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ORIGINAL CONTRIBUTION

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

Summary *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/s) than for 5-CH_3-H_4folate ($P_{app} = 1.4*10^{-6}$ cm/s) after 2 h 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/s) but lower ($P < 0.05$) than that of caffeine ($P_{app} = 34*10^{-6}$ cm/s). 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.

Key words folate – intestinal transport – folate-binding protein – Caco-2 – bioavailability

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Introduction

An adequate folate intake is preventive against megaloblastic anemia [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

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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 semipermeable 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 was 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) (Fig. 1). The integrity of the monolayers was tested by measuring the transepithelial electrical resistance (TEER) using the Millicell-ERS epithelial volt-ohm meter (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 $\Omega \cdot cm^2$.

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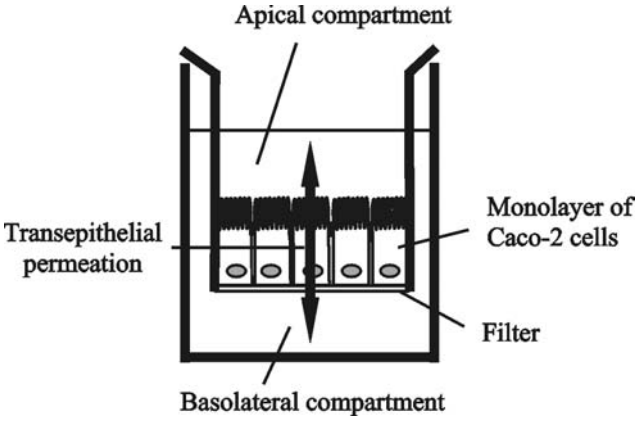

Fig. 1 The two-compartment cell culture system. The system consists of a permeable cell culture (filter) insert which is placed in a well of a cell culture plate. After reaching confluence the Caco-2 cell layer forms a barrier between the apical and basolateral compartment (0.5 and 1.8 mL, respectively)

Intestinal transport and cellular accumulation

The transport experiments with folate compounds were performed after culturing the cells for 18–22 days at 37 °C 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., manuscript in preparation] 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 the dark for 1 h at ca 20 °C 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/s) 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/s), where dQ/dt = permeability rate (mol/s), 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]; 1 mL of these test solutions was 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 intervals 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 three 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 (Fig. 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 to 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 to 0.5 ± 0.1 % of the dose.

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, Fig. 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/s) 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/s), the Ap > Bl transport of 5-CH_3-H_4folate ($1.4 \pm 0.2 * 10^{-6}$ cm/s) and the Bl > Ap transport of 5-CH_3-H_4folate ($1.4 \pm 0.1 * 10^{-6}$ cm/s). 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/s), which is a transport marker for a low absorption in humans. On the other hand, the transport of both folate compounds

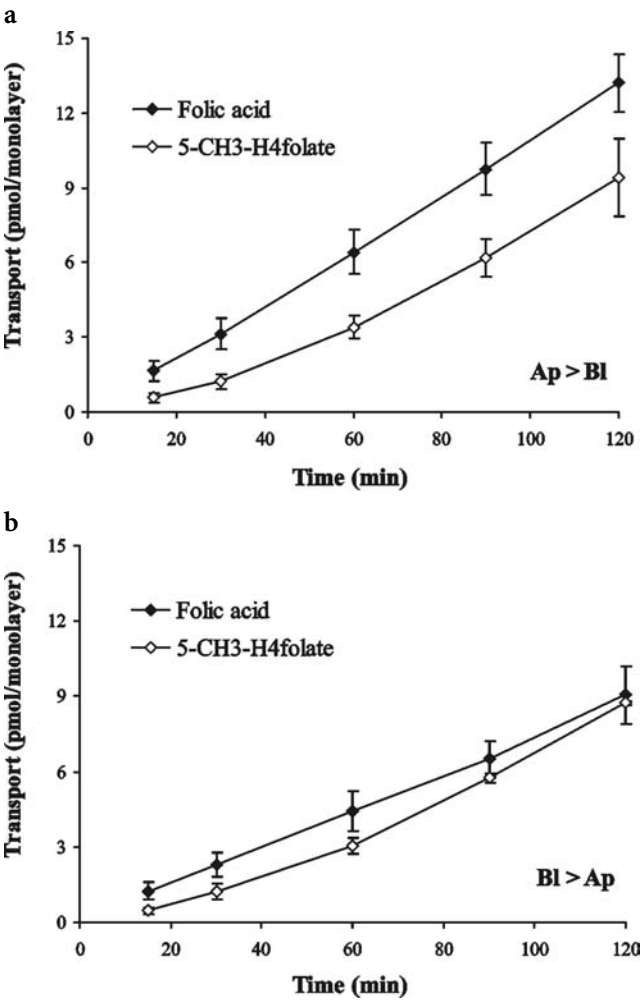

Fig. 2 Transport of folic acid and 5-CH_3-H_4folate from **a** the apical to the basolateral side (Ap > Bl) and **b** the basolateral to the apical side (Bl > Ap) of a monolayer of Caco-2 cells (1.13 cm^2). Samples were taken from the receiver compartment at 15, 30, 60, 90 and 120 min after the addition 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

was significantly ($P < 0.05$) lower than that of caffeine ($34 \pm 4 * 10^{-6}$ cm/s), 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 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 1 Transport and cellular accumulation of folic acid and 5-CH_3-H_4folate, given as % of dose, after 2 h apical incubation to folate/FBP test solutions (pH 7) containing 1 µM folic acid or 5-CH_3-H_4folate with 0 to 2 µM FBP[1, 2]

FBP (µM)	Transport (% of dose)		Cell. accumulation (% of dose)	
	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}	$0.4 \pm 0.1^{b,c}$	0.8 ± 0.2^{b}
0.75	$1.8 \pm 0.1^{b,c}$	1.0 ± 0.1^{c}	$0.3 \pm 0.1^{b,c}$	$0.5 \pm 0.1^{b,c}$
1	1.5 ± 0.1^{c}	0.5 ± 0.1^{d}	$0.3 \pm 0.1^{b,c}$	0.4 ± 0.2^{c}
1.5	1.5 ± 0.2^{c}	0.5 ± 0.1^{d}	$0.3 \pm 0^{b,c}$	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 a common letter differ significantly ($P < 0.05$)

The effect of FBP on intestinal transport