witthöft C, Schaafsma G (2003) Folic acid and 5-Methyltetrahydrofolate in fortified milk are bioaccessible as determined in a dynamic in vitro gastrointestinal model. J Nutr 133: 2377-2383
15. Arkbåge K, Verwei M, Havenaar R, Witthöft C (2003) Addition of folate-binding protein lowers the bioaccessibility of folic acid and 5-methyltetrahydrofolate from fortified yogurt as studied in a dynamic in vitro gastrointestinal model. J Nutr 133: 3678-3683
16. Verwei M, Arkbåge K, Mocking H, Havenaar R, Groten J (2004) The FBP binding characteristics during gastric passage are different for folic acid and 5-CH_3H_4folate as studied in a dynamic in vitro gastrointestinal model. J Nutr 134: 31-37
17. Said HM, Grishan FK, Murrell JE (1985) Ontogenesis of the intestinal transport of 5-methyltetrahydrofolate in the rat. Am J Physiol 249: 567-571
18. Said HM, Strum WB (1983) A pH-dependent, carrier-mediated system for transport of 5-methyltetrahydrofolate in rat jejunum. J Pharmacol Exp Ther 226: 95-99
19. Selhub J, Powell GM, Rosenberg IH (1984) Intestinal transport of 5-methyltetrahydrofolate. Am J Physiol 246: G515-G520
20. Said HM, Grishan FK, Redha R (1987) Folate transport by human intestinal brush-border membrane vesicles. Am J Physiol 252: 229-236

21. Colman N, Hettiarachchy N, Herbert V (1981) Detection of a milk factor that facilitates folate uptake by intestinal cells. Science 211: 1427-1429
22. Salter D, Blakeborough P (1988) Influence of goat's-milk folate-binding protein on transport of 5-methyltetrahydrofolate in neonatal-goat small intestinal brush-border-membrane vesicles. Br J Nutr 59: 497-507
23. Said HM, Horne DW, Wagner C (1986) Effect of human folate binding protein on folate intestinal transport. Archiv Biochem Biophys 251: 114-120
24. Tani M, Fushiki T, Iwai K (1983) Influence of folate binding protein from bovine milk on the absorption of folate in gastrointestinal tract of rat. Biochim Biophys Acta 757: 274-281
25. Hidalgo IJ, Raub TJ, Borchardt RT (1989) Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. Gastroenterology 96: 736-749
26. Hillgren KM, Kato A, Borchardt RT (1995) In vitro systems for studying intestinal drug absorption. Med Res Rev 15: 82-109
27. Vincent ML, Russell RM, Sasak V (1985) Folic acid uptake characteristics of a human colon carcinoma cell line Caco-2. A newly described cellular model for small intestinal epithelium. Human Nutrition: Clinical Nutrition 39C: 355-360
28. Artusson P, Karlsson J (1991) Correlation between oral drug absorption in humans and apparent drug permeability coefficients in human intestinal epithelial (Caco-2) cells. Biochem Biophys Res Com 175: 880-885
29. Yazdanian M, Glynn SL, Wright JL, Hawi A (1998) Correlating partitioning and Caco-2 cell permeability of structurally diverse small molecular weight compounds. Pharm Res 15: 1490-1494
30. Yee S (1997) In vitro permeability across Caco-2 cells (colonic) can predict in vivo (small intestinal) absorption in man – fact or myth. Pharm Res 14: 763-766
31. Grès M-C, Julian B, Bourrié M, Meunier V, Roques C, Berger M, Boulenc X, Berger Y, Fabre G (1998) Correlation between oral drug absorption in humans, and apparent drug permeability in TC-7 cells, a human epithelial intestinal cell line: comparison with the parental Caco-2 cell line. Pharm Res 15: 726-733
32. Rubas W, Jezyk N, Grass GM (1993) Comparison of the permeability characteristics of human colonic epithelial (Caco-2) cell line to colon of rabbit, monkey, and dog intestine and human drug absorption. Pharm Res 10: 113-118
33. Van den Berg H, Finglas PM, Bates C (1994) FLAIR intercomparison on serum and red cell folate. Int J Vitam Nutr Res 64: 288-293
34. Faassen F, Kelder J, Lenders J, Onderwater R, Vromans H (2003) Physicochemical properties and transport of steriods across Caco-2 cells. Pharm Res 20: 177-186
35. Duizer E, Penninks AH, Stenhuis WH, Groten JP (1997) Comparison of permeability characteristics of the human colonic Caco-2 and rat small intestinal IEC-18 cell lines. J Contr Rel 49: 39-49
36. Forssén KM, Jägerstad MI, Wigertz K, Witthöft CM (2000) Folates and dairy products: a critical update. J Am Coll Nutr 19: 100S-110S
37. Shane B (1995) In: Bailey LB (ed) Folate in Health and Disease, Marcel Dekker, Inc., New York, pp 1-22
38. Cichowicz DJ, Shane B (1987) Mammalian folylpoly-γ-glutamate synthetase. 2. substrate specificity and kinetic properties. Biochem 26: 513-521

7

Transport of folic acid and 5-methyltetrahydrofolate across Caco-2 cells occurs via the reduced folate carrier and multi-drug resistance proteins

Submitted for publication

Miriam Verwei
Karin van Ede
Heleen M. Wortelboer
John P. Groten

TNO Nutrition and Food Research, Zeist, The Netherlands

Abstract

In many countries average folate intake was found to be lower than recommended. The actual intake can be enhanced by a higher consumption of folate-rich food products or by the intake of supplements or fortified food products. The main folate compound in non-fortified food products is 5-methyltetrahydrofolate (5-CH_3-H_4folate), while in supplements and fortified products folic acid is mostly used. The aim of this study was to investigate the pathways involved in the intestinal transport of folic acid and 5-CH_3-H_4folate. For this purpose, Caco-2 cells, cultured in a two-compartment system, were used as an *in vitro* model for human intestinal absorption. The contribution of individual pathways for the intestinal transport of folic acid and 5-CH_3-H_4folate, such as transport via the reduced folate carrier (RFC), folate receptor (FR), P-glycoprotein (Pgp) and multi-drug resistance proteins (MRPs), was studied in the presence or absence of inhibitors of these pathways. In presence of inhibitors of RFC- and/or MRP-mediated transport (probenecid, methotrexate and MK571), the transport of folic acid and 5-CH_3-H_4folate across monolayers of Caco-2 cells was reduced with 40-63% and 60-75%, respectively. The compounds colchicine and PSC833 did not affect the transport of both folate compounds across Caco-2 cells, which indicates that receptor-mediated uptake and Pgp-mediated efflux are not involved in the intestinal transport of 5-CH_3-H_4folate and folic acid. Thus, folate transport is found to occur mainly via RFC- and MRP-transporters across intestinal cells, whereas the relative contribution of these pathways to the overall transport appeared to be higher for 5-CH_3-H_4folate than for folic acid.

Introduction

Folate is an essential nutrient required for many reactions of one-carbon metabolism, including methionine regeneration and the synthesis of nucleic acids. In many countries, the daily folate intake should be enhanced to recommended levels (1,2) which can be reached by the consumption of folate-rich food products, supplements and/or fortified food products. The main natural folate compound in non-fortified food products is 5-methyltetrahydrofolate (5-CH_3-H_4folate), while synthetic folic acid is mostly used in supplements and fortified products. To bridge the gap between actual and recommended folate intake, there is a need for more information on the bioavailability of 5-CH_3-H_4folate and folic acid from the diet.
After the ingestion of food products, folate is partly or totally released from the food matrix due to digestion during passage through the gastrointestinal tract. Previous studies have shown that 60-80% of folate was released from folic acid- or 5-CH_3-H_4folate-fortified milk products during transit through a dynamic *in vitro* gastrointestinal model (3,4). Next step in the bioavailability process is the intestinal absorption of the released folate compounds. The intestinal transport can be studied using human colon carcinoma, Caco-2, cells as an *in vitro* model for human intestinal absorption (5-7). Several transport mechanisms are present in the intestinal cells as illustrated in Figure 7.1. Recently, we reported a slightly higher transport of folic acid across Caco-2 cells compared with the transport of 5-CH_3-H_4folate (8). The permeability of both folate compounds across Caco-2 monolayers appeared to be three-fold higher than that of mannitol (a marker for low absorption) but twenty-fold lower than that of caffeine (a marker for high absorption) (8). This indicates that the absorption of both folate compounds from the intestinal lumen is likely to be incomplete which might limit their bioavailability. Information about the transport pathways underlying the intestinal transport of folic acid and 5-CH_3-H_4folate could contribute to the development of a dietary strategy to enhance the bioavailability of folate with the aim of optimizing the folate status of the population.
It has been shown that the intestinal transport of folate, within physiological concentrations (<10 µmol/L), was found to occur, at least partly, by a pH-dependent, active, carrier-mediated system (9-11). In this regard, carrier-mediated uptake of folate occurs via the reduced folate carrier (RFC), which functions as an anion exchanger and is located in both apical and basolateral membrane of the epithelial cells (12). In several epithelial cells (e.g. in kidney, placenta) folate was found to be taken up by folate receptors (FR) via an endocytotic process (13,14). Whether receptor-mediated uptake of folate contributes to the net transport across intestinal cells is unclear as conflicting results have been published considering the presence of folate receptors in the small intestine (15,16). Besides these uptake mechanisms, several ATP-dependent efflux transporters, such as P-glycoprotein (Pgp) and multidrug resistance proteins (MRPs), which are present in the apical (e.g. Pgp and MRP2) or basolateral (e.g. MRP1) membranes of the enterocytes, could contribute to the net transport of folic acid and 5-CH_3-H_4folate across the intestinal wall. The aim of the present study was to investigate the contribution of carrier- and receptor-mediated uptake and efflux via MRP and Pgp transporters to the overall intestinal transport of folic acid and 5-CH_3-H_4folate.

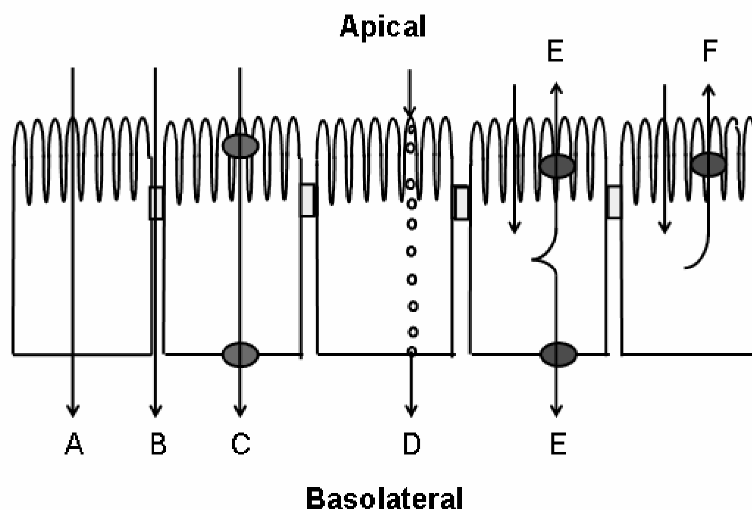


Figure 7.1. Transport mechanisms of the small intestine (Caco-2 cells). Transcellular diffusion (A), Paracellular diffusion (B), Transcellular carrier-mediated transport (C), Transcellular receptor-mediated transport via endocytose (D), Efflux transport via apical or basolateral located multi-drug resistance proteins (MRPs) (E), Efflux transport via P-glycoprotein (Pgp) (F).

Materials and methods

Chemicals

Radiolabeled folate compounds, [^{3}H]-folic acid (888 GBq/mmol; 37 MBq/mL) and [^{14}C]-(RS)- 5-CH_3-H_4folate (2.11 GBq/mmol; 3.7 MBq/mL), were obtained from Amersham Pharmacia (Buckinghamshire, UK) and checked for their purity. The non-radiolabeled standard solutions of folic acid, 5-CH_3-H_4folate and methotrexate (MTX), were obtained from Schirck's laboratories (Jona, Switzerland). The reference compound [^{3}H]-mannitol was obtained from ICN Biomedicals (Irvine, CA) and the scintillation liquids (High Ionic Fluor and Ultima Gold) were from Amersham Pharmacia. Probenecid and colchicine were obtained from Sigma (St. Louis, MO) and MK571 was obtained from Biomol (Phymouth Meeting, PA). PSC833 was a kind gift from Novartis Pharma AG (Basel, Switserland).

Cell culture

The human colon carcinoma cell line, Caco-2, was obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in 75 cm^2 flasks (Corning-Costar, Cambridge, MA) and passaged weekly at a split ratio of 1:10 using 0.05% trypsin in PBS with 0.022% EDTA. Caco-2 cells were used at passages 35-42. The Caco-2 cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 in culture medium, i.e. HEPES-buffered DMEM containing 4.5 g/L glucose, supplemented with 1% (v:v) MEM non-essential amino acids, 6 mmol/L L-glutamine, 50 µg/mL gentamycin and 10% (v:v) FCS (all from Gibco, Paisley, Scotland). For transport experiments

approximately $1*10^5$ cells/cm^2 were seeded on Transwell polycarbonate cell culture inserts (12 well) with a mean pore size of 0.4 µm (Corning-Costar, Cambridge, MA). The integrity of the monolayers was tested just before start of the transport studies by measuring the transepithelial electrical resistance (TEER) using the Millicell-ERS epithelial voltohmmeter (17) and during the studies by measuring the permeability of mannitol, a paracellular transport marker, across the Caco-2 cells. All Caco-2 monolayers used in the transport studies showed TEER values of at least 400 Ω.cm^2 and apparent permeability coeffients of mannitol of less than 6.0 x 10^{-7} cm/sec, which indicates an intact barrier function.

Intestinal transport of folic acid and 5-CH_3-H_4folate

The transport experiments with folate compounds were performed after culturing the cells for 18-22 days in an atmosphere of 5% CO_2 at 37ºC in transwell inserts with 0.5 mL and 1.8 mL culture medium in the apical and basolateral compartments, respectively, corresponding to the luminal and serosal side of the enterocytes. For apical exposure, culture medium was removed from the filter insert before moving them to a new 12-well plate containing 1.8 mL transport medium (HBBS, pH 7.0). The transport study started by filling the apical chambers with 0.5 mL folic acid (0.02-10 µmol/L) or 5-CH_3-H_4folate (1-10 µmol/L) dose solutions (folic acid or 5-CH_3-H_4folate dissolved in transport medium). For basolateral exposure, culture medium at the apical side was replaced by 0.5 mL transport medium and the transport study started by transferring the filter inserts to new 12-well plates containing 1.8 mL of folic acid (0.02-10 µmol/L) or 5-CH_3-H_4folate (1-10 µmol/L) dose solutions. Due to the low specific radioactivity of 5-CH_3-H_4folate, the concentrations in the basolateral medium after apical incubation of the cells to dose solutions lower than 1 µmol/L 5-CH_3-H_4folate could not be measured.

All cultures were incubated on a rotating platform device (approximately 60 rpm) in a humidified incubator containing 5% CO_2 in air at 37°C. Transepithelial folate transport from the apical to the basolateral compartment (Ap>Bl) and from the basolateral to the apical compartment (Bl>Ap) was measured by taking 500 µL or 200 µL samples from the basolateral or apical compartment, respectively, after 30, 60 and 120 min. After sampling, the original volume was restored by adding 500 µL or 200 µL of transport medium to the corresponding compartment. Directly after sampling, 4 mL scintillation liquid (Ultima Gold) was added to the collected samples and measured in a scintillation counter (Wallac 1409, Perkin Elmer).

Stability of folic acid and 5-CH_3-H_4folate

In brief, monolayers of Caco-2 cells were apically exposed to 0.02, 0.20 and 5 µmol/L folic acid or 5 µmol/L 5-CH_3-H_4folate. The stability of both folate compounds was followed in time by studying the profiles of the dose solutions (0 h) added to the apical compartment and the basolateral media samples sampled after 1, 2, 3, 4 and 24h. The dose solutions and basolateral media samples were analyzed by RP-HPLC (Agilent Technologies, Palo Alto, CA) following a gradient as previously described (3,18) with parallely UV (290 nm) and radiochemical detection.

Effect of inhibitors on intestinal transport of folic acid and 5-CH_3-H_4folate

To investigate the individual transport pathways possibly involved in the transport of folate across Caco-2 cells, the transport (Ap>Bl or Bl>Ap) of folic acid (1 µmol/L) and 5-CH_3-H_4folate (1 µmol/L) was studied in absence and in presence of inhibitors of several transport pathways. Carrier-mediated uptake and MRP-mediated efflux was studied measuring the transport in absence or presence of probenecid (2 mM) or MTX (1-100 µmol/L). MTX is a structural folate analog and a known substrate for RFC and MRPs (19,20) and, therefore, MTX (0.1-100 µmol/L) was used to study possible inhibition due to competition with folic acid and 5-CH_3-H_4folate for these transport pathways. In addition to probenecid and MTX, which both non-specifically inhibit the RFC and MRP-transport pathways, the agent MK571 (0.01-25 µmol/L) was studied on its effect on the transport of folic acid and 5-CH_3-H_4folate as MK571 specifically blocks the MRP-efflux transport. The contribution of receptor-mediated transport was studied by the addition of colchicine (1-100 µmol/L), which inhibits the activity of the receptors by disrupting the endocytosic pathway. Finally, the role of Pgp was studied by measuring the transport of both folate compounds in absence or presence of PSC833 (0.1-10 µmol/L), a known specific inhibitor of Pgp-mediated efflux.

Statistical analysis

Data in the figures and text are expressed as mean ± SD of at least three determinations. Comparisons were performed by a Student's two-tailed t-test. Differences were considered significant at $P < 0.05$.

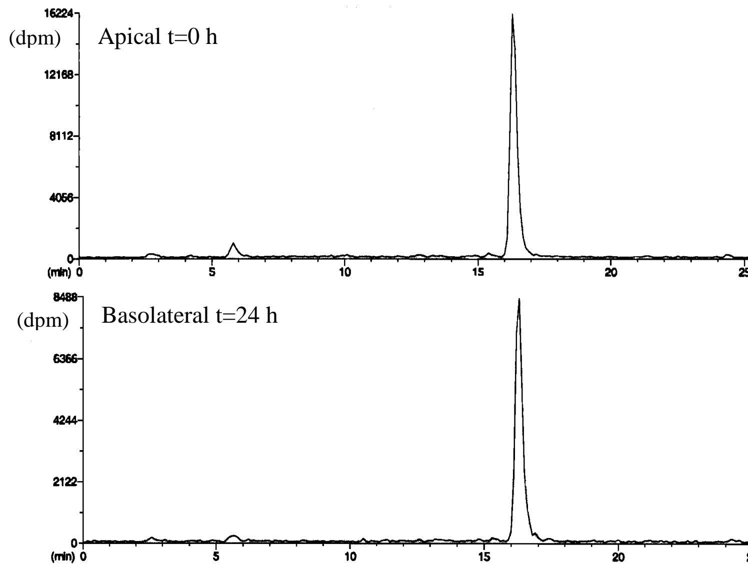
Results

Stability of folic acid and 5-CH_3-H_4folate

In order to study whether folic acid and 5-CH_3-H_4folate are metabolized during transport across human intestinal cells, Caco-2 cells were apically exposed to several dose solutions of folic acid and 5-CH_3-H_4folate and the basolateral samples were analyzed with HPLC.

The HPLC chromatograms of the dose solutions revealed a single peak with a retention time of 16-17 min for folic acid and 10-11 min for 5-CH_3-H_4folate, respectively (Figure 7.2). No metabolites were found in the basolateral samples collected over time (0-24 h) after exposure to the dose solutions of folic acid or 5-CH_3-H_4folate. The HPLC chromatograms of the basolateral samples after 24h exposure to 0.2 µmol/L folic acid or 5 µmol/L 5-CH_3-H_4folate are shown in Figure 7.2 as representative chromatograms for the basolateral samples collected over time (0-24h) after exposure to different dose solutions (0.02, 0.20 and 5 µmol/L folic acid or 5 µmol/L 5-CH_3-H_4folate). This indicates that the folate compounds are stable in the transport medium and that the parent compounds, folic acid and 5-CH_3-H_4folate, are not metabolized during transport across monolayers of Caco-2 cells.

A) Folic acid



B) 5-CH_3-H_4folate

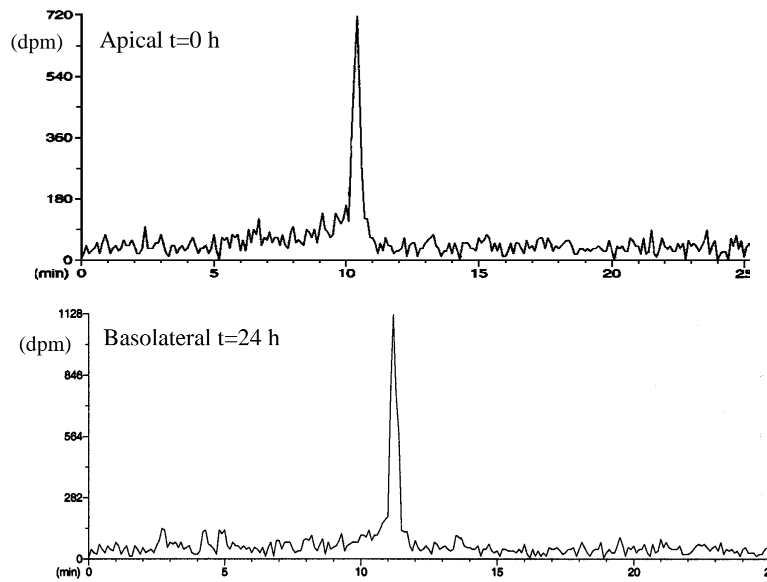
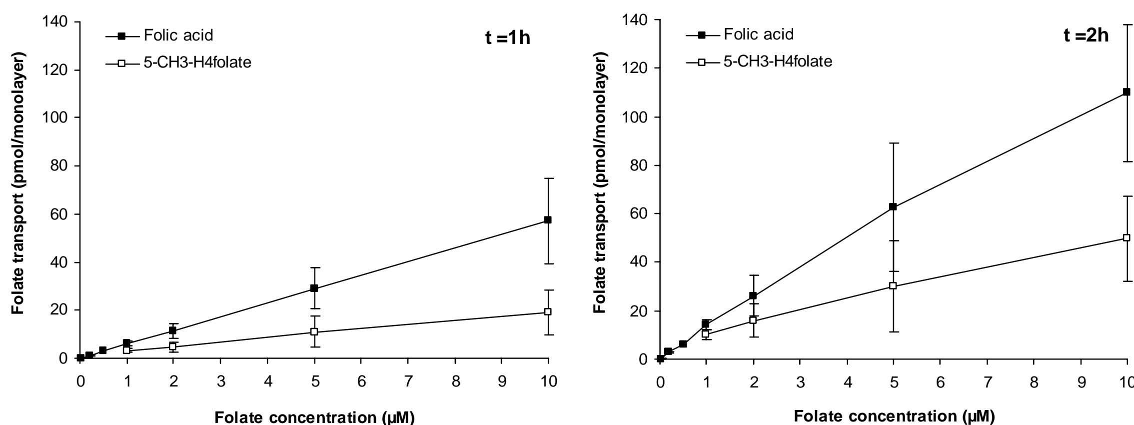


Figure 7.2. Chromatograms of A) folic acid (0.02 µmol/L) and B) 5-CH_3-H_4folate (5 µmol/L) at t=0 (dose, apical compartment) and t=24 h (basolateral compartment).

A) Ap > Bl



B) Bl>Ap

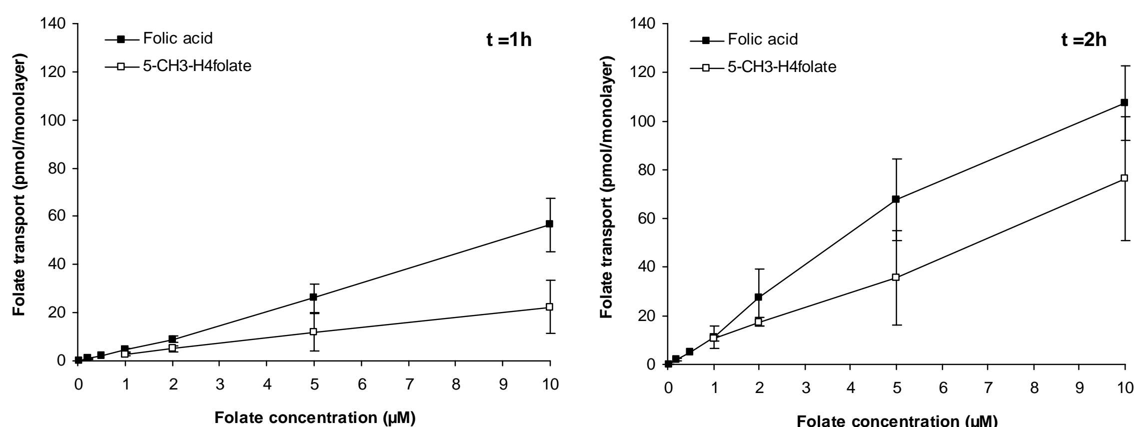


Figure 7.3. Transport of folic acid and 5-CH_3-H_4folate, given as absolute amount (pmol) per monolayer (1.13 cm^2) of Caco-2 cells, from A) the apical to the basolateral side of the Caco-2 cells (Ap>Bl) and B) vice versa (Bl>Ap). Cells were exposed to (0.02-10 µmol/L) folic acid or (1-10 µmol/L) 5-CH_3-H_4folate for 1-2 h. Values are means ± SD of at least three determinations. * indicate a significant ($P<0.05$) difference between the transport of folic acid and of 5-CH_3-H_4folate.

Transport of folic acid and 5-CH_3-H_4folate

The bidirectional transport (Ap>Bl and Bl>Ap) of folic acid and 5-CH_3-H_4folate appeared to be linear in time (1h, 2h) within the dose range of 0.02-10 µmol/L and 1-10 µmol/L, respectively (Figure 7.3). Within this concentration range, the transport (Ap>Bl and Bl>Ap) of folic acid appeared to be higher as compared to the transport of 5-CH_3-H_4folate. The Ap>Bl transport of folic acid and 5-CH_3-H_4folate (Figure 7.3A) equaled their Bl>Ap transport (Figure 7.3B). In the subsequent transport studies, in which the role of the individual transport mechanisms in the transport of folic acid and 5-CH_3-H_4folate was investigated, we used 1 µmol/L dose solutions of folic acid and 5-CH_3-H_4folate.

Effect of inhibitors on transport of folic acid and 5-CH_3-H_4folate

To distinguish the pathways underlying the intestinal transport of folic acid and 5-CH_3-H_4folate, the transport (Ap>Bl or Bl>Ap) of both folate compounds was studied in time in absence and in presence of inhibitors of several transport pathways (Figure 7.4). In absence of inhibitors, the Ap>Bl transport of folic acid appeared to be slightly higher than that of 5-CH_3-H_4folate while an equal transport (Bl>Ap) for folic acid and 5-CH_3-H_4folate was found. MTX, probenecid and MK571 were added to the dose solutions to study whether the transport of the folate compounds is RFC- and/or MRP-mediated. MTX and MK571 were found to inhibit the transport of both folate compounds dose-dependently with a maximum inhibition at 50 µmol/L MTX and 25 µmol/L MK571 (data not shown). These concentrations were used in the inhibition studies. In the presence of MTX (50 µmol/L), probenecid (2 mM), MK571 (25 µmol/L), the transport of folic acid (Ap>Bl and Bl>Ap) was significantly reduced with 40-47%, 49-63% and 48-55%, respectively (Figure 7.5). The transport of 5-CH_3-H_4folate (Ap>Bl and Bl>Ap) was decreased to a higher extent than the transport of folic acid as the agents MTX, probenecid and MK571 significantly inhibited the transport of 5-CH_3-H_4folate with 60-67%, 75% and 69-72%, respectively (Figure 7.5).

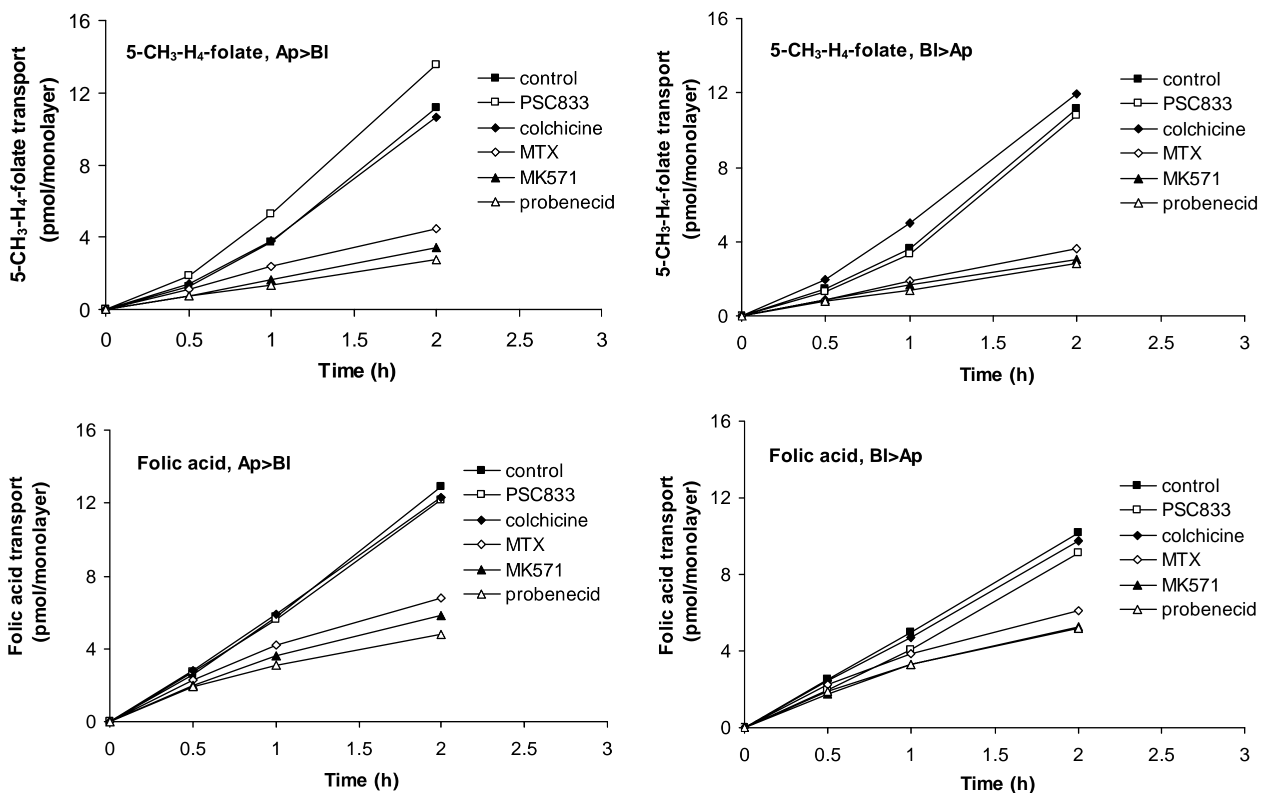


Figure 7.4. Effect of inhibitors on the Ap>Bl and Bl>Ap transport of folic acid and 5-CH_3-H_4folate, given as absolute amount (pmol) per monolayer (1.13 cm^2) of Caco-2 cells, over time (0.5, 1, 2 h). The permeability of folic acid and 5-CH_3-H_4folate across Caco-2 cells was tested in absence (control) and presence of inhibitors of several transport pathways (10 µmol/L PSC833, 100 µmol/L colchicine, 25 µmol/L MK571, 2 mmol/L probenecid, 50 µmol/L MTX). The values are means of three determinations

Colchicine was added to the dose solutions to study whether the transport of folic acid and 5-CH_3-H_4folate is receptor-mediated. Within the concentration range (1-100 µmol/L) studied, no effect of colchicine was found on the transport of folic acid and of 5-CH_3-H_4folate across Caco-2 cells. In presence of PSC833 (0.1-10 µmol/L), an inhibitor of Pgp-mediated efflux, the transport of folic acid and 5-CH_3-H_4folate was found to be similar to the transport in absence of PSC833.

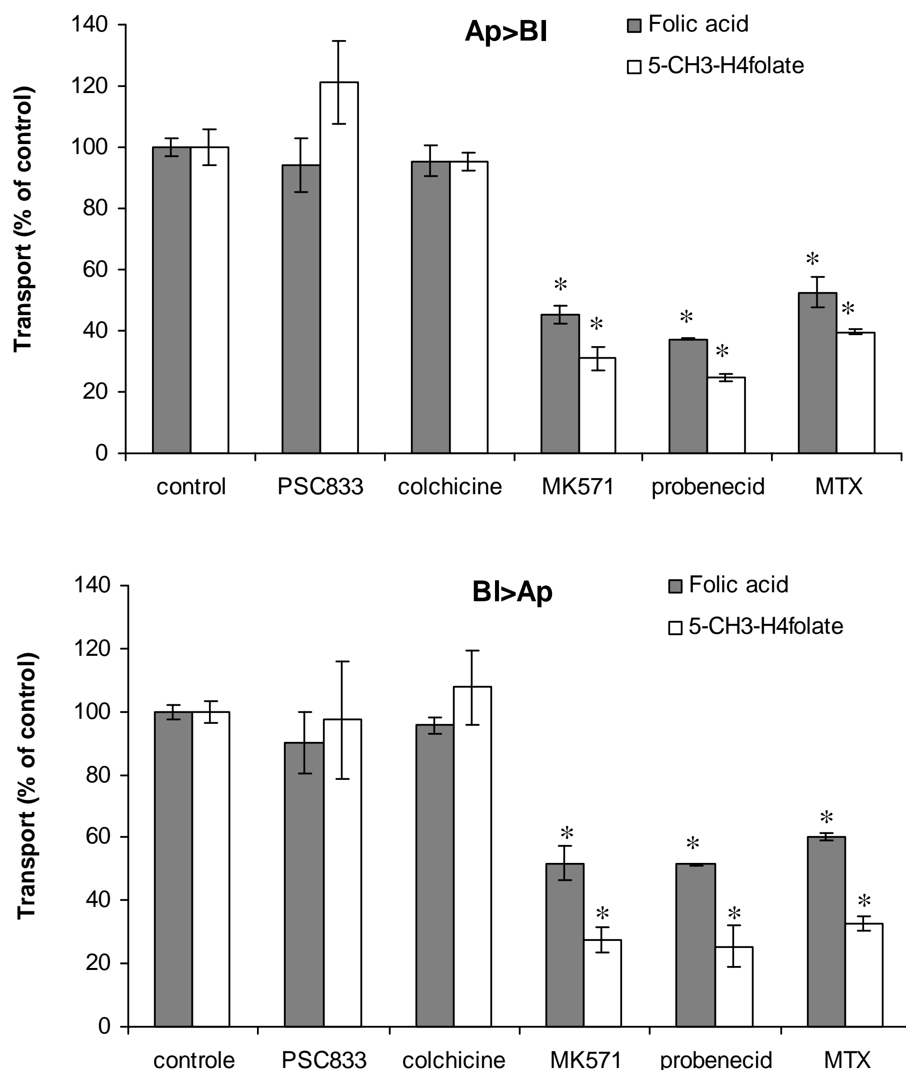


Figure 7.5. Ap>Bl and Bl>Ap transport of folic acid and 5-CH_3-H_4folate, given as percentage of transport in absence of inhibitors (control), after 2 h incubation to 1 µmol/L folic acid or 5-CH_3-H_4folate in absence or presence of inhibitors (10 µmol/L PSC833, 100 µmol/L colchicine, 25 µmol/L MK571, 2 mmol/L probenecid, 50 µmol/L MTX). The values (±SD) are means of three determinations. * indicate a significant ($P<0.05$) difference in the transport of folic acid or 5-CH_3-H_4folate in presence of inhibitors compared with the transport in absence of inhibitors.

Discussion

In the present study, the transport of folic acid and 5-CH_3-H_4folate across human Caco-2 cells was investigated. The relative contribution of individual pathways to the overall intestinal transport of folic acid and 5-CH_3-H_4folate, such as transport via the reduced folate carrier (RFC), folate receptor (FR), P-glycoprotein (Pgp) and multi-drug resistance proteins (MRPs), was studied in the presence or absence of inhibitors of these pathways in the dose solutions of folic acid (1 µmol/L) and 5-CH_3-H_4folate (1 µmol/L). The transport mechanisms underlying the intestinal transport of the folate compounds should be studied under unsaturated conditions. These test conditions were established prior to the inhibition studies by investigating the transport of folic acid and 5-CH_3-H_4folate over a concentration range of 0.02-10 µmol/L. As illustrated in Figure 7.3, a linear relation was found between transport and concentration of folate indicating that the transport mechanisms involved in the transport of folate were not saturated till 10 µmol/L folic acid or 5-CH_3-H_4folate. These results are in line with previous studies (9,10,21) which demonstrated a linear relation between transport and mucosal concentrations till 5 µmol/L and a high contribution of non-saturable transport of folate at higher concentrations (>20 µmol/L).

As folate is highly hydrophilic, passive transcellular transport of folate across the lipid bilayer membrane is not expected (Figure 7.1, route A). Therefore, folic acid and 5-CH_3-H_4folate might be partly transported via passive paracellular transport (Figure 7.1, route B). In addition, uptake via FR and/or RFC and efflux via MRPs or Pgp might contribute to the net intestinal transport of folate (Figure 7.1, routes C-F). The folate receptor, anchored to the cell surface by a glycosyl phosphatidylinositol (GPI) adduct, has a high affinity for binding folate but a low capacity for facilitating folate uptake by endocytosis (13). Previous studies showed that Caco-2 cells express folate receptors, both apically and basolaterally (22,23), but the contribution of FR-mediated uptake to the net transport of folic acid and 5-CH_3-H_4folate has not been investigated before. In the present study we focused on the activity of the folate receptors without measuring the expression of the receptors in the Caco-2 cells. Earlier studies with cultured human Caco-2 (22) and KB (nasopharyngeal epidermoid carcinoma) cells (24) showed that the expression of FR was inversely related with the folic acid content in the culture medium. As a result, the expression of FR in the Caco-2 cells used in the present studies is expected to be low as the cells were cultured in medium with a high folic acid content (approximately 2.3 µmol/L). A low expression of FR in the Caco-2 cells seems to be in line with the physiological conditions as the expression of FR in the human small intestine *in vivo* is also expected to be low (15). The contribution of receptor-mediated uptake to the overall transport of folic acid and 5-CH_3-H_4folate across Caco-2 cells was studied by the addition of colchicine, which inhibits the activity of the receptors by disrupting the endocytosic pathway (25). Colchicine was found not to reduce the transport of folic acid and 5-CH_3-H_4folate across Caco-2 cells. Thus, these studies indicate that receptor-mediated transport of folic acid and 5-CH_3-H_4folate is most probably not involved in the intestinal transport of folate.

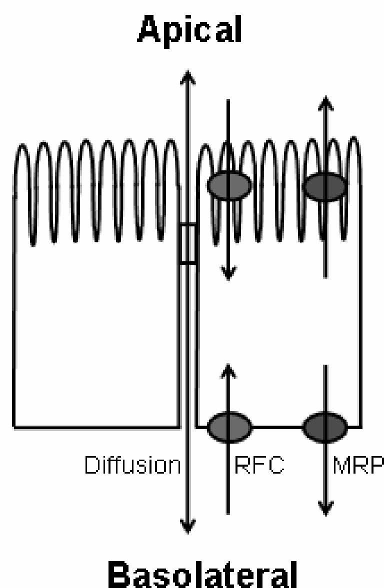


Figure 7.6. Hypothesis for the transportmechanisms involved in the intestinal transport (Ap>Bl and Bl>Ap) of folic acid and 5-CH_3-H_4folate

RFC-mediated uptake of folic acid and 5-CH_3-H_4folate was studied with known inhibitors (probenecid and MTX) of this transport pathway. In presence of probenecid or MTX, the transport of folic acid and 5-CH_3-H_4folate was reduced with 40-63% and 60-75%, respectively. These agents, however, do not only inhibit RFC-mediated uptake of folate but also the efflux via MRPs. Thus, RFC-mediated transport was not investigated separately. But, in presence of MK571, which is known to be a specific inhibitor of MRP1 and MRP2-mediated efflux, the transport of folic acid and 5-CH_3-H_4folate was also significantly reduced with 48-55% and 69-72%, respectively (Figure 7.5). These results indicate that the intestinal transport of folic acid and 5-CH_3-H_4folate was found to be highly dependent on the activity of MRP-efflux transporters. As a result, we hypothesize that, beside paracellular diffusion of folate, the transport (Ap>Bl and Bl>Ap) of folate is dependent on RFC-mediated uptake and efflux via MRP transporters (Figure 7.6). As the transport of 5-CH_3-H_4folate was decreased to a higher extent in presence of inhibitors than that of folic acid, we conclude that the contribution of RFC-mediated uptake and MRP-mediated efflux to the net transport of 5-CH_3-H_4folate is higher than their contribution to the net transport of folic acid.
In this study, MK571, an inhibitor of efflux via MRP1 and MRP2, was used to investigate the contribution of MRP transporters to the intestinal transport of folate. These efflux transporters belong to the superfamily of ATP binding cassette (ABC) transporter proteins and mediate the ATP-driven unidirectional transport of a broad range of compounds across cellular membranes. Till now, nine members of the MRP family of exporters have been identified (26), of which MRP1-4 were found to transport folate and anti-folates (27-29). There is no evidence to date that MRP5-9 transport folates (30). Caco-2 cells have shown to express at least MRP1-5 (31), of which the expression of MRP2 is found to be most abundant (32). The transcript levels of 9 of 10 of the ABC transporters were found to correlate well between human jejunum and Caco-2 cells (32). This indicates that the Caco-2 cell line is a useful model to study the jejunal efflux of nutrients and drugs. Next to MRPs, Pgp, another ABC transport protein, is recognized as an important barrier for

drug absorption. Pgp is an efflux transporter with wide substrate specificity and is expressed on the brush-border membrane of enterocytes (including Caco-2 cells) (33). Folic acid and 5-CH_3-H_4folate appeared not to be substrates for the Pgp efflux transporter as PSC833, which specifically blocks this transport route, had no effect on the transport of both folate compounds.

After folic acid is absorbed from the intestinal lumen, it is converted to metabolically active folate derivatives (such as 5-CH_3-H_4folate) in the body. Whether intestinal cells contribute to the metabolism of folate compounds is unknown. In present study, the profiles of the transport solutions with folic acid or 5-CH_3-H_4folate prior and after incubation to Caco-2 cells were studied by HPLC analysis. Folic acid and 5-CH_3-H_4folate were unaltered transported across Caco-2 cells even after 24 h of incubation (Figure 7.2). Based upon these results, we conclude that folic acid and 5-CH_3-H_4folate most probably reach the hepatic portal vein in the parent form and, thus, no contribution of the intestinal cells to the metabolism of folate in the human body is expected.

The transport of folic acid and 5-CH_3-H_4folate appeared to be highly dependent on the intestinal transport mechanisms (RFC and MRP). If we extrapolate these *in vitro* results to the human situation *in vivo*, it becomes clear that the activity of the transporters in the small intestine might be an important determinant for the absorption of folate from the intestinal lumen. Previous *in vitro* studies have shown interactions of drug and food compounds with folate uptake (34-36). For instance, it is shown that sulfasalazine, a drug widely used in the treatment of inflammatory bowel diseases, impairs the intestinal transport of folic acid in the human small intestine (34). In addition, dietary flavonoids might also decrease the absorption of folate as these food ingredients are known inhibitors of the ABC transporters (including MRP transporters) (37,38). Information on interactions of food ingredients with folate absorption can be used in the development of a dietary strategy leading to an optimal bioavailability of folate.

In conclusion, folic acid and 5-CH_3-H_4folate are transported across Caco-2 cells via RFC-mediated uptake and efflux via MRPs. The FR- and Pgp-transporters were found not to be involved in the intestinal transport of folate. The relative contribution of the RFC and MRP-mediated transport to the net intestinal transport appeared to be higher for 5-CH_3-H_4folate than for folic acid. Furthermore, we have found no evidence for intestinal metabolism of folate in the studies with monolayers of Caco-2 cells.

References

1. De Bree, A., Van Dusseldorp, M., Brouwer, I.A., Van het Hof, K.H. & Steegers-Theunissen, R.P. (1997) Folate intake in Europe: recommended, actual and desired intake. Eur. J. Clin. Nutr. 51: 643-660.
2. Konings, E.J.M., Roomans, H., Dorant, E., Goldbohm, R., Saris, W. & van den Brandt, P. (2001) Folate intake of the Dutch population based newly established liquid chromatography data for foods. Am. J. Clin. Nutr. 73: 765-776.
3. Verwei, M., Arkbåge, K., Havenaar, R., van den Berg, H., Witthöft, C. & Schaafsma, G. (2003) Folic acid and 5-Methyltetrahydrofolate in fortified milk are bioaccessible as determined in a dynamic in vitro gastrointestinal model. J. Nutr. 133: 2377-2383.
4. Arkbåge, K., Verwei, M., Havenaar, R. & Witthöft, C. (2003) Addition of folate-binding protein lowers the bioaccessibility of folic acid and 5-methyltetrahydrofolate from fortified yogurt as studied in a dynamic in vitro gastrointestinal model. J. Nutr. 133: 3678-3683.
5. Hidalgo, I.J., Raub, T.J. & Borchardt, R.T. (1989). Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. Gastroenterology, 96: 736-749.
6. Hillgren, K.M., Kato, A. & Borchardt, R.T. (1995) In vitro systems for studying intestinal drug absorption. Med.Res.Rev. 15: 82-109.
7. Artusson, P. & Karlsson, J. (1991) Correlation between oral drug absorption in humans and apparent drug permeability coefficients in human intestinal epithelial (Caco-2) cells. Biochem. Biophys. Res. Com., 175 (3): 880-885.
8. Verwei, M., van den Berg, H., Havenaar, R. & Groten, J.P. (2004) Effect of folate-binding protein on intestinal transport of folic acid and 5-methyltetrahydrofolate across Caco-2 cells. Eur. J. Nutr. (in press).
9. Said, H.M., Grishan, F.K. & Murrell, J.E. (1985) Ontogenesis of the intestinal transport of 5-methyltetrahydrofolate in the rat. Am. J. Physiol. 249: 567-571.
10. Selhub, J., Powell, G.M. & Rosenberg, I.H. (1984) Intestinal transport of 5-methyltetrahydrofolate. Am. J. Physiol. 246: G515-G520.
11. Said, H.M., Grishan, F.K. & Redha, R. (1987) Folate transport by human intestinal brush-border membrane vesicles. Am. J. Physiol. 252: 229-236.
12. Dudeja, P.K., Kode, A., Alnounou, M., Tyagi, S., Torania, S., Subramamian, V.S. & Said, H.M. (2001) Mechanism of folate transport across the human colonic basolateral membrane. Am. J. Physiol. Gastrointest. Liver Physiol., 281: G54-G60.
13. Birn, H., Selhub, J. & Christensen, E.I. (1993) Internalization and intracellular transport of folate binding protein in rat kidney proximal tubule. Am. J. Physiol. 264: C302-C310.
14. Verma R.S., Gullapalli, S. & Antony, A.C. (1992) Evidence that the hydrophobicity of isolated, in situ, and de novo-synthesized native human placental folate receptors is a function of glycosyl-phosphatidylinositol anchoring to membranes. J. Biol. Chem., 267 (6): 4119-4127.
15. Van Hoozen, C.M, Ling, E. & Halsted, C.H. (1996) Folate binding protein: molecular characterization and transcript distribution in pig liver, kidney and jejunum. Biochem. J., 319: 725-729.
16. Shoda, R., Mason, J.B., Selhub, J. & Rosenberg, I.H. (1990) Folate binding in intestinal brush border membranes: evidence for the presence of two binding activities. J. Nutr. Biochem., 1: 257-276.

17. Duizer, E., Penninks, A.H., Stenhuis, W.H. & Groten, J.P. (1997) Comparison of permeability characteristics of the human colonic Caco-2 and rat small intestinal IEC-18 cell lines. J. Contr. Rel. 49: 39-49.
18. Konings, E.J.M. (1999) A validated liquid chromatographic method for determining folates in vegetables, milk powder, liver and flour. J. AOAC Int. 82: 119-127.
19. Zhao, R. & Goldman, I.D. (2003) Resistance to antifolates. Oncogene, 22: 7431-7457.
20. Assaraf, Y.G., Rothem, L., Hooijberg, J.H., Stark, M., Ifergan, I., Kathman I., Dijkmans, B.A.C., Peters, G.J. & Jansen, G. (2003) Loss of Multidrug resistance protein 1 expression and folate efflux activity results in a highly concentrative folate transport in human leukaemia cells. J. Biol. Chem. 278 (9): 6680-6686.
21. Mason, J.B., Shoda, R., Haskell, M., Selhub, J. & Rosenberg, I.H. (1990) Carrier affinity as a mechanism for the pH-dependence of folate transport in the small intestine. Biochim. Biophys. Acta, 1024: 331-335.
22. Doucette, M.M & Stevens, V.L. (2001) Folate receptor function is regulated in response to different cellular growth rates in cultured mammalian cells. J. Nutr. 131: 2819-2825.
23. Jackman, M.R., Shurety, W., Ellis, J.A. & Luzio, J.P. (1994) Inhibition of apical but not basolateral endocytosis of ricin and folate in Caco-2 cells by cytochalasin D. J. Cell Science, 107: 2547-2556.
24. Hsueh, C.T. & Dolnick, B.J. (1993) Altered folate-binding protein mRNA stability in KB cells grown in folate-deficient medium. Biochem. Pharm., 45: 2537-2545.
25. Morshed, K.M., Ross, D.M. & McMartin, K.E. (1997) Folate transport proteins mediate the bidirectional transport of 5-methyltetrahydrofolate in cultured human proximal tubule cells. J. Nutr. 127: 1137-1147.
26. Borst, P & Elferink, R.O. (2002) Mammalian ABC transporters in health and disease. Annu.Rev.Biochem., 71: 537-592.
27. Chen, Z.S., Lee, K., Walther, S., Blanchard Raftogianis, R., Kuwano, M., Zeng, H. & Kruh, G.D. (2002) Analysis of methothrexate and folate transport by multidrug resistance protein 4 (ABCC4): MRP4 is a component of the methotrexate efflux system. Cancer Res., 62: 3144-3150.
28. Hooijberg, J.H., Broxterman, H.J., Kool, M., Assaraf, Y.G., Peters, G.J., Noordhuis, P., Scheper, R.J., Borst, P., Pinedo, H.M. & Jansen, G. (1999) Antifolate resistance mediated by the multidrug resistance proteins MRP1 and MRP2. Cancer Res., 59: 2532-2535.
29. Zeng, H., Chen, Z.S., Belinsky, M.G., Rea, P.A. & Kruh, G.D. (2001) Transport of methotrexate (MTX) and folate by multidrug resistance protein (MRP)3 and MRP1: effect of polyglutamation on MTX transport. Cancer Res., 61: 7225-7232.
30. Matherly, L.H. & Goldman, I.D. (2003) Membrane transport of folates. In vitamins and hormones, 66: 403-456.
31. Pfrunder, A., Gutmann, H., Beglinger, C. & Drewe, J. (2003) Gene expression of CYP3A4, ABC-transporters (MDR1 and MRP1-MRP5) and hPXR in three different human colon carcinoma cell lines. J. Pharmacy and Pharmacology, 55: 59-66.
32. Taipalensuu, J., Tornblom, H., Lindberg, G., Einarsson, C., Sjoqvist, F., Melhus, H., Garberg, P., Sjostrom, B., Lundgren, B. & Artursson, P. (2001) Correlation of gene expression of ten drug efflux proteins of the ATP-binding cassette transporter family in normal human jejunum and in human intestinal epithelial Caco-2 cell monolayers. J. Pharmacol. Exp. Therap. 299: 164-170.
33. Ambudkar, S.V., Dey, S.C., Hrycyna, A., Ramachandra, M., Pastan, I. & Gottesman, M.M. (1999) Biochemical, cellular, and pharmacological aspects of the multidrug transporter. Annu. Rev. Pharmacol.Toxicol., 39, 361-398.

34. Zimmerman, J. (1992) Drug interactions in intestinal transport of folic acid and methotrexate. Further evidence for the heterogeneity of folate transport in the human small intestine. Biochem. Pharm. 44: 1839-1842.
35. Amilburu, A., Idoate, I., Ponz, F. & Larralde, J. (2001) Inhibition of intestinal absorption of 5-methyltetrahydrofolate by fluoxetine. J Physiol Biochem. 57, 71-80.
36. Villanueva, J., Devlin, A.M. & Halsted, C.H. (2001) Reduced folate carrier: tissue distribution and effects of chronic ethanol intake in the micropig. Alcohol Clin Exp Res, 25, 415-420.
37. Leslie, E.M., Mao, Q., Oleschuk, C.J., Deeley, R.G. & Cole, S.P. (2001) Modulation of multidrug resistance protein 1 (MRP1/ABCC1) transport and ATPase activities by interaction with dietary flavonoids. Mol. Pharmacol., 59, 1171-1180.
38. Hooijberg, J.H., Broxterman, H.J., Heijn, M., Fles, D.L., Lankelma, J. & Pinedo, H.M. (1997) Modulation by (iso)flavonoids of the ATPase activity of the multidrug resistance protein. FEBS Lett, 413, 344-348.

8

Bioavailability of folic acid from fortified pasteurized and UHT-treated milk in humans

Submitted for publication

Rosemarijn J. de Jong[1]
Miriam Verwei[1,2]
Clive E. West[1]
Trinette van Vliet[2]
Els Siebelink[1]
Henk van den Berg[3]
Jacqueline J.M. Castenmiller[4]

[1]Department of Human Nutrition, Wageningen University, Wageningen, The Netherlands
[2]TNO Nutrition and Food Research, Zeist, The Netherlands
[3]The Netherlands Nutrition Center, Den Haag, The Netherlands
[4]Wageningen Centre for Food Sciences, Wageningen, The Netherlands

Abstract

Background Folate intake is often lower than recommended. Milk may be a suitable product for folic acid fortification to enhance the folate status of the population. In untreated and pasteurized milk, folate occurs bound to folate-binding proteins (FBP), while FBP is destroyed in ultra high temperature (UHT)-treated milk. The effect of FBP on folate bioavailability is still unclear. *Objective* The aim of the study was to investigate whether milk fortified with folic acid enhances the folate status of humans and whether the destruction of FBP by UHT treatment affects the response to folic acid from fortified milk. *Design* Healthy subjects (n=69) aged 18-49 y participated in a 4-week double blind randomized placebo controlled intervention trial. In addition to a fully controlled diet, the subjects consumed each day 500 mL of pasteurized or UHT milk, either or not fortified with 200 µg folic acid. *Results* Compared with the corresponding unfortified milk groups, consumption of fortified milk increased folate concentrations in serum and red blood cell (RBC) by 6.6–7.0 nmol/L ($P<0.001$) and 32–36 µmol/L ($P<0.01$), respectively. Similarly plasma total homocysteine concentrations were lowered by 0.88-0.89 µmol/L ($P=0.001$) in subjects who consumed fortified milk. The bioavailability of folic acid from pasteurized milk relative to that of folic acid from UHT milk was 74 - 94% (NS), depending on the parameter used. *Conclusions* Milk fortified to supply an additional 200 µg of folic acid/d substantially increased folate status, and decreased plasma total homocysteine concentrations in young, healthy subjects. No significant effect of endogenous FBP was found on the bioavailability of folic acid from milk.

Introduction

Low folate status can cause megaloblastic anaemia (1, 2), increases the risk of neural tube defects (3, 4) and is associated with increased risk of colon cancer (5). Furthermore, inadequate folate intake can enhance plasma homocysteine concentrations (6), which in turn is associated with increased risk for cardiovascular disease (7-10). In many European countries where folic acid fortification is not yet permitted, folate intake is usually lower than recommended (11). Thus, although there is currently much debate over folate requirements for optimal health and function (4, 12, 13), increasing folate intake of populations to recommended levels is desirable. It would appear that it is not feasible for the whole population to reach the recommended intake by the consumption of products naturally rich in folate such as fruits and vegetables (14-16). As a result, the consumption of fortified food products can be considered as an alternative or complementary approach to increase folate intakes (15).

Milk may be a suitable product for folic acid fortification in many Western countries. As a result of its high consumption, dairy products provide 10 to 15% of the daily folate intake in such countries, especially among the younger population (17). In addition, the presence of milk in the diet seems to enhance folate bioavailability compared to diets without milk (18). The naturally occurring form of folate in untreated milk, 5-methyltetrahydrofolate (5-CH_3-H_4folate), is bound to folate-binding proteins (FBP) (19-21). Ultra high temperature (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, as a result, folate remains FBP-bound (21). Folate bound to FBP was found to be relatively unavailable to intestinal flora (22), and consequently FBP might enhance folate absorption from the gut. FBP might also directly promote the transport of folate across the intestinal mucosa, although contradictory results have been reported (23-26). Thus, the role of FBP in milk in folate bioavailability is still a matter of debate.

The objectives of the present study were twofold: firstly, to determine whether fortified milk can enhance the folate status in humans and secondly, to determine the response to folic acid from pasteurized milk relative to that from UHT milk. For these purposes, serum folate, red blood cell folate and plasma homocysteine levels were measured prior to and after a 4-week intervention period in which 69 healthy subjects consumed pasteurized or UHT milk either or not fortified with 200 μg folic acid per day.

Subjects and Methods

Subjects

Subjects were recruited via advertisements in local and university newspapers and on posters in university buildings and student apartments. The study protocol was explained carefully to potential participants who were admitted to the study on signing an informed consent form. The study protocol was approved by the Medical Ethical Committee of Wageningen University. Subjects were eligible if they were between 18 and 50 y of age. Exclusion criteria were smoking, use of dietary supplements or yeast tablets during the 4 wk prior to the study, use of malaria prophylactics or

corticosteroids during the previous 3 mo, abnormal fasting concentrations in urine of glucose or protein, abnormal hematology, blood hemoglobin concentrations <7.4 mmol/L in women or <8.5 mmol/L in men, or total plasma homocysteine concentrations > 15 μmol/L.

Study design

The study comprised a 4-wk run-in period followed by a 4-wk double-blind intervention using four parallel dietary treatment groups. During the run-in period, subjects were allowed to choose their own diets but were instructed to avoid foods rich in folate. Before the dietary intervention period, subjects were stratified according to sex, self-reported energy intake and total plasma homocysteine concentration, and randomly allocated to one of 4 experimental groups. During the intervention period, all subjects received each day a basal diet plus 500 mL of one of the following semi-skimmed milk products: pasteurized milk, pasteurized milk fortified with 200 μg folic acid, UHT milk, or UHT milk fortified with 200 μg folic acid.

Folic acid (pteroylglutamic acid; Eprova, Schaffhausen, Switzerland) was added to unprocessed milk. After fortification, the milk was homogenized at 60-65°C and divided into two portions. One half of the milk was pasteurized (15 sec, 76°C) and one half was processed following UHT treatment (15 sec, 140°C). The unfortified milk was processed in the same way. Each milk product was assigned a color code that was used to label the 250 mL sealed milk beakers, in order to blind the subjects and researchers to their content. The milk products were manufactured and supplied twice weekly by Campina Netherlands (Woerden, The Netherlands). The milk was stored at 4ºC and distributed daily to the subjects in insulated bags with a cooling element. The subjects were not allowed to heat the milk, and were instructed to drink the milk together with their bread meals (breakfast and evening meals), in order to diminish variations in uptake due to variations in food matrices.

The menu was changed daily on a weekly cycle and based on Dutch nutrition guidelines and recommendations (27). Special care was taken to ensure that the folate and vitamin C content in the diets served each day were similar. On weekdays, the subjects received a hot meal at the Division of Human Nutrition of Wageningen University. Bread meals, milk, fruit and cookies were packed for consumption at home, as was food and milk for the weekends together with instructions on how to prepare the food. To estimate habitual energy intake, the subjects completed a food frequency questionnaire (28) prior to the start of the study. We designed menus for 19 levels of energy intake, ranging from 7 to 25 MJ/d, and allocated the subjects to an intake level close to their energy requirements. All foods were weighed or counted for every subject. We recorded the body weight of the subjects twice each week and adjusted energy intake when necessary in order to maintain stable body weight. The food distributed provided 90% of the energy required to maintain body weight. For the remaining 10% of energy intake, subjects were allowed to choose from a list of foods low in fat and containing no folate. The daily selection of free choice food items was recorded in a diary, as were illnesses and deviations from the instructions.

Analysis of diets

Each day during the intervention period, one complete portion of the controlled diet for an imaginary subject with a daily intake of approximately 11 MJ (ca. 1100 g) was collected for the analysis of macronutrients. The portions were stored at -20°C. On completion of the intervention, the seven individual day portions of each week were thawed and mixed, resulting in one portion for every week. Samples of each of these week portions were refrozen at -20°C. For the analysis of folate, another complete portion of the controlled diet, but without milk, was collected daily and blended with 1L of a freshly prepared 2% (w/v) ascorbic acid-phosphate buffer (pH 6.1) under a flow of nitrogen in order to reduce the risk of oxidation, and samples were stored at -80°C. In addition, portions (ca. 500 g) of each of the 4 milk products were collected daily, mixed with ca. 5 g sodium ascorbate (1% w/w) (Sigma, St. Louis, MO), and stored at -80°C until folate analysis.
The content of moisture and of ash in each weekly food portion was determined by heating in a vacuum oven at 85°C and then in a muffle furnace at 550°C. Protein content was assessed by the Kjeldahl method using a conversion factor of 6.25; fat was measured by the acid hydrolysis method (29), dietary fiber according to the AOAC Official Method for total dietary fiber (30), and digestible carbohydrate was calculated by difference.
The total folate content of the milk and food samples was determined using a microbiological assay based upon the growth of the folate-requiring chloroamphenical-resistent *Lactobacillus casei* (NCBI 10463, ATCC, Manassas, VA 20108 USA) (31). The samples were extracted following a tri-enzyme method. Successively, 1-5 mL of the samples was incubated for 3 h with 1 mL protease (2 g/L; Sigma), 2 h with 1 mL amylase (20 g/L; Sigma) and at least 4 h with 4 mL of chicken pancreas preparation (Amano Pharmaceuticals, Nagoya, Japan). Afterwards, the samples were added to microtiter plates and mixed with incubation medium (folic acid casei medium, 0822-15-9, Difco), containing chloramphenicol, and inoculated with the *Lactobacillus casei* strain. After an incubation period of 18-22 h at 37˚C, growth was measured by fluorescence spectrometry (at 600 nm) relative to the standard concentration curve of folic acid on each microtiter plate to measure the folate content of the samples.
The concentration of FBP in the milk products was quantified by a two-site ELISA (32) as described earlier (33). The antibody against FBP from bovine milk (rabbit anti-bovine FBP 24740) and the FBP calibrant were, respectively, obtained from the State Serum Institute, Copenhagen, Denmark and the Central Hospital, Hillerød, Denmark. Inter- and intra-assay coeffients of variation of folates and FBP did not exceed 15%.

Analysis of blood

Blood was taken from subjects after an overnight fast by venipuncture between 07.15 h and 10.30 h on days 0, 1, 27 and 28 of the dietary intervention period. Plasma samples for analysis of homocysteine concentrations were immediately placed on ice and centrifuged within 30 min (4°C, 1200 x *g*, 10 min). Serum samples for folate and vitamin B12 measurements were kept in the dark at room temperature for at least 30 min before centrifugation (4°C, 1200 x *g*, 10 min). Plasma and serum were stored at -80°C. A third tube (EDTA K3E, Venoject, Terumo Europe N.V., Leuven,

Belgium) was filled with blood and placed in the dark. For analysis of whole blood folate, one portion of this blood was diluted (1:9, v/v) with freshly prepared ascorbic acid solution (1%, w/v), mixed and immediately frozen in dry ice and stored at -80°C. A second portion of the blood from this tube was kept at room temperature for measuring the hematocrit, which was required for calculating folate concentrations in red blood cells. In addition, on the final day of the study, a portion of this blood was stored at -80°C for determination of the 5,10-methylenetetrahydrofolate reductase (MTHFR) genotype. For the determination of serum folate, whole blood folate and plasma homocysteine concentrations, all samples of each person sampled on days 0, 1, 27 and 28 were measured in the same run to eliminate inter-assay variation.

Whole blood lysate samples (400 µL) were incubated for 60 min in a waterbath at 37°C for conversion to monoglutamyl forms. Subsequently, proteins were precipitated by addition of 800 µL acetonitrile, and after mixing by vortex for 30 sec and centrifugation (4ºC, 10,000 x *g*, 10 min), the supernatant was evaporated to dryness under a flow of nitrogen at 50ºC and reconstituted in 250 µL of 0.1M phosphate buffer (with 1%, w/v ascorbic acid, pH 7.0). As no other folate compounds than 5-CH_3-H_4folate were detected in the serum and blood samples (as measured in a sub-sample, data not shown), the analytical method was directed towards the determination of the 5-CH_3-H_4folate content. Concentrations of 5-CH_3-H_4folate in serum and blood were determined using reversed phase HPLC with fluorescence (excitation 290 nm, emission 360 nm) detection. An inertsil 5 OD-3 column (2x10 cm, 3 mm id., Varian CP28308) was used in combination with a Chromsep guard column (10x2 mm, Varian CP28141). A gradient elution with 0.033 mol/L phosphate buffer (pH 2.1) and acetonitrile (HPLC-S grade) were used and the start gradient was a mixture of 95% phosphate buffer and 5% acetonitrile as described by Konings (34). Within- and between-run coefficients of variation (CVs) for serum and whole blood 5-CH_3-H_4folate were <5%. Folate concentration in red blood cells was calculated from those in whole blood and serum, and from the hematocrit.

Plasma concentrations of total homocysteine (sum of all oxidized and reduced forms of homocysteine) were measured by HPLC and fluorimetric detection as described by Ubbink et al (35). Within- and between-run CVs were 2% and 7%, respectively. Vitamin B12 concentrations in serum were determined with the Immulite 2000 Vitamin B12 Assay (DPC, Los Angeles, CA 90045-5597, USA). Within- and between-run CVs were 7 and 6%, respectively. Detection of the 677 C to T substitution in both alleles of the MTHFR gene was performed with a polymerase chain reaction, followed by restriction fragment length polymorphism analysis using the *Hinf*I enzyme (36).

Statistics and calculations

All analyses were based on the 69 subjects who completed the intervention study. For each subject, mean values for total plasma homocysteine, serum folate and red blood cell folate concentrations were calculated for day 0 and day 1 (week 0) and for day 27 and day 28 (week 4). All data are reported as mean ± SD. Serum folate and red blood cell folate concentrations were normally distributed. Total plasma homocysteine concentrations were positively skewed, so were transformed to natural logarithms to normalize their distribution for statistical analyses. For each person, the

response to treatment was calculated as the change in the measured parameter from the start to the end of the intervention period and those responses were used for statistical analyses. A 2 x2 factorial design was used to examine if addition of folic acid to milk increased the serum and erythrocyte folate concentrations and decreased the plasma homocysteine concentrations and if there was any difference in these responses between UHT and pasteurized milk (two-way analysis of variance, ANOVA). If the ANOVA indicated an overall significant effect of supplementation or type of milk ($P<0.05$), the treatment means were compared with Student's unpaired t-tests. All statistical analyses were carried out using SPSS for Windows 11.0 (SPSS Inc., Chicago, 60606 IL, USA).

We calculated the bioavailability of folic acid added to pasteurized milk relative to that of folic acid added to UHT milk using the formula:

$$\text{Relative bioavailability} = \frac{\text{Effect}_{past.}}{\text{Effect}_{UHT}} \times \frac{\text{FA}_{UHT}}{\text{FA}_{past.}} \times 100\%$$

where 'Effect $_{past.}$' corresponds to the difference in serum or red blood cell folate concentrations of the fortified pasteurized milk group compared to that in the unfortified pasteurized milk group, 'Effect $_{UHT}$' refers to the differences with respect to the UHT milk groups, while 'FA $_{UHT}$'and 'FA $_{past}$' refer to the folic acid content of the fortified UHT and pasteurized milk compared to the unfortified milks respectively. Relative bioefficacy was calculated from the ratio of the differences in plasma homocysteine concentrations.

Table 8.1. Characteristics of the subjects[1]

	Intervention group			
	UHT milk	UHT milk + folic acid	Pasteurized milk	Pasteurized milk + folic acid
N (male/female)	16 (3/13)	17 (2/15)	18 (4/14)	18 (5/13)
Age (years)[2]	21 (18 – 33)	22 (18 – 47)	21 (19 – 46)	22 (19 – 49)
Vitamin B_{12} (pmol/L serum)[2]	263 (185 – 393)	215 (100 – 521)	258 (158 – 380)	306 (160 – 654)
Body mass index (kg/m^2)[2]	23 (19 – 28)	24 (20 – 31)	23 (19 – 28)	24 (18 – 30)
MTHFR genotype (*CC*/*CT*/*TT*)	7/9/0	9/6/2	7/10/1	10/7/1

[1]Data are collected immediately before the intervention study, [2]Values are shown as median (range).

Results

Of the 72 eligible subjects who started the intervention, 7 had been unable to participate fully in the run-in period. As they were divided among the four intervention groups, we performed our analyses on data of all subjects. However, their exclusion would not have affected our conclusions. Three subjects withdrew during the intervention period: one because of illness unrelated to the intervention and two for personal reasons. Median age of the participants was 21 y and 80% were women (Table 8.1). Subject characteristics did not differ statistically significant among the four treatment groups. Their serum vitamin B_{12} concentrations were within the normal range (118 – 590 pmol/L), except for one woman in the fortified UHT milk group, who had a relatively low serum vitamin B_{12} concentration of 100 pmol/L.
The mean total intake of folate, macronutrients and energy during the intervention period are shown in Table 8.2. Compliance to the diet was very high as nearly all subjects consumed the expected 56 beakers (250 mL each) of milk. Two subjects failed to consume one beaker and one subject failed to consume 2.5 beakers of milk. The folate content of the free choice items was negligible. The energy intake from the free choice items was on average 11% of total energy. The folate concentrations in the unfortified and fortified UHT milk, 40 and 402 µg/L respectively, were slightly lower than in the unfortified and fortified pasteurized milk, 46 and 466 µg/L respectively (Table 8.3). The concentration of FBP in the pasteurized milk was 140-160 nmol/L, while no FBP was detected in the UHT milk (Table 8.3). This results in molar ratios between FBP and folate of approximately 1:0.7 and 1:7.5, respectively, in the unfortified and fortified pasteurized milk.

Table 8.2. Daily intake of nutrients and energy during the dietary intervention period.

	Mean intake
Energy (MJ/d)	10.6
Protein (energy%)	13
Fat (energy%)	34
Carbohydrates (energy%)	51
Alcohol (energy%)	2
Dietary fiber (g/MJ)	2.3
Folate (µg/day)[1]	140 ± 25

[1] Folate content of daily diet (milk excluded), given values as mean ± SD

Baseline serum folate, red blood cell folate and total plasma homocysteine concentrations were not significantly different among the four intervention groups (Table 8.4). Folic acid supplementation increased both serum and red blood cell folate and decreased plasma homocysteine concentrations. The two-factor ANOVA showed no interaction between addition of folate and type of milk. Addition of folate significantly affected all three study parameters (P<0.0001) whereas the type of milk did not significantly affect any of the study parameters. Because we found that the content of

folate was slightly different for the two supplemented milk groups, we carried out the same statistical analysis correcting for the amount of folate in the groups. The statistical analyses of the responses per unit of folate resulted in the same conclusions as described before. The bioavailability of folic acid added to pasteurized milk relative to folic acid added to UHT milk, according to serum folate concentrations was 81% (95% CI: 50 – 111%). Based on red blood cell folate concentrations, this relative bioavailability was 94% (95 CI: 28 – 160%). Relative bioefficacy based, on plasma homocysteine concentrations was 74% (95% CI: 17 – 132%).
The MTHFR-genotype distribution of subjects over homozygote wildtype (*CC*), heterozygote type (*CT*) and homozygote mutant type (*TT*) was 48%, 46% and 6%, respectively (Table 8.1). There were no differences in response of *CT* or *TT* subjects, but the number of subjects was low to expect differences to be found.

Table 8.3. Folate and FBP content of the four milk products[1]

	UHT milk	UHT milk + folic acid	Pasteurized milk	Pasteurized milk + folic acid
Folate (µg/500mL) [2]	20 ± 2	201 ± 8	23 ± 2	233 ± 7
FBP (nmol/500mL) [3]	<1	<1	80 ± 11	70 ± 8

[1] Values are mean ± SD, [2] The folate content of each milk product is different from that of the other milk products, P<0.01, [3] The FBP content of both pasteurized milks are significantly different from both UHT milks, P<0.001; pasteurized milk + folic acid is different from pasteurized milk, P=0.05.

Discussion

The present study showed that an extra daily dose of 200 µg folic acid added to milk significantly increased concentrations of folate in serum and red blood cells and decreased plasma homocysteine concentrations within four weeks in adults with a relatively low natural dietary folate intake (37). This indicates that milk is a suitable matrix to enhance the folate status in countries where milk is commonly consumed. Fortification of food products is not yet allowed in the Netherlands in contrast to some other countries such as the USA where fortification of bread and flour is found to be an effective strategy for the enhancement of folate status. In our study, folic acid from fortified milk was found to elevate serum folate concentrations and reduce plasma total homocysteine concentrations to a similar extent as folic acid from fortified breakfast cereals (38) and supplements (14, 39).

Table 8.4. Serum folate, red blood cell folate and total plasma homocysteine concentrations at start and end of dietary intervention

	Intervention groups			
	UHT milk (n=16)	UHT milk + folic acid (n=17)	Past. milk (n=18)	Past. milk + folic acid (n=18)
Serum folate (nmol/L)[1]				
Week 0	8.7 ± 3.2	8.6 ± 3.3	9.3 ± 2.1	9.1 ± 2.4
Week 4	7.2 ± 2.3	14.1 ± 5.3	7.5 ± 1.8	13.9 ± 3.9
Difference[2]	-1.5 ± 1.7	5.5 ± 4.0	-1.8 ±1.2	4.8 ± 2.9
Adjusted Difference[3]		7.0 (4.8 – 9.2)		6.6 (5.1 – 8.1)
Red blood cell folate (nmol/L)[1]				
Week 0	342 ± 93	348 ± 148	334 ± 88	365 ± 83
Week 4	322 ± 89	361 ± 127	313 ± 82	377 ± 77
Difference[2]	-19 ± 22	13 ± 39	-20 ± 30	12 ±18
Adjusted difference[3]		32 (10 – 55)		36 (20 – 52)
Plasma homocysteine (µmol/L)[1]				
Week 0	8.8 ± 1.6	8.9 ± 2.3	8.6 ± 2.1	8.7 ± 1.0
Week 4	9.2 ± 1.6	8.3 ± 2.3	9.1 ± 1.9	8.1 ± 1.2
Difference[2]	0.4 ± 0.9	-0.6 ± 1.0	0.5 ±1.1	-0.6 ±0.6
Adjusted ratio[4]		0.88 (0.82 – 0.94)		0.89 (0.84 – 0.95)

[1] Values are given as mean ± SD. [2] Significant difference between the fortified milk groups and their corresponding unfortified milk groups (P<0.0001).
[3] 95% CI in parentheses. [4] Based on the log-transformed data used for statistical analysis, thus ratio of geometric means of the fortified milk groups relative to the corresponding unfortified milk group at week 4, adjusted for baseline values. 95% CI in parentheses. Significant difference between the fortified milk groups and their corresponding unfortified milk groups (P<0.0001)

UHT treatment of milk led to a decrease in folate and FBP concentrations which is in agreement with earlier findings by Wigertz et al. (21). No FBP could be detected in UHT milk while pasteurized milk contained 140-160 nmol FBP/L. In the fortified pasteurized milk the amount of folate was 7.5 times higher than the amount of FBP (Table 8.3). According to Salter et al (40), 1 mol FBP binds 1 mol of folate at pH 7.2. Thus only a small amount of the added folic acid is expected to be bound to FBP in the fortified pasteurized milk. This might explain why no difference in bioavailability of folic acid was found from pasteurized milk compared with UHT milk. These results are in line with an earlier study with subjects with an ileostomy (41), who consumed milk products either with or without endogenous FBP together with a standardized diet daily for three weeks. No difference in total 5-CH_3-H_4folate excretion in the ileostomal effluent was found between the two groups, indicating similar absorption and thus no effect of endogenous FBP on folate bioavailability. The folic acid-fortified milk products tested in the present study were also studied in an *in vitro* dynamic gastrointestinal model (33) and a small but non-significant difference was found between the bioaccessible fraction of folic acid from pasteurized milk (58%) as compared with that from UHT milk (61%). The slightly lower bioaccessible folate fraction, corresponding to the folate fraction released from the food matrix and available for absorption in the small intestine, from pasteurized milk indicates a minor effect of endogenous FBP on folate bioaccessibility. The effect of additional FBP on the bioaccessibility of folic acid from UHT or pasteurized milk was also studied *in vitro* (33). After FBP addition, in a 1:1 molar ratio with folic acid, the bioaccessible fraction of folic acid was significantly ($P<0.05$) decreased as compared with the fortified pasteurized milk without additional FBP. Although pasteurization of milk might affect the binding characteristics of FBP, as shown by Gregory et al (42), our *in vitro* studies with the gastrointestinal model demonstrated that there is still residual binding of folic acid to FBP after gastric passage. Thus, an effect of FBP could be expected if the molar ratio between folic acid and FBP was 1:1 instead of the 7.5:1 molar ratio as tested in the present study. Therefore, in order to investigate the effect of FBP on the bioavailability of folate in humans, folate-fortified milk products with or without an equimolar amount of FBP should be tested in a human intervention study.

Until now, folic acid has been used as a supplement as this synthetic folate compound is more stable than 5-CH_3-H_4folate. However, the natural folate compound, 5-CH_3-H_4folate, might be a preferable substrate for fortification as it will not mask vitamin B_{12}-defiency. Another reason to use 5-CH_3-H_4folate as fortificant is that this natural folate compound is the metabolic active compound and does not need to be further metabolized in the body. Recent studies (43,44) have found that both 5-CH_3-H_4folate and folic acid significantly increases plasma folate concentrations and lowers homocysteine concentrations. As 5-CH_3-H_4folate was found to be higher bioaccessible (71%) than folic acid (58%) from pasteurized milk (33), 5-CH_3-H_4folate might be used as an alternative fortificant in dairy products. However, up until now no trials have been done to test whether increasing the intake of 5-CH_3-H_4folate in women immediately before and during the first trimester of pregnancy will reduce the incidence of neural tube defect in their babies. It would be worthwhile to give more attention to studying the bioavailability and the effects of 5-CH_3-H_4folate compared to folic acid from fortified food products or supplements in humans. It is interesting to note that we

were unable to detect (< 1% of total concentration) any folic acid in the plasma of those subjects who were supplemented with folic acid (R.B. van Breemen, University of Illinois at Chicago, personal communication, data not shown).
The genetic polymorphism of MTHFR in our subjects was examined as a substitution of C to T at nucleotide 677 in the gene results in the production of a MTHFR enzyme with less activity. As MTHFR is involved in the formation of 5-CH_3-H_4folate, this polymorphism could influence the outcome of the study. The proportion of the population carrying the *TT* homozygote for the C to T substitution at nucleotide 677 in the MTHFR gene is around 10% (36, 44, 45). These people usually have normal serum folate levels, but elevated homocysteine concentrations, and show enhanced response to folic acid supplementation (44, 45). In our study only 4 of the 69 subjects had the *TT* polymorphism. As this group behaved similarly to the *CC* and *CT* subjects, it was not necessary to take this polymorphism into account when analyzing our data.
Questions can be raised as to whether an extra dose of 200 µg of folic acid a day can also improve folate status in persons that have higher folate intakes from food than the subjects of our study. The daily folate intake of the subjects in the current study was below the average folate intake in the Netherlands (37) which might explain the decrease in folate concentrations in serum and red blood cells, and the increase in plasma homocysteine concentrations in the unfortified milk groups during the four weeks of study. In this study, even the subjects in the highest quartile of baseline serum folate concentrations showed the same response on fortification as the other subjects (data not shown). This study also strengthens the evidence that low doses of folate in the diet can lower plasma homocysteine levels (39, 46). Even though our subjects had a mean plasma homocysteine concentration of 8.75 µmol/L at baseline, the low dose of folate was able to lower plasma homocysteine concentrations.
In summary, milk is found to be a suitable product for folic acid fortification to increase folate status in humans. This study clearly showed that milk fortified with folic acid enhanced concentrations in serum and red blood cells, and decreased plasma total homocysteine concentrations within 4 weeks. No significant effect of endogenous FBP in pasteurized milk on the bioavailability of folic acid from fortified milk was found.

Acknowledgements

The authors thank Gertjan Schaafsma (TNO Nutrition and Food Research) for his advice and Frank Elbers and Jan Steijns (Campina Centre of Expertise Nutrition, Wageningen) for developing and supplying the milk products. We thank the participants for their cooperation; the late Peter van de Bovenkamp for his analytical advice; Tineke van Roekel, Truus Kosmeijer and Jan Harryvan (Wageningen University), Karin Arkbåge (Swedish University of Agricultural Sciences, Uppsala, Sweden), Hans Mocking (TNO Nutrition and Food Research, Zeist) and Susanna Kariluoto (University of Helsinki, Helsinki, Finland) for carrying out the analyses; and Jan Burema and Hans Verhoef for their statistical advice.

References

1. Fishman SM, Christian P, West KP. The role of vitamins in the prevention and control of anaemia. Public Health Nutr 2000;3:125-50.
2. Haurani FI, Hall CA, Rubin R. Megaloblastic anemia as a result of an abnormal transcobalamin II (Cardeza). J Clin Invest 1979;64:1253-9.
3. MRC Vitamin Study Research Group. Prevention of neural tube defects: results of the medical research council vitamin study. Lancet 1991;338:131-7.
4. Daly S, Mills JL, Molloy AM, et al. Minimum effective dose of folic acid for food fortification to prevent neural-tube defects. Lancet 1997;350:1666-9.
5. Glynn SA, Albanes D. Folate and cancer: a review of the literature. Nutr Cancer 1994;22:101-19.
6. Selhub J, Jacques PF, Wilson PWF, Rush D, Rosenberg IH. Vitamin status and intake as primary determinants of homocysteinemia in an elderly population. JAMA 1993;270:2693-98.
7. Pancharuniti N, Lewis CA, Sauberlich HE, et al. Plasma homocyst(e)ine, folate, and vitamin B-12 concentrations and risk for early-onset coronary artery disease. Am J Clin Nutr 1994;59:940-8.
8. Verhoef P, Stampfer MJ, Buring JE, et al. Homocysteine metabolism and risk of myocardial infarction: relation with vitamins B_6, B_{12}, and folate. Am J Epidemiol 1996;143:845-59.
9. Rimm EB, Willett WC, Hu FB, et al. Folate and vitamin B_6 from diet and supplements in relation to risk of coronary heart disease among women. JAMA 1998;279:359-64.
10. Boushey CJ, Beresford SAA, Omenn GS, Motulsky AG. A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. JAMA 1995;274:1049-57.
11. de Bree A, Verschuren WM, Blom HJ, Kromhout D. Association between B vitamin intake and plasma homocysteine concentration in the general Dutch population aged 20-65 y. Am J Clin Nutr 2001;73:1027-33.
12. O'Keefe CA, Bailey LB, Thomas EA, et al. Controlled dietary folate affects folate status in nonpregnant women. J Nutr 1995;125:2717-25.
13. Lewis CJ, Crane NT, Wilson DB, Yetley EA. Estimated folate intakes: data updated to reflect food fortification, increased bioavailability, and dietary supplement use. Am J Clin Nutr 1999;70:198-207.
14. Brouwer IA, van Dusseldorp M, West CE, et al. Dietary folate from vegetables and citrus fruit decreases plasma homocysteine concentrations in humans in a dietary controlled trial. J Nutr 1999;129:1135-9.
15. Riddell LJ, Chisholm A, Williams S, Mann JI. Dietary strategies for lowering homocysteine concentrations. Am J Clin Nutr 2000;71:1448-54.
16. Castenmiller JJM, van de Poll CJ, West CE, Brouwer IA, Thomas CM, van Dusseldorp M. Bioavailability of folate from processed spinach in humans. Effect of food matrix and interaction with carotenoids. Ann Nutr Metab 2000;44:163-9.
17. Forssén KM, Jägerstad MI, Wigertz K, Witthöft CM. Folates and dairy products: a critical update. J Am Coll Nutr 2000;19:100S-10S.
18. Swiatlo N, O'Connor DL, Andrews J, Picciano MF. Relative folate bioavailability from diets containing human, bovine and goat milk. J Nutr 1990;120:172-177.
19. Ghitis J. The folate binding in milk. Am J Clin Nutr 1967;20:1-4.
20. Wagner C. Folate binding proteins. Nutr Rev 1985;43:293-9.
21. Wigertz K, Hansen I, Høier-Madsen M, Holm J, Jägerstad MI. Effect of milk processing on the concentration of folate-binding protein (FBP), folate-binding capacity and retention of 5-methyltetrahydrofolate. Int J Food Sci Nutr 1996;47:315-22.

22. Ford JE. Some observations on the possible nutritional significance of vitamin B12- and folate-binding proteins in milk. Br J Nutr 1974;31:243-57.
23. Colman N, Hettiarachchy N, Herbert V. Detection of a milk factor that facilitates folate uptake by intestinal cells. Science 1981;211:1427-29.
24. Salter D, Blakeborough P. Influence of goat's-milk folate-binding protein on transport of 5-methyltetrahydrofolate in neonatal-goat small intestinal brush-border-membrane vesicles. Br J Nutr 1988;59:497-507.
25. Tani M, Fushiki T, Iwai K. Influence of folate binding protein from bovine milk on the absorption of folate in gastrointestinal tract of rat. Biochem Biophys Acta 1983;757:274-81.
26. Said HM, Horne DW, Wagner C. Effect of human milk folate binding protein on folate intestinal transport. Arch Biochem Biophys 1986;251:114-20.
27. Voedingsraad. Nederlandse voedingsnormen (Dutch nutrition guidelines, language: Dutch). The Hague: Voorlichtingsbureau voor de Voeding, 1992.
28. Feunekes GIJ, van Staveren WA, de Vries JHM, Burema J, Hautvast JGAJ. Relative and biomarker-based validity of a food-frequency questionnaire estimating intake of fats and cholesterol. Am J Clin Nutr 1993;58:489-96.
29. AOAC Official Method 920.85. AOAC International, ed. Official methods of analysis of AOAC International, vol. II, 16th ed. Arlington: Association of Official Analytical Chemists International, 1999.
30. AOAC Official method 992.16. AOAC International, ed. Official methods of analysis of AOAC International, vol. II, 16th ed. Arlington: Association of Official Analytical Chemists International, 1996.
31. Williams S (ed) (1984) Microbiological assays. In: Official Methods of Analysis of the Association of Official Analytical Chemist, fourteenth edition. AOAC Inc., Arlington, Virginia, USA, pp 862-73.
32. Høier-Madsen M, Hansen SI, Holm J. Rabbit antibodies against the folate binding protein from cow's milk. Production, characterisation and use for development of an enzyme-linked immunosorbent assay (ELISA). Biosci Rep 1986;6:895-900.
33. Verwei M, Arkbåge K, Havenaar R, van den Berg H, Witthöft C, Schaafsma G. Folic acid and 5-methyltetrahydrofolate in fortified milk are bioaccessible as determined in a dynamic in vitro gastrointestinal model. J Nutr 2003;133:2377-83.
34. Konings, E.J.M. A validated liquid chromatographic method for determining folates in vegetables, milk powder, liver and flour. J. AOAC Int. 82;1999:119-27.
35. Ubbink JB, Vermaak WJH, Bissbort S. Rapid high-performance liquid chromatographic assay for total homocysteine levels in human serum. J Chromatogr 1991;565:441-6.
36. Frosst P, Blom HJ, Milos R, et al. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat Genet 1995;10:111-3.
37. de Bree A, van Dusseldorp M, Brouwer IA, van het Hof KH, Steegers-Theunissen RPM. Folate intake in Europe: recommended, actual and desired intake. Eur J Clin Nutr 1997;51:643-60.
38. Schorah CJ, Devitt H, Lucock M, Dowell AC. The responsiveness of plasma homocysteine to small increases in dietary folic acid: a primary care study. Eur J Clin Nutr 1998;52:407-11.
39. Brouwer IA, van Dusseldorp M, Thomas CMG, et al. Low-dose folic acid supplementation decreases plasma homocysteine: a randomized trial. Am J Clin Nutr 1999;69:99-104.
40. Salter DN, Scott JK, Slade H, Andrews P. The preparation and properties of folate-binding protein from cow's milk. Biochem J 1981;193:469-76.

41. Wigertz K, Hallmans G, Sandberg A-S, Tidehag P, and Jägerstad MI. Improved apparent dietary folate absorption in ileostomy subjects with the incorporation of milk into a mixed diet. In: Wigertz K. Milk folates. Characterisation and availability. Thesis. Lund: Lund University, 1997.
42 Gregory III, JF. Denaturation of the folacin-binding protein in pasteurized milk products. J Nutr 1982;112:1329-38.
43. Venn BJ, Green TJ, Moser R, McKenzie JE, Skeaff CM, Mann J. Increases in blood folate indices are similar in women of chilbearing age supplemented with [6S]-5-methyltetrahydrofolate and folic acid. J Nutr 2002;132:3353-55.
44. Fohr IP, Prinz-Langenohl R, Brönstrup A, et al. 5,10-Methylenetetrahydrofolate reductase genotype determines the plasma homocysteine-lowering effect of supplementation with 5-methyltetrahydrofolate or folic acid in healthy young women. Am J Clin Nutr 2002;75:275-82.
45. Malinow MR, Nieto FJ, Kruger WD, et al. The effects of folic acid supplementation on plasma total homocysteine are modulated by multivitamin use and metylenetetrahydrofolate reductase genotypes. Arterioscler Thromb Vasc Biol 1997;17:1157-62.
46. van Oort FVA, Melse-Boonstra A, Brouwer IA, et al. Folic acid and reduction of plasma homocysteine concentrations in older adults: a dose-respons study. Am J Clin Nutr 2003;77:1318-23.

9

General discussion: integration of in vitro and in vivo results

Bioavailability of folate from fortified milk products

The work described in this thesis was carried out to study the bioavailability of folate from fortified milk products, which will provide information about the suitability of the milk products for folate fortification. The bioavailability of folate from fortified milk products was investigated with five research questions, posed in Chapter 1, which are discussed in this chapter. The research questions are answered by integrating the results of the *in vitro* and *in vivo* studies described in the Chapters 2 to 8 following a step-wise approach as illustrated in Figure 1.3.

1. What is the bioaccessibility of folic acid and 5-CH_3-H_4folate from fortified milk products and does it differ from the bioaccessibility of natural folate from unfortified food products?

The bioaccessibility of folate from several natural or fortified food products was studied with an *in vitro* dynamic gastrointestinal model as described in Chapters 2 to 4. Several fortified fermented (yogurt and filmjölk) and unfermented (UHT-treated and pasteurized) milk products were tested in the *in vitro* model to investigate the effect of the milk matrix on the release of folic acid and 5-CH_3-H_4folate during transit through the gastrointestinal tract (Figure 9.1).

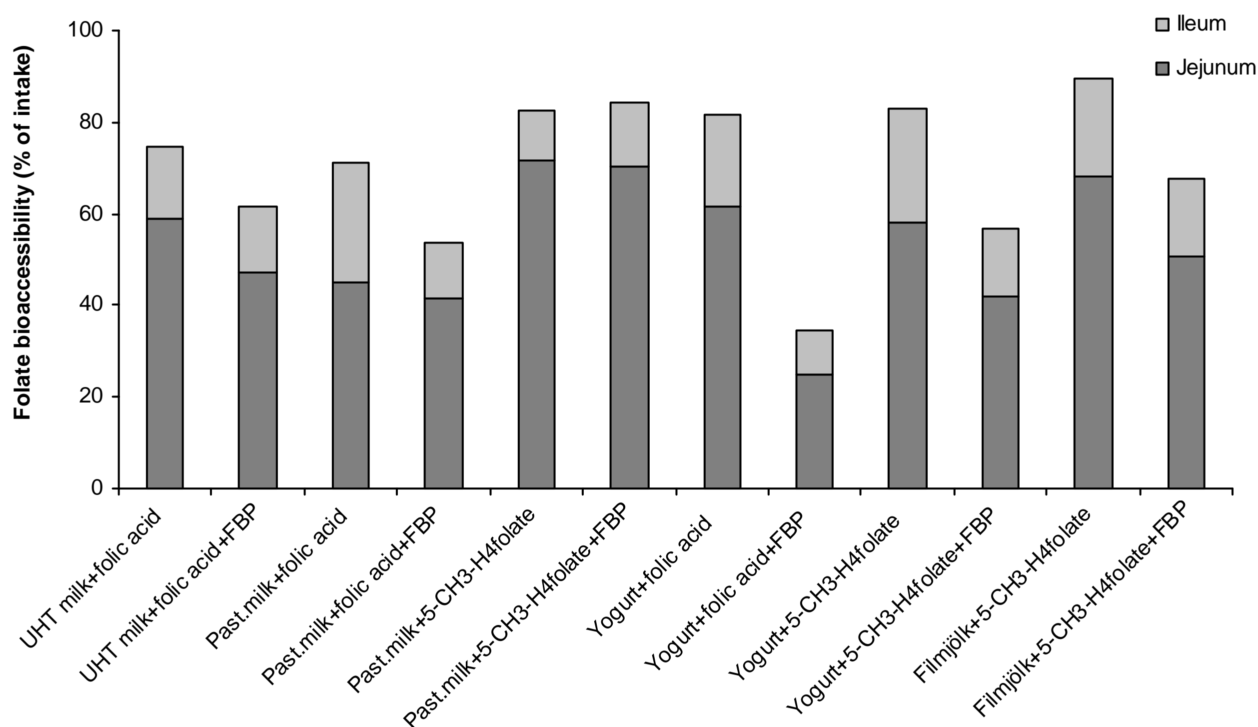


Figure 9.1. Overview of the results from Chapters 2,3 and 4 on bioaccessibility of folic acid or 5-CH_3-H_4-folate from fortified milk products.

To enable a direct comparison of the bioaccessible fractions of folate from the different experiments, the results were corrected for recovery, as a lower recovery of folate was found for the experiments with UHT and pasteurized milk of which the samples were analyzed with HPLC (80-90%) than for the experiments with the fermented milk products of which the samples were analyzed with MA or RPBA methods (~100%). A large amount of the supplemental folate (71-90%) appeared to be bioaccessible from the fortified milk products (in absence of additional FBP). The bioaccessible fraction of 5-CH_3-H_4-folate from pasteurized milk (82%) was higher than the bioaccessible fraction of folic acid from pasteurized milk (71%) and UHT milk (75%). No difference was found between the bioaccessible fractions (82%) of 5-CH_3-H_4-folate and folic acid from fortified yogurt.

The bioaccessibility of supplemental folate was similar to that of native folate as no difference was found between the bioaccessible folate fractions from fortified milk products and the fractions from knäckebröd (87%), orange juice (87-91%), beer (88%), spinach (80-83%), peas (78-79%), gazpacho (94%) and (unfortified) milk (86%) (Figure 9.2). Although no difference was found in the bioaccessibility of native folate (mixture of mono- and polyglutamates) and supplemental folate (monoglutamates), a difference in bioavailability can not be excluded as the deconjugation of polyglutamates as a potential limiting step in the bioavailability of dietary (native) folate was not investigated in these *in vitro* studies.

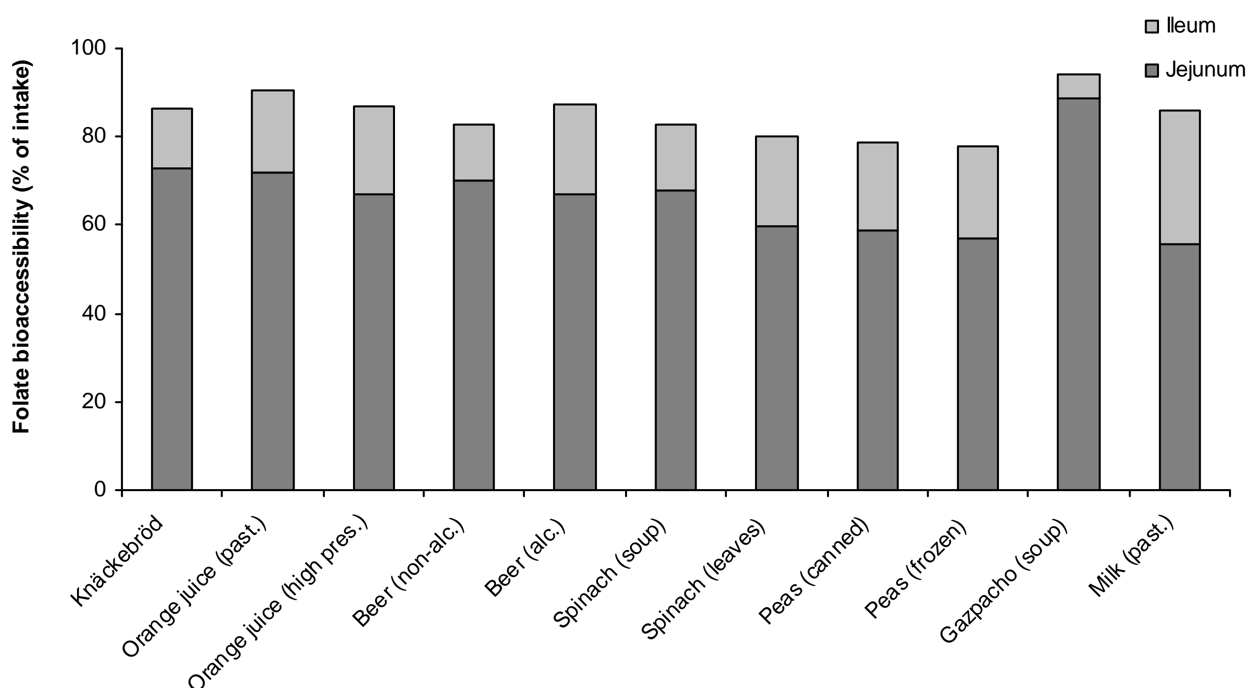


Figure 9.2. Overview of results from Chapter 4 on bioaccessibility of folate (mainly 5-CH_3-H_4-folate) from natural (unfortified) milk products.

The studies with the natural products tested in the *in vitro* gastrointestinal model showed that the bioaccessibility of folate from spinach leaves and peas was slightly lower than that of folate from orange juice, alcoholic beer, gazpacho, knäckebröd and fortified filmjölk, but comparable to that from milk, spinach soup and non-alcoholic beer. This indicates that the matrix of vegetables only minimally limits the release of natural folate. Some food products (e.g. gazpacho and milk) showed a difference in kinetics of folate release. For example, folate from the tomato-vegetable (gazpacho) soup was almost exclusively released in the jejunal compartment while folate from milk was released more gradually during gastrointestinal passage (Figure 9.2). This difference in kinetic profile of folate release could result in a difference in kinetics of folate absorption from the intestinal lumen and, therefore, might affect the bioavailability of folate.

2. What is the effect of FBP on the bioaccessibility of folic acid and 5-CH3-H4folate from fortified milk products?

In unprocessed bovine milk, most of the folate is bound to FBP (1-3). FBP was thought to enhance the bioavailability of folate from milk. This hypothesis is based on observations that FBP protects folate from bacterial uptake and degradation (4-5), which might indirectly lead to a higher folate absorption, and could enhance the absorption of folate by mucosal cells (6-7). However, the effect of FBP on the bioavailability of folic acid and 5-CH_3-H_4folate from milk products in humans is unclear. Whether FBP is digested by human intestinal enzymes during passage through the gastrointestinal tract and if folate still occurs bound to FBP in the human small intestine has not been studied before.

In our *in vitro* studies, the stability of FBP (Chapters 2 and 3), the extent of binding to FBP for folic acid and 5-CH_3-H_4folate (Chapter 5) and the bioaccessibility of folic acid and 5-CH_3-H_4folate from milk products with additional FBP (Chapters 2, 3 and 4) was investigated during gastrointestinal passage. In these *in vitro* studies, fortified milk products with equimolar ratios between FBP and folic acid or 5-CH_3-H_4-folate were tested which is in line with the equimolar ratio between FBP and native folate in unfortified milk. After gastric passage of folic acid and 5-CH_3-H_4-folate fortified milk products, approximately 70% of the initial FBP content could be retained (Chapter 5). FBP was found to be further digested by the intestinal enzymes during passage through the small intestinal tract. The extent of digestion of FBP appeared to be dependent on the type of milk product (fermented or unfermented milk) and on the folate compound present in the milk product (Chapters 2 and 3). FBP was more stable (13-16%) in folic acid-fortified milk than in 5-CH_3-H_4-folate-fortified milk (0-1%). This difference in FBP stability was also found in the studies with yogurt as 34% of the FBP in folic acid-fortified yogurt and 17% of the FBP in 5-CH_3-H_4-folate-fortified yogurt was recovered after gastrointestinal passage. In a study of Kaarsholm et al. (8) it was shown that FBP is more stable in complex with folate as compared with the free FBP molecule. Thus, an explanation for the higher FBP retention after gastrointestinal passage of the folic acid-fortified milk products could be the higher amount of FBP-folic acid complexes as compared with FBP-5-CH_3-H_4folate complexes. The occurrence of FBP-folate complexes in the gastrointestinal tract reduces the bioaccessible fractions of folic acid or 5-CH_3-H_4-folate. It was indeed found that FBP had a more pronounced inhibitory effect on the bioaccessibility of folic acid compared to that of 5-

CH_3-H_4folate both in fermented and unfermented milk products. This difference can be explained by the lower binding affinity of FBP for 5-CH_3-H_4folate compared to folic acid at the pH range 5-7.4 (9). Studies with the gastrointestinal model showed that the addition of FBP to fortified (pasteurized) milk reduced the bioaccessibility of folic acid but not from 5-CH_3-H_4-folate (Figure 9.1). Also in the studies with fortified yogurt, a difference between 5-CH_3-H_4-folate and folic acid was found in the decrease of their bioaccessible fractions in presence of additional FBP (Figure 9.1). However, the addition of FBP to fortified yogurt led to a higher decrease in bioaccessibility of both folic acid and of 5-CH_3-H_4-folate as compared with results from the studies with pasteurized milk. Thus, although no difference in folate bioaccessibility was found between folate-fortified yogurt and pasteurized milk, the lowering effect of FBP was significantly stronger in yogurt compared to pasteurized milk. This could be related to a difference in extent of digestion of FBP during transit through the gastrointestinal tract.

Important findings from these *in vitro* studies with fortified milk products was that FBP was partially stable during gastrointestinal passage and that the effect of FBP was different for folic acid and 5-CH_3-H_4-folate (Chapter 2 and 3). We performed additional studies to measure the extent of binding to FBP of folic acid and 5-CH_3-H_4-folate during gastric passage of fortified milk products (Chapter 5). The passage through the stomach was studied in more detail because it was shown in a rat study (10) that under acidic gastric conditions ($pH < 4.5$) folic acid was released from FBP but recombined in the small intestine (pH 6-7). Whether folic acid and 5-CH_3-H_4-folate occur in complex with FBP in the human duodenal lumen was not investigated so far, and therefore included in this research. Before gastric passage, folic acid and 5-CH_3-H_4-folate were mainly bound to FBP (76-79%) while 7% was free. After gastric passage, folic acid remained bound to FBP to a similar extent (80-81%). For 5-CH_3-H_4-folate the FBP-bound fraction gradually decreased from 79% to 5% and the free fraction increased from 7% to 93%. So, while folic acid enters the proximal part of the small intestine bound to FBP, 5-CH_3-H_4-folate appears to be mainly present as free folate in the duodenal lumen. These results are in line with our findings that FBP decreased the bioaccessibility of folic acid to a higher extent than the bioaccessibility of 5-CH_3-H_4-folate. The difference in binding of folic acid and 5-CH_3-H_4-folate to FBP in the intestinal lumen results in a difference in free luminal folate concentration and this might lead to a different intestinal absorption from folic acid- and 5-CH_3-H_4-folate-fortified milk products. The intestinal absorption of folate, the second step in bioavailability, was studied using monolayers of human colon carcinoma (Caco-2) cells representing an *in vitro* model for human intestinal absorption.

3. Is there a difference in transport across human intestinal cells between folic acid and 5-CH_3-H_4folate?

The intestinal transport of folate was studied using monolayers of Caco-2 cells grown on semi-permeable inserts in a two-compartment system. Caco-2 cells can be used to predict human intestinal absorption (11-14) as they display, after differentiation, both biochemical and morphological characteristics of small intestinal enterocytes (15,16). The permeability of folic acid and 5-CH_3-H_4-folate across Caco-2 monolayers was found to be three-fold higher than that of mannitol (marker for low permeability) but twenty-fold lower than that of caffeine (marker for high

permeability). This indicates that the relatively low permeability of folic acid and 5-CH_3-H_4-folate across intestinal cells could limit the bioavailability of folate. The transport from the luminal (apical) to the serosal (basolateral) side of the Caco-2 cells appeared to be slightly higher for folic acid than for 5-CH_3-H_4-folate (Chapter 6). Thus, a small difference in intestinal transport was found between the synthetic and natural folate. Whether the transport mechanisms underlying the intestinal transport differ between folic acid and 5-CH_3-H_4folate was studied by investigating the transport across Caco-2 cells in presence of inhibitors of individual transport pathways (Chapter 7). These studies showed that the transport of folic acid and 5-CH_3-H_4-folate across Caco-2 cells mainly occurs via carrier-mediated uptake (via the reduced folate carrier, RFC) and efflux via multi-drug resistance proteins (MRPs). The transport of both folate compounds across Caco-2 cells was found not to occur via receptor-mediated transport, i.e., endocytosis via the folate receptor (FR), as colchicine, a known inhibitor of endocytosis, did not affect the transport to the apical or basolateral side of the Caco-2 cells. The relative contribution of RFC and MRP-mediated transport to the overall transport appeared to be higher for 5-CH_3-H_4-folate than for folic acid. This indicates that the transport of 5-CH_3-H_4-folate is more dependent on the activity of the reduced folate carriers (RFC) and multi-drug resistance proteins (MRPs) than the transport of folic acid.

4. What is the effect of FBP on the intestinal absorption of folic acid and 5-CH_3-H_4folate?

The *in vitro* studies with the gastrointestinal model showed that folic acid occurs mainly bound to FBP while 5-CH_3-H_4-folate occurs mainly unbound in the small intestine. Whether the degree of binding to FBP affects the absorption of folate from the intestinal lumen was studied using monolayers of Caco-2 cells as described in Chapter 6. The transport and cellular accumulation of folic acid and 5-CH_3-H_4-folate was studied in absence and in presence of FBP in the dose solution added to the apical side of the Caco-2 cells. Dose solutions with different molar ratios between FBP and folic acid or 5-CH_3-H_4-folate were tested to study the transport of folic acid and 5-CH_3-H_4-folate after exposure to solutions with different FBP-bound fractions of folic acid and 5-CH_3-H_4-folate. In presence of FBP, the intestinal transport and cellular accumulation of folic acid and 5-CH_3-H_4-folate was decreased. The effect of FBP appeared to be dependent on the extent of binding to FBP of folic acid and 5-CH_3-H_4-folate at the luminal side of the cells as an inverse correlation was found between extent of binding to FBP and intestinal transport. Thus, these studies indicate that the occurrence of FBP-folate complexes in the intestinal lumen will lead to a lower absorption and, as a result, a lower bioavailability of folate from milk. As the studies with the *in vitro* gastrointestinal model (Chapters 2, 3 and 5) showed that more folic acid-FBP complexes occur in the intestinal lumen than 5-CH_3-H_4-folate-FBP complexes, the inhibitory effect of FBP on the bioavailability of folic acid is expected to be more pronounced than on that of 5-CH_3-H_4-folate.

5a. Will the consumption of fortified milk products lead to an enhanced folate status in humans?

The folate levels in the blood of human volunteers were measured prior to and after a 4 week human intervention study in which the subjects consumed milk fortified with folic acid (Chapter 8). This study showed that the daily consumption of 200 μg folic acid added to milk, in addition to a

standard diet, significantly increased concentrations of folate in serum and red blood cells and decreased plasma homocysteine concentrations. This indicates that milk is a suitable matrix for fortification with folic acid to enhance the folate status in countries where milk is commonly consumed.

In the human intervention study, described in Chapter 8, the effect of FBP on the bioavailability of folic acid was studied by comparing the response in blood folate levels after consumption of fortified pasteurized milk (with endogenous FBP) with the levels after consumption of fortified UHT milk (no FBP). The results tended to a higher response in blood folate levels after consumption of fortified UHT milk, although this difference was not significant. This observation is in line with the small difference in bioaccessible fractions of folic acid from pasteurized and UHT milk as found in the *in vitro* studies with the gastrointestinal model (Chapter 2). The molar ratio between folic acid and FBP in the folic acid-fortified pasteurized milk was found to be 8:1. As Salter et al (17) found that 1 mol FBP binds 1 mol of folate at pH 7.2, only a small amount of the added folic acid is expected to occur bound to FBP in the fortified pasteurized milk in which folic acid is present in excess. Our *in vitro* binding studies also show that almost all folic acid (~80%) occurred FBP-bound at a 1:1 molar ratio between folic acid and FBP, while only 45% of folic acid occurred FBP-bound at a molar ratio of 2:1 (Chapters 5 and 6). The *in vitro* studies with Caco-2 cells further demonstrate that the effect of FBP on the bioavailability of folate is dependent on the binding of folic acid or 5-CH_3-H_4-folate to FBP (Chapter 6). These studies show that at a 4:1 molar ratio between folic acid and FBP, the transport of folic acid was only slightly affected. A higher effect of FBP was found at the 2:1, 1.5:1 and 1:1 molar ratios between folic acid and FBP. No further decrease in intestinal transport of folic acid and 5-CH_3-H_4-folate was found at the 1:2 molar ratio between folate and FBP. This is explained by the optimal binding of folate to FBP at an equimolar ratio as supported by the findings of Salter et al (17). The research described in this thesis underline the importance of performing *in vitro* bioavailability studies prior to an *in vivo* human intervention study. Following an approach to describe the kinetic behavior by making use of *in vitro* data on bioavailability of the test compound, which will be described in the next section, the test conditions (matrix, dose, frequency, duration) of the food products will be optimized to test more efficiently the research question in a human intervention study avoiding the need to perform multiple extensive *in vivo* studies.

5b. Which combination of milk product, FBP content, and supplement is most effective in enhancing the plasma folate levels in humans?

In the human intervention study described in Chapter 8, UHT and pasteurized milk fortified with folic acid, without additional FBP, were tested. Besides these two fortified milk products, many milk products (fermented or unfermented) could be used as carrier for supplemental folic acid or 5-CH_3-H_4-folate whether or not in combination with additional FBP. However, not all these variables can be tested in a single human intervention study.

An alternative approach which allows the testing of several variables (matrix, FBP content, supplement) in the milk products is demonstrated in current section. In the studies with the *in vitro* gastrointestinal model, several fermented (yogurt and filmjölk) and unfermented (UHT and

pasteurized) milk products fortified with folic acid or with 5-CH_3-H_4-folate and with or without additional FBP were tested (as described in Chapters 2 to 4). These studies gave information about the bioaccessibility of folic acid and 5-CH_3-H_4-folate from the milk matrix which is the first step in bioavailability. The bioaccessibility data should be combined with the intestinal absorption of folic acid and 5-CH_3-H_4-folate (Chapter 6) to be able to predict the blood folate response in humans. Recently, we set up a kinetic model, which integrates the *in vitro* results about kinetics of folate bioaccessibility and absorption and extrapolates these *in vitro* results to the human situation *in vivo*. As described in the next section, with this approach blood folate levels can be predicted after the consumption of various fortified milk products without testing each individual milk product in an extensive *in vivo* study.

Plasma folate levels in humans: An *in silico* approach to combine *in vitro* and *in vivo* data in a kinetic model (Manuscript in preparation)

A kinetic model was used as an alternative approach to study the response in blood folate levels after the consumption of milk products fortified with folic acid or 5-CH_3-H_4-folate and with or without additional FBP. This approach was followed to answer the research question "which combination of milk matrix, FBP content and folate supplement is most effective in enhancing blood folate levels?".

Description of the kinetic model to predict blood folate levels

Recently, we have set up a computational kinetic model, which describes the processes involved in the bioavailability of folate, to predict blood folate levels based on *in vitro* data. The kinetic model consists of mathematical descriptions of the processes and interactions involved in the bioavailability of folate in the human body. After consumption, the first step determining the bioavailability of folate is the release of folate from the food products which are successively transported through the stomach, duodenum, jejunum and ileum before reaching the colon. The bioaccessible fractions of folate in the small intestinal segments were experimentally determined in studies with the *in vitro* dynamic gastrointestinal model as described in Chapters 2 to 4. These *in vitro* results were incorporated in the computational model to describe the kinetics of folate release during transit through the gastrointestinal tract. During the gastrointestinal transit, the bioaccessible fractions of folate from the foods can be absorbed by the intestinal cells (in duodenum, jejunum and ileum). Whether all bioaccessible folate compounds are absorbed during passage through the gastrointestinal tract is dependent on the transport rate of folate across the intestinal cells. The transport rate of folic acid and 5-CH_3-H_4-folate across intestinal cells was experimentally determined in studies with monolayers of human Caco-2 cells (Chapter 6). Thus, the kinetic data of folate release are combined with the kinetic data of folate absorption *in silico* as illustrated in Figure 9.3. After absorption, folate enters the portal vein and after passing the liver, folate is distributed among all tissues in the human body. The human body is mathematically described *in silico* as a plasma compartment and a fast and slow compartment based on the different turnover rates found for folate in time in the human body (18,19). The compartments are also illustrated in the schematic

representation of the kinetic model (Figure 9.3) which shows the interactions between the individual compartments including the elimination rate constant (e.g. urinary excretion). The present model describes mathematically the processes in the human body based upon information from literature about the kinetics of folate distribution and elimination. This is not a physiologically realistic presentation of all individual tissues in the human body.

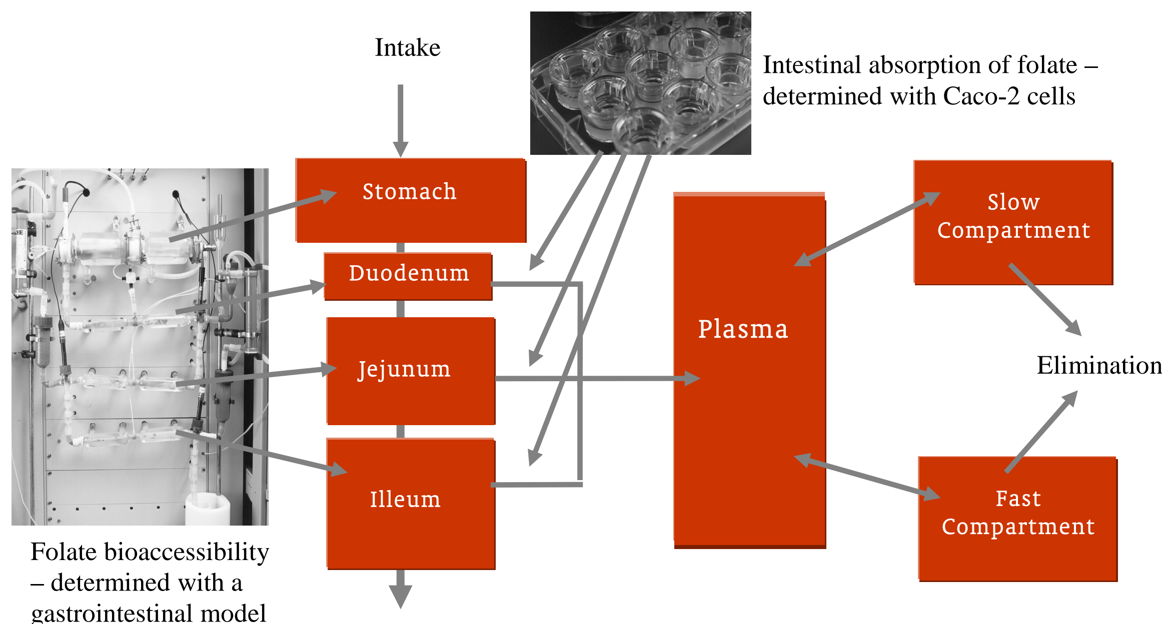


Figure 9.3 Integration of *in vitro* data derived from studies with the *in vitro* gastrointestinal model and monolayers of Caco-2 cells in a kinetic model, with describes *in silico* the distribution of folate in the human body.

Comparison of the serum folate levels measured in a human intervention study and predicted by kinetic modeling after consumption of folic acid-fortified milk

In our human intervention study, described in Chapter 8, the subjects received unfortified milk (20-23 µg folate) or folic acid-fortified milk (201-233 µg folate) in combination with a diet with 140 µg dietary folate. After four weeks of intervention, the folate response in serum levels was determined in the human volunteers. To clarify the validity of the kinetic model to predict plasma (or serum) folate levels after the consumption of folate-containing products for a certain time period, a comparison was made between the serum folate levels measured in the human intervention study (Chapter 8) and the model simulations constructed with the *in vitro* data (release and intestinal transport) and *in vivo* data from literature (distribution within the body). Similar fortified UHT and pasteurized milk products were tested in the human intervention study (Chapter 8) and in the studies with the *in vitro* gastrointestinal model (Chapter 2). The bioaccessible fraction of folic acid was found to be slightly lower from pasteurized milk than from UHT milk. The kinetics of folate release from the UHT-treated and pasteurized milk (Chapter 2) were incorporated in the model together with the kinetics of folate transport described with the permeability rate (Papp value in cm/sec) across intestinal cells (Chapter 6). By changing the parameters in the kinetic model based on the *in*

vitro data that are specific for these test products, the change in plasma (or serum) folate levels could be predicted after consumption of fortified UHT and pasteurized milk. The serum folate levels of the volunteers participating in the 4-week intervention study were determined by the intake of the (fortified) milk product plus the intake of the diets consumed over 24 h (background folate intake). This background folate intake is modeled by the amount of dietary folate multiplied by a factor of 0.7 describing the bioavailability of folate from meals which is estimated to be 70% based upon available *in vivo* data (ranging between 50 and 90%).

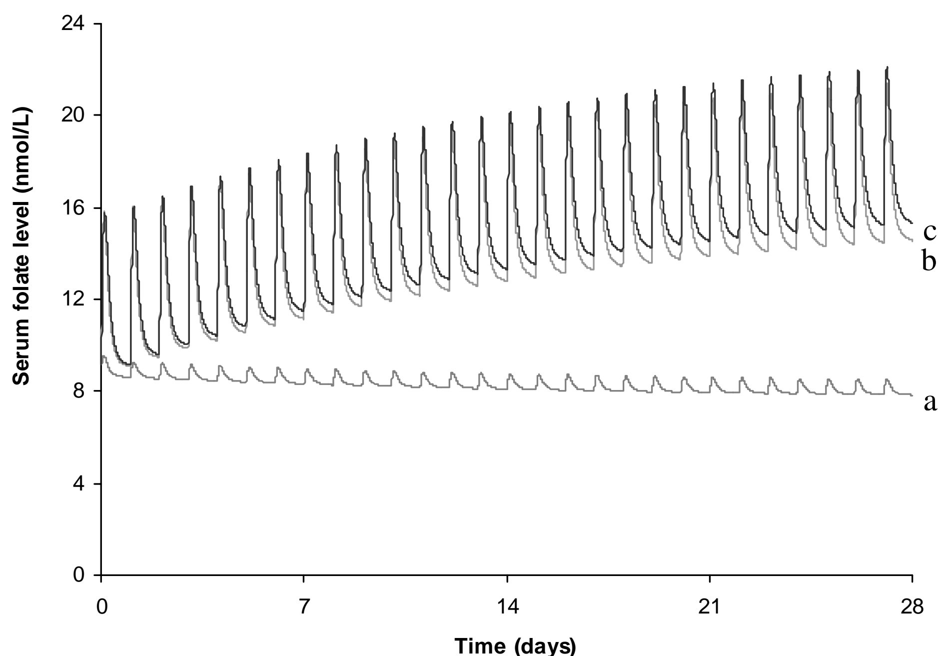


Figure 9.4. *In silico* prediction of serum folate levels after the consumption for four weeks of a diet (140 µg folate) in combination with a) unfortified milk (20 µg folate), b) fortified UHT milk (201 µg folate) or c) fortified pasteurized milk (233 µg folate).

In the kinetic model, the resulting bioavailable amount of folate enters the blood evenly over the day and creates a baseline plasma level in the population. The baseline plasma level is the result of the distribution of folate over the tissues in the human body. Prior to the human intervention study, the subjects had a baseline folate level in serum of approximately 9 nmol/L (Chapter 8). In the kinetic model, this baseline folate level is described by calibrating the size (and interactions) of the compartments to a baseline plasma level of approximately 9 nmol/L at a average daily intake of 200 µg folate.

The response in serum levels was predicted by the kinetic model after integrating the information about the intake (diet and test product), bioaccessibility and intestinal transport of folate in the model describing the distribution of folate in the human body (Figure 9.4). The serum level responds to the combination of supplemental folate in the test product and the natural folate from the diet. As illustrated in Figure 9.4, the serum folate levels increase directly after consumption of

the test product after which it levels off during the day till the next (daily) event of consumption. After each day on which the test product is consumed, the serum level slightly increases till a new steady-state level in serum folate is reached (Figure 9.4). These *in silico* results for the folate levels in serum after four weeks of intervention are summarized in Figure 9.5. The serum levels of the subjects who consumed unfortified milk for four weeks were slightly decreased. The decrease in serum folate level was a result of the lower dietary folate intake (140 µg) by the meals and drinks during the study as compared with the period prior to the study (approximately 200 µg). This decline in serum levels after consumption of unfortified milk for 4 weeks was correctly predicted with *in silico* modeling (Figure 9.5).

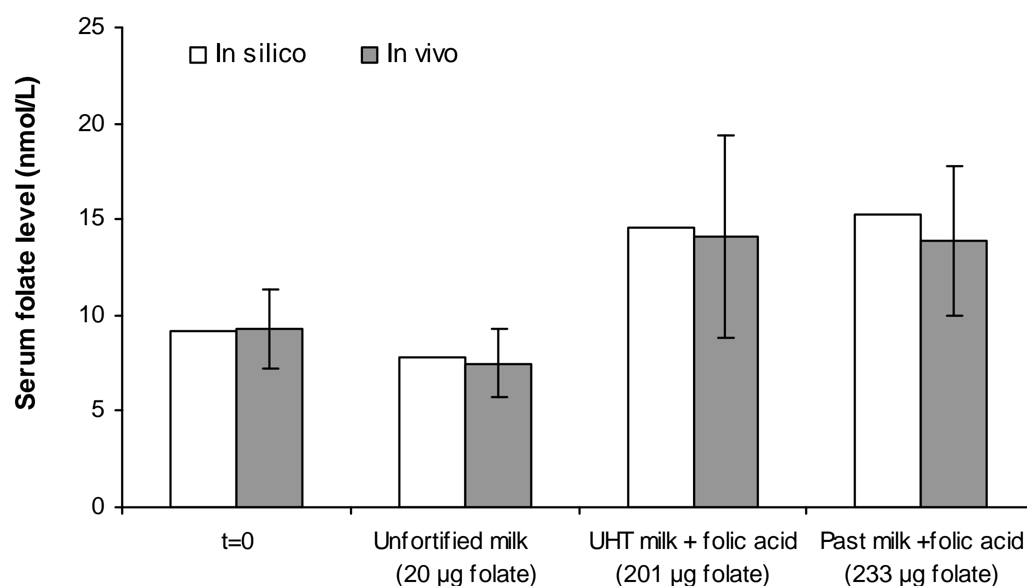


Figure 9.5. Folate levels in serum prior to (t=0) and after four weeks of consumption of unfortified or folic acid- fortified milk products either predicted (light bars) with a kinetic model or measured (dark bars) in a 4-week human intervention study.

The serum levels measured in the human volunteers increased from 9.3 ± 2.1 nmol/l prior to the study to 13.9 ± 3.9 nmol/l or 14.1 ± 5.3 nmol/l, respectively, after four weeks of consumption of fortified UHT or pasteurized milk (Chapter 8). The actual serum levels after consumption of fortified milk were correctly predicted with the kinetic model based on the *in vitro* results with folate release from studies with the gastrointestinal model and folate transport from studies with the Caco-2 cells.

Comparison of the plasma response measured in humans or predicted by kinetic modeling after a single or repeated dose of supplemental folic acid or 5-CH_3-H_4-folate

The validity of the kinetic model was not only checked with *in vivo* data from the human intervention study described in this thesis, but also with literature data from other *in vivo* studies. Two model simulations are described and compared with plasma levels of human volunteers after a single or repeated dose of folic acid or 5-CH_3-H_4-folate supplements.

In a short term study of Wright et al. (20), single oral doses of 280 µg folic acid were given as a capsule to adult volunteers. Subsequently, the rise in plasma folate levels was monitored over 8h (Figure 9.6). The parameters (intake, bioaccessibility and intestinal absorption) within the kinetic model were adjusted to predict the rise in plasma level after a single oral dose of 280 µg folic acid which was given as a capsule in absence of a meal. As illustrated in Figure 9.6, the rise in plasma level as measured *in vivo* was correctly predicted with the kinetic model.

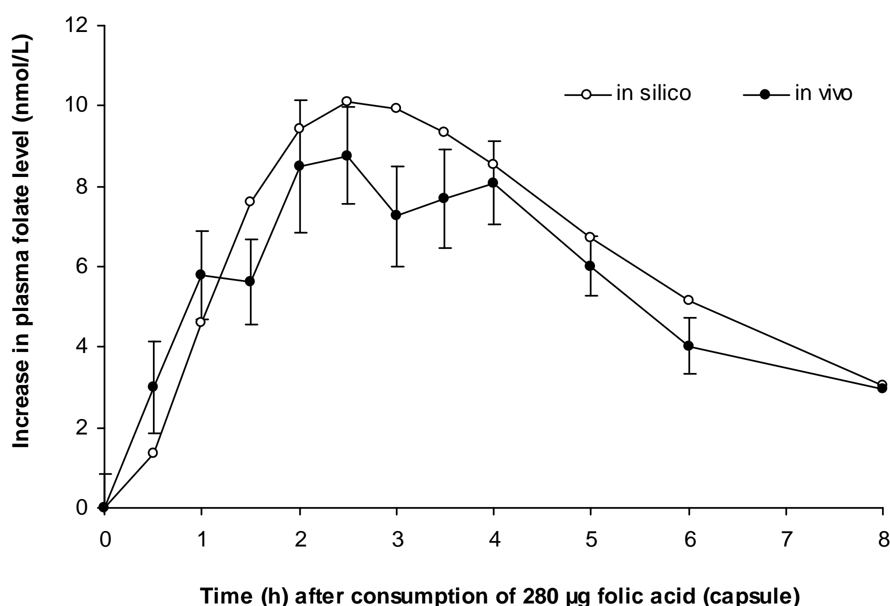


Figure 9.6. Plasma folate levels predicted by kinetic modeling and determined in a short-term study in which human volunteers received a single dose of 280 µg folic acid.

The second literature study was from Lamers et al (21) in which the plasma response of human volunteers was measured at a 4-week interval for a period of 24 weeks. We performed a model simulation for the change in plasma level after repeated (daily) doses of 208 µg 5-CH_3-H_4-folate, 416 µg 5-CH_3-H_4-folate or 400 µg folic acid in addition to a standard diet. Although after 4 weeks the predicted responses in plasma levels after consumption of 208-416 µg 5-CH_3-H_4-folate appeared to be higher than the actual values, the plasma responses ('steady-state') after 8, 12, 16, 20 and 24 weeks of daily consumption of folic acid or 5-CH_3-H_4-folate supplements in combination with a standard diet were correctly predicted with kinetic modeling (Figure 9.7).

These results derived from modeling indicate that a kinetic profile of a compound in the human body can be constructed based upon *in vitro* data on the release of the ingredient from the food product and on its transport across the intestinal wall. This integrated *in silico* approach offers the opportunity to predict plasma folate levels after the consumption of a variety of products after a single dose or repeated doses over a certain time period without the need to perform multiple extensive *in vivo* studies. This approach can be used to predict steady-state plasma levels after a dietary change and can be used to develop (and recommend) a dietary strategy to enhance the folate status of the population. Within this research, computational kinetic modeling was used to answer the question which combination of milk matrix, FBP content and supplement (folic acid or 5-CH_3-H_4-folate) leads to the highest increase in folate status of the population.

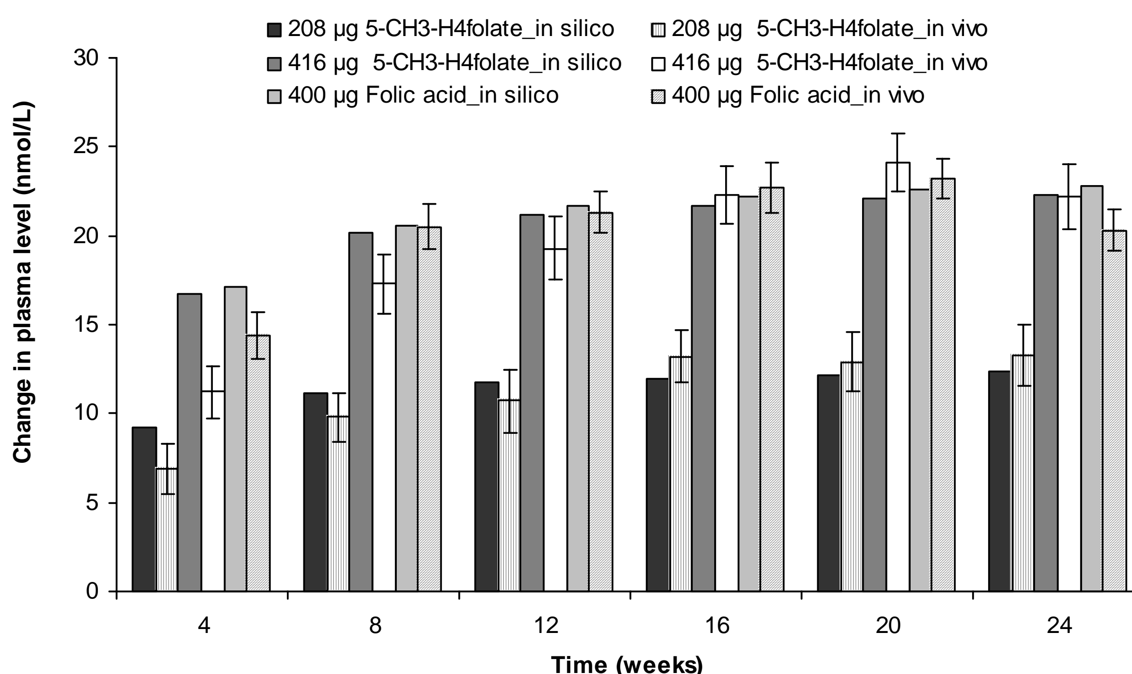


Figure 9.7. Change in plasma folate levels after 4, 8, 12, 16, 20 and 24 weeks either predicted with a kinetic model or actual values determined in a 4-wk human intervention study in which the subjects consumed daily (repeated dose) 208 µg 5-CH_3-H_4-folate, 416 µg 5-CH_3-H_4-folate or 400 µg folic acid in addition to a standard diet.

Application of the kinetic model to select a suitable milk product for fortification purposes

To select the combination of milk product, FBP content and supplement leading to the highest increase in plasma folate response, model simulations were performed based upon the data from the *in vitro* studies. In general, these results from the kinetic model show that the consumption of fortified milk products (200 µg folic acid or 5-CH_3-H_4-folate) in combination with a standard diet of 200 µg for four weeks leads to a 70-89% increase in plasma folate (steady-state) level (Table 9.1).

Table 9.1. Plasma folate levels predicted with the kinetic model after the consumption of fermented or unfermented milk fortified with folic acid (200 µg) or 5-CH_3-H_4-folate (200 µg) with or without supplemental FBP for four weeks in combination with an average Dutch diet (200 µg dietary folate) and the rise in plasma level (%) from a baseline level of 9.2 nmol/l.

Fortified milk products	Plasma folate level (nmol/L)	Rise in plasma level (%)
Pasteurized milk + 5-CH_3-H_4-folate	16.9	84
Pasteurized milk + 5-CH_3-H_4-folate + FBP	16.9	84
Pasteurized milk + folic acid	17.1	86
Pasteurized milk + folic acid + FBP	16.6	80
UHT milk + folic acid	17.4	89
UHT milk + folic acid + FBP	16.8	83
Yogurt + folic acid	17.4	89
Yogurt + folic acid + FBP	15.6	70
Yogurt + 5-CH_3-H_4-folate	16.8	83
Yogurt + 5-CH_3-H_4-folate + FBP	15.8	72
Filmjölk+5-CH_3-H_4-folate	16.8	83
Filmjölk+5-CH_3-H_4-folate + FBP	16.2	76

From the *in silico* simulations, it can be concluded that the consumption of UHT milk or yogurt fortified with folic acid leads to the highest increase (89%) in plasma folate level. Due to processing of these milk products, endogenous FBP is no longer present in UHT milk and yogurt. Thus, the bioavailability of folic acid is not decreased due to its binding to FBP. Addition of FBP to yogurt fortified with folic acid leads to a lower rise (19%) in plasma folate levels. FBP also decreased the bioavailability of folic acid from pasteurized or UHT-treated milk. These results make clear that supplementation of yogurt, UHT milk but also pasteurized milk with folic acid is an efficient strategy to enhance the folate status. However, based on our studies, it is recommended not to add FBP to the folic acid-fortified milk products as this will lead to a lower bioavailability of folic acid.
Whether 5-CH_3-H_4-folate can be used as an alternative supplement to fortify milk products was not studied in the *in vivo* study but was studied *in silico* by combining the *in vitro* data about kinetics of folate release and absorption. The rise in plasma level is almost equal after the consumption of 5-CH_3-H_4-folate-fortified pasteurized milk (84%) and folic acid-fortified pasteurized milk (86%). This indicates that 5-CH_3-H_4-folate could be used as supplement in fortified milk to enhance the plasma folate levels of the population. However, it is not allowed until now to use 5-CH_3-H_4-folate in supplements or fortified products in the Netherlands. In addition, its use also has the disadvantages of higher (production/isolation) costs and a lower stability as compared with the use of synthetic folic acid as supplement. If the use of 5-CH_3-H_4-folate as supplement is allowed, e.g. based on the fact that no upper level exists for the intake of this natural folate, our *in vitro* studies indicate that it is recommendable to add the supplemental 5-CH_3-H_4-folate to a milk matrix. The *in*

vitro studies show a high bioaccessibility of 5-CH_3-H_4-folate from the milk matrix and although the transport rate of 5-CH_3-H_4-folate is slightly lower than that of folic acid, the model simulations show that the rise in plasma level is almost equal after the consumption of unfermented milk with folic acid or 5-CH_3-H_4-folate. Our *in vitro* studies showed that additional FBP did not lower the bioavailability of 5-CH_3-H_4-folate from pasteurized milk which is due to the low FBP-bound 5-CH_3-H_4-folate fraction in the intestinal lumen, and as a result no inhibition of FBP on the intestinal absorption of 5-CH_3-H_4-folate is to be expected. Moreover, it might even be beneficial to add FBP to 5-CH_3-H_4-folate-fortified milk as FBP stabilizes 5-CH_3-H_4-folate during processing and storage (22). Thus, FBP fortification of 5-CH_3-H_4-folate-fortified pasteurized milk could be beneficial for the enhancement of the folate status. In contrast, model simulations showed that FBP fortification of 5-CH_3-H_4-folate-fortified fermented milk products (filmjölk and yogurt) will lead to a lower plasma folate level. These *in silico* results are in agreement with results from a recently performed *in vivo* study in which the effect of additional FBP on the bioavailability of 5-CH_3-H_4folate from fermented milk (filmjölk) was studied (23). Nine ileostomists consumed fermented milk fortified with 5-CH_3-H_4folate in absence or presence of additional FBP. In almost all volunteers the addition of FBP led to a lower bioavailability of 5-CH_3-H_4folate from fermented milk (10). In case of these fermented milk products, it should be investigated whether the benefits of FBP (which is stabilization of 5-CH_3-H_4-folate during storage and processing) weigh up against the inhibitory effect of FBP on the bioavailability of 5-CH_3-H_4-folate from fortified fermented milk.

Future research

The *in vitro* studies and *in silico* modeling described in this thesis focus on the bioavailability of folate monoglutamates (folic acid and 5-CH_3-H_4-folate) from fortified food products. As a result, the deconjugation of polyglutamates as a potential limiting step in the bioavailability of folate from natural food products was not included in this thesis. Future studies should focus on the deconjugation activity of the brush border enzymes in the human small intestine to elucidate whether deconjugation is a critical step in the bioavailability of polyglutamates from natural food products. If so, this step could be incorporated into kinetic modeling to optimize the *in silico* prediction of plasma folate levels after the consumption of natural food products (single products or mixed diets).

Another aspect that is worth further studying is the impact of ingredients in the diet on the bioavailability of folate. In the folate-containing products, other food ingredients might interact with the brush-border enzymes which could lead to a lower deconjugation activity of these enzymes. This may result in a lower bioavailability of natural folate. Food ingredients might also interact with the activity of the transport mechanisms present in the human small intestinal cells. The *in vitro* studies in this thesis showed that the intestinal absorption of folate (especially 5-CH_3-H_4-folate) was highly dependent on the activity of transporters (reduced folate carrier and multi-drug resistance proteins) in the intestinal cells. Information about interactions of food ingredients on the absorption of folate could be used to optimize the bioavailability of folate from the diet.

The *in vitro* studies described in this thesis show that the bioavailability of 5-CH_3-H_4-folate is comparable to that of folic acid. This was also observed in recent human studies after a single dose or repeated doses of supplements of 5-CH_3-H_4-folate and folic acid. Thus, the *in vitro* and *in vivo* bioavailability studies indicate that 5-CH_3-H_4-folate can be used as alternative fortificant instead of folic acid for the fortification of food products. However, information about the bioefficacy of 5-CH_3-H_4-folate is less or lacking. Future research should investigate whether 5-CH_3-H_4-folate is as effective as folic acid in the prevention of neural tube defects. However, for ethical reasons a clinical trial to prove the effectiveness of 5-CH_3-H_4-folate in the prevention of neural tube defects in humans is not feasible. An alternative approach would be to perform observational studies on the bioefficacy of 5-CH_3-H_4-folate.

From a scientific point of view, it is interesting to perform additional studies to increase the knowledge about the bioavailability and bioefficacy of natural and synthetic folate. It is, however, important that the increased knowledge is applied for the development of recommendations that lead to an optimal risk reduction of several diseases.

Conclusions

The *in vitro* and *in vivo* studies described in this thesis show that milk is an appropriate food matrix for folate fortification. In the *in vitro* studies with the gastrointestinal model was found that folic acid and $5\text{-}CH_3\text{-}H_4$-folate were highly bioaccessible from fortified milk. In addition the suitability of the milk matrix for fortification with folic acid was shown in a human intervention study, as the consumption of folic acid-fortified milk for four weeks led to an increase in plasma folate levels of the volunteers. The *in vitro* studies with the gastrointestinal model and monolayers of intestinal cells showed that FBP reduced, respectively, the bioaccessibility and intestinal absorption of folate. Thus, additional FBP in the milk matrix will decrease the bioavailability of folate (especially folic acid) from fortified milk products. As a result, FBP fortification of fortified milk is not recommended. Folic acid-fortified milk products were found to be effective in enhancing the folate status. Although the use of $5\text{-}CH_3\text{-}H_4$-folate as a supplement has a few practical limitations, $5\text{-}CH_3\text{-}H_4$-folate is proven to be a suitable alternative for folic acid in milk products to enhance the folate status of the population. In conclusion, milk fortified with folic acid or $5\text{-}CH_3\text{-}H_4$-folate might be used to bridge the gap between actual folate intake and recommended folate intake to optimize the folate status of the population.

This thesis also shows that the application of *in vitro* models is a useful approach to study the individual processes involved in the bioavailability of folate from various food products. The *in vitro* results on the bioaccessibility and intestinal absorption of folate were integrated in a kinetic model to predict (*in silico*) blood folate levels in humans. Following this approach the most suitable milk matrix and supplement can be selected which might be incorporated in an alternative dietary strategy to enhance the folate status of the population. Such an *in silico* approach can be efficiently integrated in a test strategy to identify critical steps in bioavailability and to predict the effect of various exposure scenarios (dose, frequency, duration). In addition, based on *in vitro* studies and *in silico* modeling, the optimal supplements and test conditions can be established for an efficient design for a human trial avoiding the need to perform multiple human intervention studies.

References

1. Wigertz, K., Hansen, S.I., Høier-Madsen, M., Holm, J., Jägerstad, M. (1996) Effect of milk processing on the concentration of folate binding protein (FBP), the folate binding capacity and the retention of 5-methyltetrahydrofolate. Int. J. Food Sci. & Nutr. 47: 315-322.
2. Ghitis, J. (1967) The folate binding in milk. Am. J. Clin. Nutr. 20: 1-4.
3. Wagner, C. (1985) Folate Binding Proteins. Nutr. Rev. 43: 293-299.
4. Ford, J.E. (1974) Some observations on the possible nutritional significance of vitamin B_{12}- and folate binding proteins in milk. Br. J. Nutr. 31: 243-257.
5. Tani, M., Iwai, K. (1984) Some nutritional effects of folate binding protein in bovine milk on the bioavailability of folate to rats. J. Nutr. 114: 778-785.
6. Colman, N., Hettiarachchy, N., Herbert, V. (1981) Detection of a milk factor that facilitates folate uptake by intestinal cells. Science 211: 1427-1429.
7. Salter, D., Blakeborough, P. (1988) Influence of goat's-milk folate-binding protein on transport of 5-methyltetrahydrofolate in neonatal-goat small intestinal brush-border-membrane vesicles. Br. J. Nutr. 59: 497-507
8. Kaarsholm, N.C., Kolstrup, A., Danielsen, S.E., Holm, J., Hansen, S.I. (1993) Ligand-induced conformation change in folate-binding protein. Biochem. J. 292: 921-925.
9. Holm, J., Hansen, S.I. (2001) Binding of radiolabeled folate and 5Methyltetrahydrofolate to cow's milk folate binding protein at pH 7.4 and 5.0. Relationship to concentration and polymerization equilibrium of the purified protein. Biosci. Rep. 21: 733-743.
10. Tani, M., Fushiki, T., Iwai, K. (1983) Influence of folate binding protein from bovine milk on the absorption of folate in gastrointestinal tract of rat. Biochim. Biophys. Acta 757: 274-281.
11. Artusson P, Karlsson J (1991) Correlation between oral drug absorption in humans and apparent drug permeability coefficients in human intestinal epithelial (Caco-2) cells. Biochem. Biophys. Res. Com. 175: 880-885
12. Yazdanian M, Glynn SL, Wright JL, Hawi A (1998) Correlating partitioning and Caco-2 cell permeability of structurally diverse small molecular weight compounds. Pharm. Res. 15: 1490-1494
13. Yee S (1997) In vitro permeability across Caco-2 cells (colonic) can predict in vivo (small intestinal) absorption in man – fact or myth. Pharm. Res. 14: 763-766
14. Rubas W, Jezyk N, Grass GM (1993) Comparison of the permeability characteristics of human colonic epithelial (Caco-2) cell line to colon of rabbit, monkey, and dog intestine and human drug absorption. Pharm Res 10: 113-118
15. Hidalgo IJ, Raub TJ, Borchardt RT (1989) Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. Gastroenterology 96: 736-749
16. Hillgren KM, Kato A, Borchardt RT (1995) In vitro systems for studying intestinal drug absorption. Med. Res. Rev. 15: 82-109
17. Salter DN, Scott JK, Slade H, Andrews P. The preparation and properties of folate-binding protein from cow's milk. Biochem. J. 1981;193:469-76.
18. Gregory III, J.F., Williamson, J., Liao, J.-F, Bailey, L.B., Toth, J.P. (1998) Kinetic model of folate metabolism in nonpregnant women consuming [$^{2}H_2$]folic acid: isotopic labeling of urinary folate and the catabolite *para*-acetamidobenzoylglutamate indicates slow, intake-dependent, turnover of folate pools. J.Nutr. 128: 1896-1906.
19. Gregory III, J.F., Caudill, M.A., Opalko, J., Bailey, L.B. (2001) Kinetics of folate turnover in pregnant women (second trimester) and nonpregnant controls during folic acid supplementation: stable-isotopic

labeling of plasma folate, urinary folate and folate catabolites shows subtle effects of pregnancy on turnover of folate pools. J.Nutr. 131: 1928-1937.

20. Wright, A.J.A., Finglas, P.M., Dainty, J.R., Hart, D.J., Wolfe, C.A., Southon, S., Gregory, J.F. (2003) Single oral doses of ^{13}C forms of pteryolglutamic acid and 5-formyltetrahydrofolic acid elicit differences in short-term kinetics of labelled and unlabelled folates in plasma: potential problems in interpretation of folate bioavailability studies. Br.J.Nutr., 90, 363-371.
21. Lamers, Y., Prinz-Langenohl, R., Moser, R., Pietrzik, K. (2004) Supplementation with [6S]-5-methyltetrahydrofolate or folic acid equally reduces plasma total homocysteine concentrations in healthy women. Am.J.Clin.Nutr., 79, 473-478.
22. Jones, M.L., Nixon, P.F. (2002) Tetrahydrofolates are greatly stabilized by binding to bovine milk folate-binding protein. J.Nutr. 132: 2690-2694.
23. Arkbåge K (2003) Vitamin B_{12} folate and folate-binding proteins in dairy products. Analysis, process retention and bioavailability. Doctoral Thesis, Swedish University of Agricultural Sciences, Uppsala, Sweden.

Summary

In many countries, the actual folate intake is lower than recommended for risk reduction of diseases such as anemia and neural tube defects. An enhanced folate intake can be realized following one (or a combination) of the following strategies: 1) consumption of folate-rich food products such as orange juice and spinach, 2) taking folate supplements (e.g. tablets), or 3) consumption of folate-fortified food products. The first and second strategies have shown to improve the folate status in an experimental setting. However, compliance to a diet high in fruits and vegetables or to the daily intake of supplements of the (free-living) population appears to be relatively low and not efficacious to improve the folate status for the whole population on the longer term. An alternative approach to bridge the gap between actual and recommended folate intake is the consumption of folate-fortified food products. If food fortification would be allowed in the Netherlands, the next step could be to establish the most suitable food matrix for fortification to enhance the folate status of the population. In this respect, milk products should be considered as potential matrix for fortification because they are consumed by a large part of the Dutch population. Folic acid is currently the only allowed folate form for addition to foods and in supplements, but 5-CH_3-H_4folate, the natural form present in milk, might be an interesting alternative. Milk is also considered as a candidate food matrix for folate fortification due to the presence of folate-binding proteins (FBP). FBP was thought to enhance the bioavailability of folate from milk. This was based on observations that FBP protects folate from bacterial uptake and degradation and could enhance the absorption of folate by mucosal cells. However, the effect of FBP on the bioavailability of folic acid and 5-CH_3-H_4folate from milk products in humans has not been investigated before.

The aim of the work described in this thesis was to investigate the bioavailability of folate from milk products in order to study whether the milk matrix is a suitable carrier for fortification with folic acid or 5-CH_3-H_4folate. In addition, the bioavailability of folate from milk supplemented with additional FBP was studied to investigate whether FBP enhances the bioavailability of folic acid and 5-CH_3-H_4folate from milk products. The individual processes in folate bioavailability were investigated with *in vitro* models to study the bioaccessibility and intestinal absorption of folate. The first step is the bioaccessibility of folate. This was studied using a dynamic *in vitro* gastrointestinal model. The results obtained with this model showed that most of the supplemental folate (71-90%) was bioaccessible from the fortified milk products (as described in Chapters 2 to 4). The bioaccessible fraction of 5-CH_3-H_4-folate from pasteurized milk (82%) was higher than the bioaccessible fraction of folic acid from pasteurized milk (71%) and UHT milk (75%). No difference was found between the bioaccessible fractions (82%) of 5-CH_3-H_4-folate and folic acid from fortified yogurt. Besides fortified milk products, several natural (unfortified) food products (such as orange juice, spinach, beer, peas, knäckebröd and milk) were tested to study the bioaccessibility of native folate. These studies showed that the bioaccessibility of supplemental folate was similar to that of native folate (Chapter 4). Although no difference was found in the bioaccessibility of native folate (mixture of mono- and polyglutamates) and supplemental folate (monoglutamates), a difference in bioavailability can not be excluded as the deconjugation of

polyglutamates as a potential limiting step in the bioavailability of dietary (native) folate was not investigated in these *in vitro* studies.

In unprocessed milk, most of the (natural) folate is bound to FBP. Whether FBP is digested by human intestinal enzymes during passage through the gastrointestinal tract, and if folate still occurs bound to FBP in the human small intestine was studied using the *in vitro* gastrointestinal model. In these *in vitro* studies, fortified milk products with equimolar ratios between FBP and folic acid or 5-CH_3-H_4-folate were tested, which is in line with the equimolar ratio between FBP and native folate in unfortified milk. After gastric passage of folic acid- and 5-CH_3-H_4-folate-fortified milk products, approximately 70% of the initial FBP content could be retained (Chapter 5). The extent of digestion of FBP during passage through the small intestine appeared to be dependent on the type of milk product (fermented or unfermented milk) and on the folate compound present in the milk product (Chapters 2 and 3). FBP was more stable (13-16%) in folic acid-fortified milk than in 5-CH_3-H_4-folate-fortified milk (0-1%). This difference in FBP stability was also found in the studies with yogurt as 34% of the FBP in folic acid-fortified yogurt and 17% of the FBP in 5-CH_3-H_4-folate-fortified yogurt was recovered after gastrointestinal passage. In presence of additional FBP, the bioaccessibility of folic acid from pasteurized milk was reduced while no effect on the bioaccessibility of 5-CH_3-H_4-folate was found. Also in the studies with fortified yogurt, a difference between 5-CH_3-H_4-folate and folic acid was found in the decrease of their bioaccessible fractions in presence of additional FBP. Thus, FBP has a more pronounced inhibitory effect on the bioaccessibility of folic acid as compared with 5-CH_3-H_4-folate. This difference in binding characteristics of FBP for folic acid and 5-CH_3-H_4-folate in the intestinal lumen was investigated in more detail in studies with *in vitro* gastrointestinal model (Chapter 5). The extent of binding of folic acid and 5-CH_3-H_4-folate to FBP during gastric passage was studied in order to investigate whether folic acid and 5-CH_3-H_4-folate occur in complex with FBP in the human duodenal lumen. Before gastric passage, folic acid and 5-CH_3-H_4-folate were mainly bound to FBP (76-79%) while 7% was free. After gastric passage, folic acid remained bound to FBP to a similar extent (80-81%). For 5-CH_3-H_4-folate, the FBP-bound fraction gradually decreased from 79% to 5% and the free fraction increased from 7% to 93%. So, while folic acid enters the proximal part of the small intestine bound to FBP, 5-CH_3-H_4-folate appears mainly to be present as free folate in the duodenal lumen. These results are in line with our findings that FBP decreased the bioaccessibility of folic acid to a higher extent than the bioaccessibility of 5-CH_3-H_4-folate. The difference in binding of folic acid and 5-CH_3-H_4-folate to FBP in the intestinal lumen could result in a different intestinal absorption from folic acid- and 5-CH_3-H_4-folate-fortified milk products.

The intestinal absorption of folate is the second step in folate bioavailability. This was studied using monolayers of human colon carcinoma (Caco-2) cells representing an *in vitro* model for human intestinal absorption. The permeability of folic acid and 5-CH_3-H_4-folate across Caco-2 monolayers was found to be three-fold higher than that of mannitol (marker for low permeability) but twenty-fold lower than that of caffeine (marker for high permeability). This indicates that the relatively low permeability of folic acid and 5-CH_3-H_4-folate across intestinal cells could limit the bioavailability of folate. In Chapter 7 the transport mechanisms underlying the intestinal transport of folic acid and 5-CH_3-H_4folate were investigated. These studies showed that the transport of folic acid and 5-CH_3-

H_4-folate across Caco-2 cells mainly occurred via carrier-mediated uptake (via the reduced folate carrier, RFC) and efflux via multi-drug resistance proteins (MRPs). The relative contribution of RFC- and MRP-mediated transport to the overall transport appeared to be higher for 5-CH_3-H_4-folate than for folic acid. The transport from the luminal (apical) to the serosal (basolateral) side of the Caco-2 cells was slightly higher for folic acid than for 5-CH_3-H_4-folate (Chapter 6). Thus, a small difference in intestinal transport, in rate and underlying transport mechanisms, was found between the synthetic and natural folate.

Whether the degree of binding to FBP affects the absorption of folate from the intestinal lumen was studied using monolayers of Caco-2 cells. In presence of FBP, the intestinal transport and cellular accumulation of folic acid and 5-CH_3-H_4-folate was decreased. The effect of FBP appeared to be dependent on the extent of binding to FBP of folic acid and 5-CH_3-H_4-folate at the luminal side of the cells, as an inverse correlation was found between extent of binding to FBP and intestinal transport. Thus, these studies indicate that the presence of FBP-folate complexes in the intestinal lumen will result in a lower absorption and, thus, in a lower bioavailability of folate from milk. As the studies with the *in vitro* gastrointestinal model (Chapters 2, 3 and 5) showed that more folic acid-FBP complexes occur in the intestinal lumen than 5-CH_3-H_4-folate-FBP complexes, a higher effect of FBP on the bioavailability of folic acid than on that of 5-CH_3-H_4-folate is expected.

The human intervention study, described in Chapter 8, showed that the consumption of 200 μg folic acid added to milk, in addition to a standard diet, significantly increased concentrations of folate in serum and red blood cells and decreased plasma homocysteine concentrations. This indicates that milk is a suitable matrix for fortification with folic acid to enhance the folate status in countries where milk is commonly consumed. The effect of FBP on the bioavailability of folic acid was studied by comparing the response in blood folate levels after consumption of fortified pasteurized milk (with endogenous FBP) with the levels after consumption of fortified UHT milk (no FBP). The results tended to a higher response in blood folate levels after consumption of fortified UHT milk, although this difference was not significant. This observation is in line with the small difference in bioaccessible fractions of folic acid from pasteurized and UHT milk as found in the *in vitro* studies with the gastrointestinal model (Chapter 2). This can be explained by the molar ratio (8:1) between folic acid and FBP in the folic acid-fortified pasteurized milk due to the excess of added folic acid. Only a small part of the folic acid occurred bound to FBP under these conditions, which will not lead to a limiting effect on the bioavailability of folic acid in humans.

Two milk products fortified with folic acid, but without additional FBP, could be tested in a human intervention study to measure the blood folate levels after four weeks of consumption. However, several fermented (yogurt and filmjölk) and unfermented milk (UHT and pasteurized milk) products fortified with folic acid or with 5-CH_3-H_4-folate, and with or without additional FBP, were tested in the studies with the *in vitro* gastrointestinal model (as described in Chapters 2 to 4). All these studies gave information about the bioaccessibility of folic acid and 5-CH_3-H_4-folate from the milk matrix which is the first step in bioavailability. The bioaccessibility data should be combined with the intestinal absorption of folic acid and 5-CH_3-H_4-folate to be able to predict the blood folate response in humans. For this purpose, a kinetic model was used which integrates the *in vitro* results about kinetics of folate bioaccessibility and absorption and extrapolates these *in vitro* results to the

human situation *in vivo*. With this approach blood folate levels can be predicted after the consumption of various fortified milk products without testing each individual milk product in an extensive *in vivo* study. From the model simulations (*in silico*), it can be concluded that supplementation of yogurt, UHT milk or pasteurized milk with folic acid is an efficient strategy to enhance the folate status. In addition, 5-CH_3-H_4-folate might be used as an alternative supplement to fortify milk products. The use of 5-CH_3-H_4-folate has at least some technological limitations such as its instability during storage and processing. Additional FBP might be recommendable in 5-CH_3-H_4-folate-fortified milk to stabilize 5-CH_3-H_4-folate and thus to enhance the folate intake, as FBP has only a small inhibitory effect on the bioavailability of 5-CH_3-H_4-folate from fortified milk products. However, based on the studies described in this thesis, it is recommended not to add FBP to the folic acid-fortified milk products as this will lead to a lower bioavailability of folic acid.
In conclusion, the *in vitro* and *in vivo* studies described in this thesis show that milk is an appropriate food matrix for folate fortification. Thus, a dietary strategy with fortified milk products can be recommended to bridge the gap between actual and recommended folate intake to optimize the folate status of the population.

Samenvatting

De consumptie van foliumzuur, vitamine B_{11}, verlaagt het risico op bloedarmoede en op de geboorte van een kind met een neurale buisdefect ('open ruggetje'). Daarnaast komen er steeds meer aanwijzingen dat een hoge foliumzuurinname geassocieerd is met een lager risico op hart- en vaatziekten en (darm)kanker. Het is daarom belangrijk dat men dagelijks voldoende foliumzuur binnenkrijgt. Belangrijke bronnen van foliumzuur in de voeding zijn groente en fruit, zoals spinazie en citrusvruchten. De gemiddelde foliumzuurinname van de Nederlandse bevolking is ongeveer 200 µg per dag. Dit is echter lager dan de aanbevolen dagelijkse foliumzuurinname in Nederland (300 µg per dag). Het is dus wenselijk dat de foliumzuurinname van de Nederlandse bevolking wordt verhoogd.

De dagelijkse foliumzuurinname kan worden verhoogd door een hogere consumptie van foliumzuurrijke voedingsproducten. Het blijkt echter moeilijk te zijn om verhoging van de foliumzuurinname bij de gehele bevolking te bereiken via het advies om meer groente en fruit te eten. Voor vrouwen die zwanger willen worden geldt in Nederland een aparte aanbeveling. Zij worden geadviseerd om dagelijks een foliumzuursupplement te slikken vanaf vier weken voor tot en met acht weken na de bevruchting. In de praktijk blijkt dat deze aanbeveling niet door een ieder wordt opgevolgd en in veel gevallen worden de supplementen pas geslikt in de periode na het vaststellen van de zwangerschap. Om het risico op neurale buisdefecten bij baby's optimaal te verlagen is in verschillende landen, waaronder de Verenigde Staten, ervoor gekozen om voedingsmiddelen met foliumzuur te verrijken. In Nederland is dit tot op heden niet toegestaan. De verwachting is wel dat in de toekomst ook in Nederland en andere Europese landen foliumzuur-verrijkte producten op de markt zullen verschijnen. Indien foliumzuur-verrijkte producten op de Nederlandse markt worden toegestaan, is de vraag welk voedingsproduct het meest geschikt is om de foliumzuurstatus van een zo groot mogelijk gedeelte van de Nederlandse bevolking te verhogen. Aangezien het lastig is gebleken om mensen hun dagelijkse voeding te laten wijzigen, is het verrijken van een product dat reeds onderdeel is van de dagelijkse voeding waarschijnlijk een effectievere manier om de foliumzuurstatus van de bevolking te verhogen. Gezien de hoge melkconsumptie in Nederland en enkele andere Europese landen, kan melk worden beschouwd als een potentieel geschikt product voor verrijking met foliumzuur. Een extra reden is de aanwezigheid van foliumzuur-bindende eiwitten (FBP) in melk. Eerdere studies hebben aangetoond dat FBP de stabiliteit en mogelijk ook de biobeschikbaarheid van foliumzuur verhoogt. De term biobeschikbaarheid verwijst naar het gedeelte dat na consumptie wordt opgenomen vanuit het darmkanaal naar de bloedbaan en beschikbaar is voor processen in het lichaam.

Het doel van het onderzoek beschreven in dit proefschrift is het bestuderen van de biobeschikbaarheid van foliumzuur uit verschillende foliumzuur-verrijkte melkproducten. Daarbij is de biobeschikbaarheid van synthetisch foliumzuur (PGA) en van een natuurlijke foliumzuurverbinding (5MTHF) uit verrijkte melkproducten met elkaar vergeleken. In de voeding komt foliumzuur in verschillende vormen voor, waarvan 5MTHF de belangrijkste verbinding is. PGA komt van nature niet in de voeding voor, maar wordt gebruikt in verrijkte voedingsmiddelen

(in o.a. de Verenigde Staten) en supplementen (in o.a. Nederland). Tevens is bij dit onderzoek het effect van FBP op de biobeschikbaarheid van PGA en 5MTHF bestudeerd.
In de hoofdstukken 2 tot en met 5 zijn studies beschreven waarin werd onderzocht welk deel van de hoeveelheid foliumzuur (PGA of 5MTHF) aanwezig in de verrijkte melkproducten vrijkomt gedurende maag-darmpassage. Na consumptie zal de melkmatrix (gedeeltelijk) worden afgebroken door blootstelling aan verteringsenzymen in het maag-darmkanaal. Een gedeelte van het aanwezige foliumzuur komt hierbij vrij en is beschikbaar voor opname uit het darmlumen. De mate van vrijkomen van foliumzuur uit de melk is onderzocht in studies met een dynamisch *in vitro* maag-darmmodel (afgekort TIM), dat de fysiologische condities in het maag-darmkanaal van de mens nabootst. Verschillende melkproducten (gesteriliseerde melk, gepasteuriseerde melk en yoghurt) met toegevoegd 5MTHF of PGA zijn getest in dit *in vitro* model. Een groot gedeelte (71-90%) van het toegevoegde foliumzuur bleek vrij te komen uit de melkproducten tijdens de passage door het maag-darmkanaal, zoals beschreven in de hoofdstukken 2, 3 en 4. De studies beschreven in hoofdstuk 2 tonen aan dat 5MTHF in vergelijking met PGA beter beschikbaar komt voor absorptie uit verrijkte gepasteuriseerde melk. Er werd geen verschil waargenomen tussen de vrijgekomen fracties van PGA en 5MTHF uit verrijkte yoghurt (hoofdstuk 3). Naast verrijkte producten zijn ook natuurlijke producten zoals spinazie, erwten, sinaasappelsap en bier getest in het *in vitro* maag-darmmodel. Er bleek geen verschil te zijn tussen de mate van vrijkomen van het 5MTHF dat van nature in de voeding aanwezig was en het 5MTHF dat toegevoegd was aan de melkproducten (hoofdstuk 4).
In onbehandelde melk is bijna al het van nature aanwezige foliumzuur (5MTHF) gebonden aan FBP. Tot nu toe was het onbekend of het FBP afgebroken wordt gedurende passage door het maag-darmkanaal door blootstelling aan verteringsenzymen. Tevens was het niet bekend of het aan FBP-gebonden foliumzuur in (verrijkte) melk ook gebonden blijft gedurende maag-darmpassage, of dat het vrijkomt in de dunne darm en beschikbaar is voor transport door de darmwand. Deze onderzoeksvragen zijn onderzocht in studies met het eerder beschreven *in vitro* maag-darmmodel (hoofdstukken 2 tot en met 5). In deze studies zijn PGA- of 5MTHF-verrijkte melkproducten getest met en zonder extra FBP. De hoeveelheid FBP toegevoegd aan de melkproducten was gelijk aan de hoeveelheid toegevoegd PGA of 5MTHF in de melk. Voor deze verhouding (1:1) was gekozen omdat dit overeenkomt met de natuurlijke verhouding tussen foliumzuur en FBP in onbehandelde melk. Na het passeren van het maagcompartiment van het *in vitro* maag-darmmodel bleek 70% van de initiële hoeveelheid FBP intact het begin van de dunne darm te bereiken. FBP werd verder afgebroken gedurende passage door de dunne darm. Slechts 13-16% van de FBP in PGA-verrijkte melk en 0-1% van de FBP in 5MTHF-verrijkte melk was nog intact na passage door de maag en de dunne darm. Dit verschil in stabiliteit tussen FBP in PGA- of in 5MTHF-verrijkte melk werd ook gevonden tussen de hoeveelheid intact FBP na passage door het maag-darmmodel van PGA-verrijkte yoghurt (34%) en 5MTHF-verrijkte yoghurt (17%).
Niet alleen de stabiliteit van FBP, maar ook het effect van FBP op het beschikbaar komen voor opname van PGA en 5MTHF uit de melkproducten is bestudeerd in de studies beschreven in de hoofdstukken 2 tot en met 5. In aanwezigheid van extra FBP werd een lagere beschikbare PGA fractie gevonden uit PGA-verrijkte gesteriliseerde en gepasteuriseerde melk. FBP had geen effect

op het vrijkomen van 5MTHF uit verrijkte gepasteuriseerde melk. Dit verschil in effect van FBP op het vrijkomen van PGA en 5MTHF uit melk werd ook gevonden in de studies met verrijkte yoghurt. De resultaten uit deze studies tonen dus aan dat FBP een sterker verlagend effect heeft op de beschikbaarheid voor absorptie van PGA dan van 5MTHF uit de verrijkte melkproducten. Dit zou kunnen worden verklaard door een verschil in mate van binding aan FBP tussen PGA en 5MTHF. Dit is nader onderzocht in studies beschreven in hoofdstuk 5, waarin de mate van FBP binding is gemeten voor en na passage door het maagcompartiment van het *in vitro* model. De aanwezigheid van FBP-PGA of FBP-5MTHF complexen in het eerste deel van de dunne darm (duodenum) is gemeten aangezien dit de absorptie van foliumzuur uit het darmlumen zou kunnen beïnvloeden. De gebonden en ongebonden PGA en 5MTHF fracties zijn gemeten in melkproducten waarin PGA of 5MTHF in gelijke hoeveelheid (1:1 ratio) voorkomen ten opzichte van het aanwezige FBP. Bij deze 1:1 ratio tussen foliumzuur en FBP was 76-79% van het foliumzuur, zowel PGA als 5MTHF, in de melkproducten gebonden aan FBP. Na passage door de maag, was PGA nog steeds voor 80-81% gebonden aan FBP. PGA komt dus in het begin van de dunne darm nog steeds voor als FBP-PGA complex. Daarentegen bleek dat de fractie FBP-gebonden 5MTHF na maagpassage sterk afnam. De percentages FBP-gebonden 5MTHF in de monsters verzameld uit het duodenum gedurende 0 tot 2 uur namen af van 79% tot 5%. De vrije fractie 5MTHF in de melkproducten was gedurende deze tijd toegenomen van 7% naar 93%. Deze resultaten tonen aan dat PGA gebonden aan FBP het eerste gedeelte van de dunne darm bereikt, terwijl 5MTHF ongebonden voorkomt in het lumen van de dunne darm na consumptie van (verrijkte) melkproducten. Dit is tevens de verklaring voor het verschil in effect van FBP op het vrijkomen van PGA en 5MTHF uit verrijkte gepasteuriseerde melk en yoghurt zoals gevonden in de studies beschreven in de hoofdstukken 2 en 3. Het verschil in mate van binding aan FBP tussen PGA en 5MTHF in het darmlumen zou kunnen leiden tot een verschil in absorptie van PGA en 5MTHF na het consumeren van verrijkte melkproducten.

De absorptie van foliumzuur uit het darmlumen is bestudeerd met een humane dikke darmkanker cellijn (Caco-2) die gekweekt als cellaag op een membraan als *in vitro* model gebruikt kan worden. In de hoofdstukken 6 en 7 zijn deze Caco-2 cellen gebruikt om het transport van PGA en 5MTHF over de darmwand te bestuderen. Verschillende FBP concentraties in de PGA en 5MTHF oplossingen zijn getest om te bestuderen of een verschil in mate van binding aan FBP leidt tot een verschil in transport van PGA en 5MTHF. Uit de studies beschreven in hoofdstuk 6 bleek dat het transport van het transport van PGA en 5MTHF over de Caco-2 cellen afneemt in aanwezigheid van FBP. Deze afname was afhankelijk van de concentratie FBP in de foliumzuuroplossingen. Deze resultaten tonen aan dat de mate van binding aan FBP de mate van absorptie van foliumzuur uit het darmlumen bepaalt. Gezien de eerdere observaties dat PGA grotendeels gebonden aan FBP voorkomt na maagpassage, in tegenstelling tot 5MTHF, zal FBP een sterker limiterend effect hebben op de absorptie van PGA dan op de absorptie van 5MTHF. Uit deze *in vitro* resultaten kan worden geconcludeerd dat FBP de biobeschikbaarheid van foliumzuur en met name PGA uit melkproducten verlaagd.

In de studies met Caco-2 cellen hebben we tevens de transportsnelheid (hoofdstuk 6) en de mechanismen betrokken bij het transport van PGA en 5MTHF (hoofdstuk 7) onderzocht. Naast het

meten van de transportsnelheid van PGA en 5MTHF over de Caco-2 cellen is in dezelfde experimenten de transportsnelheid van mannitol en caffeine, voorbeeldstoffen voor respectievelijk lage en hoge absorptie, onderzocht. De transportsnelheid van beide foliumzuurverbindingen was veel lager dan die van caffeine maar wel iets hoger dan die van mannitol, wat duidt op een relatief lage transportsnelheid van foliumzuur. Dit kan erop wijzen dat het transport van foliumzuur over de darmwand een limiterende factor kan zijn in de biobeschikbaarheid van foliumzuur. De resultaten uit deze studies tonen ook aan dat het transport van PGA iets hoger is dan het transport van 5MTHF over de Caco-2 cellen. Aangezien foliumzuur een sterk hydrofiel karakter heeft, zal het niet of nauwelijks via passief, transcellulair transport de darmwand passeren. Hierdoor is het waarschijnlijk dat PGA en 5MTHF voor hun opname afhankelijk zijn van transporteiwitten, aanwezig in de membranen van de darmcellen. Door gebruik te maken van stoffen die bepaalde transporteiwitten blokkeren kon worden onderzocht via welke mechanismen foliumzuur over de Caco-2 cellen wordt getransporteerd. Deze studies tonen aan dat zowel PGA als 5MTHF voornamelijk via een transport carrier (zgn. reduced folate carrier of afgekort RFC) worden opgenomen in de darmcellen en vervolgens uit de cellen worden gepompt via efflux pompen (zgn. multi-drug resistance pumps of afgekort MRP). De bijdrage van het transport via RFC en MRP transportmechanismen aan het totale transport over de Caco-2 cellen bleek hoger te zijn voor 5MTHF dan voor PGA.
De biobeschikbaarheid van foliumzuur uit melk werd niet alleen bestudeerd in *in vitro* studies met behulp van een maag-darmmodel en Caco-2 cellen maar ook in een humane interventiestudie (hoofdstuk 8). In deze studie kregen humane vrijwilligers (n=69) gesteriliseerde of gepasteuriseerde melk te drinken die wel of niet was verrijkt met 200 µg PGA. Het doel van deze studie was om te onderzoeken of de consumptie van verrijkte melk leidt tot een verhoogde concentratie foliumzuur in het bloed. Tijdens deze vier weken durende studie kregen de vrijwilligers een standaard voeding met daarnaast dagelijks twee bekers (totaal 500 ml) melk met of zonder PGA. De gesteriliseerde melk bevatte door de hittebehandeling geen intact FBP meer. In de gepasteuriseerde melk was echter nog wel enig FBP aanwezig. De tweede onderzoeksvraag van deze studie was of het FBP, van nature aanwezig in de gepasteuriseerde melk, een effect had op de biobeschikbaarheid van PGA uit de verrijkte melkproducten. De consumptie van foliumzuur-verrijkte melkproducten gedurende vier weken leidde tot een verhoogde foliumzuurstatus van de consumenten. Deze resultaten tonen aan dat melk een geschikt voedingsproduct is voor verrijking met foliumzuur om de foliumzuurstatus te verhogen. Daarnaast bleek dat FBP een klein, niet significant, limiterend effect had op de biobeschikbaarheid van PGA uit de verrijkte melkproducten. Dit kleine effect van FBP op de biobeschikbaarheid van PGA kan worden verklaard door het enorme verschil in hoeveelheden FBP en PGA (verhouding 1:8) in de verrijkte gepasteuriseerde melk. De *in vitro* studies hebben namelijk aangetoond dat FBP de biobeschikbaarheid van PGA verlaagt bij een gelijke verhouding tussen het aanwezige FBP en PGA (1:1).
In de humane studie werden twee met PGA-verrijkte melkproducten, maar niet verrijkt met FBP, getest om de toename van de foliumzuurconcentraties in het bloed te meten. In de *in vitro* studies met het *in vitro* maag-darmmodel zijn daarnaast nog verschillende andere melkproducten getest, verrijkt met PGA of 5MTHF, met of zonder extra FBP. Uit deze *in vitro* studies is informatie verkregen over het vrijkomen van PGA en 5MTHF uit de melkproducten gedurende maag-

darmpassage. Door deze resultaten te combineren met de resultaten uit de *in vitro* studies met de Caco-2 cellen, waarin de transportsnelheid over de darmwand is gemeten, is het mogelijk om de (verandering in de) foliumzuurconcentraties in de tijd te voorspellen. Met een computer model dat de kinetische data uit de *in vitro* studies combineert met de informatie over de processen in het menselijke lichaam zijn de foliumzuurconcentraties in het bloed voorspeld na consumptie van verschillende verrijkte melkproducten. Met deze strategie kan een geschikt melkproduct worden geselecteerd dat effectief de foliumzuurstatus verhoogt. Deze selectie geschiedt op basis van de combinatie van een melkproduct (gesteriliseerd, gepasteuriseerd of gefermenteerd), supplement (PGA of 5MTHF) en de aanwezigheid van FBP die leidt tot de meest effectieve toename in de foliumzuurstatus van de consumenten. Het volgen van deze strategie heeft als voordeel dat niet elk product individueel getest hoeft te worden in een humane interventiestudie. Dit levert dus een reductie op van het aantal *in vivo* studies. Simulaties met het computermodel, gebaseerd op de *in vitro* data uit dit proefschrift, hebben aangetoond dat zowel PGA als 5MTHF gebruikt kan worden als supplement in melkproducten. Melkproducten zonder additioneel FBP leiden tot een hogere toename in foliumzuurstatus van de consumenten in vergelijking met FBP-verrijkte melkproducten.

Samenvattend tonen de studies beschreven in dit proefschrift aan dat melk een geschikt product is om te verrijken met foliumzuur (PGA of 5MTHF) om de foliumzuurstatus van de consumenten te verhogen. Het is niet aan te raden om extra FBP aan de verrijkte melkproducten toe te voegen daar dit zal leiden tot een lagere biobeschikbaarheid van foliumzuur (met name PGA).

Curriculum vitae

Miriam Verwei werd geboren op 26 december 1974 te ‘s-Heer Arendskerke. Na het behalen van het VWO-diploma aan het ‘Goese Lyceum’ te Goes in 1994, is ze begonnen aan de studie Biologie (afstudeerrichting milieubiologie) aan de Universiteit Utrecht. Tijdens deze studie heeft ze twee stage onderzoeken verricht, waarvan de eerste is uitgevoerd bij het RITOX (afdeling Milieuchemie). Tijdens deze stage heeft ze onderzoek gedaan naar de accumulatie van surfactanten in Regenboogforellen. Vervolgens heeft ze haar opleiding afgerond met een stage en scriptie bij TNO Voeding (afdeling Verklarende Toxicologie) te Zeist. Hier heeft ze onderzoek verricht naar het gebruik van darmsegmenten en celsystemen (stage) en proteomics (scriptie) in het toxicologisch onderzoek. Na het behalen van haar doctoraal diploma in november 1998 is ze als projectmedewerker begonnen bij het RIVM te Bilthoven waar ze onderzoek heeft gedaan naar de mogelijkheden tot het verminderen van proefdiergebruik in het toxicologisch onderzoek.
Op 1 november 1999 trad zij in dienst als assistent in opleiding bij de afdeling Humane voeding van de Wageningen Universiteit. Het onderzoek beschreven in dit proefschrift werd uitgevoerd in samenwerkingsverband tussen TNO Voeding en Wageningen Universiteit en was onderdeel van het EU project ‘From Food to Functionality and Optimal Health’. Vanaf 1 december 2003 is Miriam in dienst bij TNO Voeding te Zeist, afdeling Physiological Sciences.

List of publications

Peer-reviewed papers:

Verwei M, Arkbåge K, Havenaar R, van den Berg H, Witthöft C, Schaafsma G. Folic acid and 5-methyltetrahydrofolate in fortified milk are bioaccessible as determined in a dynamic in vitro gastrointestinal model. Journal of Nutrition 2003, 133: 2377-2383.

Arkbåge K, Verwei M, Havenaar R, Witthöft C. Bioaccessibility of folic acid and (6S)-5-methyltetrahydrofolate decreases after the addition of folate-binding protein to yogurt as studied in a dynamic in vitro gastrointestinal model. Journal of Nutrition 2003, 133: 3678-3683.

Verwei M, Arkbåge K, Mocking H, Havenaar R and Groten JP. The binding of folic acid and 5-methyltetrahydrofolate to folate-binding proteins during gastric passage differs in a dynamic in vitro gastrointestinal model. Journal of Nutrition 2004, 134: 31-37.

Verwei M, van den Berg H, Havenaar R, Groten JP. Effect of folate-binding protein on intestinal transport of folic acid and 5-methyltetrahydrofolate across Caco-2 cells. European Journal of Nutrition, *In press*.

Verwei M, Olivares AB, van Vliet T, van den Berg H, Havenaar R. Bioaccessibility of folate from several liquid and solid food products as studied in a dynamic in vitro gastrointestinal model. *Submitted for publication*

Verwei M, van Ede K, Wortelboer HM, Groten JP. Transport of folic acid and 5-methyltetrahydrofolate across Caco-2 cells occurs via the reduced folate carrier and multi-drug resistance proteins. *Submitted for publication.*

De Jong RJ, Verwei M, West CE, van Vliet T, Siebelink E, van den Berg H, Castenmiller JJM. Bioavailability of folic acid from fortified pasteurized milk and UHT-treated milk in humans. *Submitted for publication.*

Verwei M, Freidig AP, Havenaar R, Groten JP. An in silico approach to integrate in vitro data on folate release and absorption in a kinetic model to predict plasma folate levels in humans. *In preparation.*

Abstracts:

Verwei M, Groten JP, Havenaar R, van den Berg H. Oral bioavailability of folate using in vitro models of GI tract. Proceedings of the scientific meeting of the Netherlands Society of Toxicology, p58, Kerkrade, December 2000.

Verwei M, van den Berg H, Groten JP, Havenaar R, West CE, Schaafsma G. Assessment of folate transport and bioavailability using in vitro models of the gastrointestinal tract. Abstract book PhD Study Tour Switzerland, Italy and Germany, p40, September 2001.

Verwei M, Arkbåge K, Havenaar R, van den Berg H, Witthöft C, Schaafsma G. Folate bioaccessibility from dairy products and the effect of FBP on the folate retention studied in a dynamic *in vitro* gastrointestinal model. FASEB Summer Research Conference, p5, Snowmass Village, Colorado, August 2002.

Verwei M, Arkbåge K, Havenaar R, van den Berg H, Witthöft C, Schaafsma G. Folate bioaccessibility from milk products studied with a dynamic *in vitro* gastro-intestinal model. 26th IDF World Dairy Congress, Paris, September 2002.

Havenaar R, Verwei M, Olivares AB, Arkbåge K, Ros G, Witthöft C, Walker C, Carnovale E, Kariluoto S, Finglas P. Folate bioaccessibility from various food products studied in a dynamic *in vitro* gastrointestinal model. International Research Conference on Food, Nutrition and Cancer, Washington DC, July 2003. Abstract published: Journal of Nutrition 2003, 133: 3862S-3863S.

Verwei M, Arkbåge K, Witthöft C, Havenaar R. Folate bioaccessibility from fortified dairy products and effect of folate binding protein studied in a dynamic *in vitro* gastrointestinal model. 9th European Nutrition Conference, Rome, October 2003. Abstract published: Annals of Nutrition & Metabolism 2003, 47: 625.

Verwei M, Arkbåge K. Witthöft C, van den Berg H, Havenaar R. The effect of folate-binding proteins of bioavailability of folate from milk products. First International Conference on Folates, Analysis, Bioavailability and Health, Warsaw, p77-78, February 2004. Abstract submitted: Trends in Food Science.

Arkbåge K, Verwei M, Havenaar R, Witthöft C. Addition of folate-binding proteins to yoghurt reduces the bioaccessibility of folate as studied in a dynamic in vitro gastrointestinal model. First International Conference on Folates, Analysis, Bioavailability and Health, Warsaw, p112, February 2004.

Havenaar R, Verwei M, Olivares AB, Arkbåge K, Ros G, Witthöft C, Walker C, Carnovale E, Kariluoto S, Finglas P. Folate bioaccessibility from liquid and solid food products during passage through a dynamic gastrointestinal model. First International Conference on Folates, Analysis, Bioavailability and Health, Warsaw, p167, February 2004.

De Jong RJ, Verwei M, West CE, van Vliet T, Siebelink E, van den Berg H, Castenmiller JJM. Bioavailability of folic acid added to pasteurized milk and UHT-treated milk in humans. First International Conference on Folates, Analysis, Bioavailability and Health, Warsaw, p217-218, February 2004.

Other publications:

Verwei M, Havenaar R. Bioaccessibility of folate from food products during passage through a dynamic gastrointestinal model (TIM). Newsletter FolateFuncHealth, June 2001, Issue 3.

Verwei M, Havenaar R, Arkbåge K. Folate bioaccessibility from dairy foods using TIM. Newsletter FolateFuncHealth, July 2002, Issue 5.

Freidig AP, Verwei M. Integration of in vitro data in kinetic models for pharmaceuticals and nutrients. Newsletter Netherlands Centre for Alternatives to Animal Use, March 2004, Issue 16.

Dankwoord

Eindelijk kan ik beginnen met het schrijven van het allerlaatste hoofdstuk van mijn proefschrift: het dankwoord. Het boekje is nu echt af en dat geeft een erg goed gevoel. Ik wil iedereen bedanken die bij het werk betrokken was of op een andere manier tot steun is geweest.

Om te beginnen wil ik graag mijn promotoren Gertjan Schaafsma en Clive West bedanken. Als begeleiders ‘op afstand’ hielden jullie de onderzoekslijn in de gaten en attendeerden jullie er mij op dat ik echt maar vier jaar had om te promoveren. Gertjan, bedankt voor het papieren regelwerk en het nakijken van de stukken tekst vooral in de eindfase. Clive, in het begin was je behoorlijk kritisch maar ik ben erg blij dat je hebt aangegeven zeer tevreden te zijn met dit proefschrift. Bedankt voor het doorlezen en corrigeren van met name de inleiding en discussie. Ondanks je verschrikkelijke ziekte, was je vastbesloten om me te helpen met de laatste onderdelen van mijn proefschrift. Ik heb enorm veel bewondering voor je energie en doorzettingsvermogen.
Uiteraard wil ik ook in het bijzonder mijn co-promotoren John Groten en Rob Havenaar bedanken. John, jouw enthousiasme werkte enorm stimulerend: het was elke keer weer leuk om nieuwe resultaten te laten zien. Ik ben ook erg blij dat je me hebt aangemoedigd om een ‘in silico-gedeelte’ aan mijn aio-project toe te voegen. Rob, als begeleider van al het TIM-werk binnen het project heb je veel resultaten gezien en veel stukken tekst doorgelezen. Jouw enthousiasme over TIM werkt erg aanstekelijk en ik ben dan ook blij dat ik TIM heb leren kennen en nog geen afscheid van hem (?) hoef te nemen. Bedankt voor het elke keer ‘even tijd’ voor me vrijmaken. Niet alleen voor het bespreken van resultaten of manuscripten maar zeker ook op momenten dat ik twijfelde of het mij wel ging lukken en of de resultaten wel goed genoeg waren. En natuurlijk wil ik je ook bedanken voor de gezelligheid tijdens de leuke reizen naar Rome, Hannover, Uppsala en Warschau.
En eigenlijk heb ik altijd drie co-promotoren gehad, want ook Henk van den Berg was jarenlang mijn co-promotor tijdens dit project. Henk, bedankt voor het delen van al je kennis over foliumzuur! Je liet me wel schrikken toen je na anderhalf jaar aankondigde weg te gaan bij TNO om in Den Haag te gaan werken. Maar gelukkig kon ik ook via de email of telefoon af en toe een beroep doen op jouw enorme foliumzuurkennis. Het bleek elke keer weer erg nuttig te zijn om jouw commentaar (vanuit een ‘in vivo’ oogpunt) te verwerken in de artikelen over het in vitro werk. Bedankt voor al je hulp en adviezen de afgelopen jaren en ik hoop dat we contact houden.

Tijdens de projectbesprekingen waren niet alleen mijn vijf (co-)promotoren aanwezig maar het eerste anderhalf jaar ook Jacqueline Castenmiller en Jan Steijns en de laatste 2-3 jaar Trinette van Vliet en Frank Elbers. Bedankt voor jullie bijdrage aan de discussies tijdens dit project! En Trinette: bedankt voor de gezelligheid tijdens de reizen naar Rome en Warschau!

Dit onderzoek maakte deel uit van een EU project en ik wil daarom zeker ook alle partners uit dit EU project bedanken. Dear colleagues of the FolateFuncHealth project: I really enjoyed the collaboration with all of you!

I especially want to thank the folate colleagues from the SLU (Uppsala, Sweden): Karin Arkbåge, Cornelia Witthöft and Margaretha Jägerstad. I really enjoyed our collaboration and also the visits to Uppsala. The nice discussions about folate, milk and FBP were very fruitful and contributed to our joint publications. Karin: thank you for all the mails in which we discussed the results, and talked about all kinds of non-folate topics, and our nice discussions by phone or during the meetings of the EU project. I really liked your visit to Zeist, although we planned a lot of TIM experiments in a very short period which was not very relaxed. I wish you all the best and I hope we will keep in touch!
And I also want to thank the Spanish folate colleagues for our collaboration: Ana Belén Olivares and Gaspar Ros. Ana, TIM received a lot of food products (spinach, peas, bread, orange juice, beer and of course your Spanish Gazpacho soup) during your stay in Zeist. It was nice to feed TIM with Gazpacho, but apparently TIM did not like the Gazpacho that much since he spat out the soup now and then (as you will remember….). I hope to see you again, maybe in Murcia!

Na het bedanken van alle begeleiders en partners van het EU project wil ik heel graag de TNO collega's bedanken met wie ik de afgelopen jaren heb samengewerkt.
Allereerst de TIM-mers: Rob, Mans, Evelijn, Mark, Jeffrey, Jan, Koen, Marleen, Annet en Cyrille. Ik vond het erg leuk om bij het TIM-team te horen, bedankt voor jullie hulp bij de experimenten en uiteraard de gezellige etentjes!
En natuurlijk ook iedereen van het vitamine-analyselab: ik vond het erg gezellig op het 'gele lab', bedankt! En in het bijzonder wil ik Hans en Wouter bedanken. Wouter: bedankt dat ik jarenlang mijn foliumzuurmonsters (uit TIM) heb mogen analyseren op het lab met behulp van HPLC of microbiologische analyse. Hans, bedankt voor je hulp bij het opzetten van de HPLC methode en het maken van de FBP- en Sephadex-kolommen. Jouw humor en mooie verhalen zorgden ervoor dat ik met veel plezier met jou heb samengewerkt. En ik zal ook zeker nooit vergeten hoe je uit een paar liter wei FBP isoleert, en dat we de wei van ons gezicht moesten wassen nadat het filter verstopt was geraakt! Bedankt voor de leuke tijd!
Ik wil ook erg graag de stagiaires Corjan en Karin bedanken voor jullie enorme inzet en enthousiasme.
Heleen, bedankt voor het delen van je kennis van de transporters en je hulp bij het schrijven van het artikel, maar zeker ook voor de steun op de momenten dat ik er even doorheen zat.
Andreas, jouw enthousiasme over het in silico werk is erg aanstekelijk en ik vind het heel leuk om met je samen te werken. Bedankt voor al je hulp!

En ik wil ook erg graag alle andere collega's bedanken die er zeker ook voor hebben gezorgd dat ik een leuke AIO-periode heb gehad! Het was af en toe lastig om 'een eigen plekje' te vinden als AIO in een centrum tussen de afdeling Humane Voeding van Wageningen Universiteit en afdeling Voedingsfysiologie van TNO Voeding, werkend bij de TIM-mers, de vitamine-analysegroep en de ABMS-ers. Inmiddels zijn de afdelingen Voedingsfysiologie en ABMS samengevoegd tot de afdeling Physiological Sciences. Ik wil iedereen van de afdeling Physiological Sciences bedanken voor jullie interesse de afgelopen jaren. In het bijzonder wil ik de collega's van ABMS bedanken

omdat jullie er zeker voor hebben gezorgd dat, ook al hoorde ik er officieel niet bij, ik me een echte ABMS-er heb gevoeld. De afdelingsuitjes waren erg leuk! En zeker ook iedereen bedankt voor de gezellige lunchpauzes, maar ook de 'vrijmiddag-thee' (Dimitri en Johan), de bakkies koffie (Maria), het 'drie-uur toetje' (Richard en Rianne) en de 'half-negen-koffie-om-nog-even-rustig-wakker-te-worden' (Wilfred en Richard). En Birol: het was erg gezellig tijdens de TNO-introductiedagen!
Tenslotte wil ik mijn (ex-)collega's van de (ex-)'in vitro'groep speciaal bedanken voor jullie interesse en steun: Han, Rianne, Rob, Wilfred, Johan, Azz-Eddine, Sonia en Richard. Heel even hoorde ik officieel bij de in vitro-groep, maar altijd heb ik het gevoel gehad dat ik er een beetje bij hoorde. Kamergenoten Johan en Azz-Eddine: sorry dat ik niet erg gezellig was de afgelopen maanden, ik vind het erg leuk om met jullie een kamer te delen. Han: bedankt dat ik even bij 'jouw groep' mocht horen, helaas ging je zelf een paar maanden later naar een andere afdeling. Ik wil je bedanken voor het veelvuldig uitspreken van de legendarische woorden 'het komt allemaal goed', zeker ook omdat het allemaal goed is gekomen. Bedankt voor je vertrouwen en steun het afgelopen jaar.

Alle mede-AIO's bij TNO en WU: bedankt voor de leuke etentjes en succes met jullie boekjes!
En ik wil ook graag iedereen op de vakgroep Humane Voeding (WU) bedanken voor de gezelligheid in de periodes dat ik op de vakgroep heb gewerkt. Rosemarijn: bedankt voor de leuke tijd (o.a. de FOLMELK studie) en veel succes met al je buitenlandse avonturen!

Bedankt alle fietsers voor de gezelligheid tijdens de fietstochten tussen Utrecht en Zeist: Cyrille, Wilfred, Richard, Rianne, Evelijn, Anne, Florence en Han (heel af en toe)! En natuurlijk de fietsclub: Menk, Han, André, Wilfred, Cyrille, Erna: bedankt voor de leuke borrels en het is zeker tijd voor een nieuwe borrel!

Ik wil ook graag alle vrienden en familie bedanken die mijn promotie van een afstand hebben gevolgd. Lieve pap en mam, Paul en Femke, eindelijk zien jullie 'mijn boekje' waar ik al die tijd mee bezig was en waarom het niet altijd lukte om naar Zeeland te komen. Bedankt voor jullie begrip en steun! Iedereen bedankt voor de gezelligheid en de nodige ontspanning in Utrecht, Goes, 's-Heer Arendskerke, Spijkenisse, Amsterdam, Nijmegen, Breda en andere delen van Nederland.

Mijn paranimfen, Cyrille en Erna, wil ik heel erg bedanken. Bedankt voor jullie vriendschap, de ontspanning op de tennisbaan en vele andere gezellige momenten. Erna, dankzij jouw goede zorg voor Goof en Loek ga ik altijd relaxed op vakantie! En Cyrille ik wil je niet alleen bedanken voor de sportieve en gezellige tijd, maar zeker ook voor al je steun en hulp de afgelopen jaren!

En tenslotte niet te vergeten: Eric. Lieve Eric, bedankt voor je hulp bij de laatste (zware) loodjes van dit proefschrift, je steun, maar zeker ook je begrip dat ik vele zondagen op TNO mijn proefschrift aan het schrijven was. Het is nu tijd om op vakantie te gaan!

List of abbreviations

5-CH_3-H_4-folate	5-methyltetrahydrofolate
5-HCO-H_4folate	5-formyltetrahydrofolate
5MTHF	5-methyltetrahydrofolate
Ap	Apical
Bl	Basolateral
Caco-2	Human colon carcinoma
DHFR	Dihydrofolate reductase
ELISA	Enzyme-linked immunosorbent assay
FBP	Folate-binding proteins
FR	Folate receptor
H_4folate	Tetrahydrofolate
HPLC	High performance liquid chromatography
MRP	Multi-drug resistance proteins
MTHFR	5,10-methylenetetrahydrofolate reductase
MTX	Methotrexate
PGA	Pteroylglutamic acid (folic acid)
Pgp	P-glycoprotein
RBC	Red blood cell
RDA	Recommended daily allowances
RFC	Reduced folate carrier
RPBA	Radio protein-binding assay
TEER	Transepithelial electrical resistance
TIM	in vitro gastrointestinal model
UHT	Ultra-high temperature

The studies described in this thesis were conducted within a collaborative project of TNO Nutrition and Food Research and Wageningen University. The research was financially supported by Campina BV and the European Union project "Folate: From Food to Functionality and Optimal Health" (QLK1-1999-00576).

Publication of this thesis was financially supported by TNO Nutrition and Food Research, Wageningen University and Campina BV.

Cover design: Erna Hissink, Cyrille Krul, Mark Müting & Miriam Verwei

Printing: Ponsen & Looijen BV, Wageningen, The Netherlands.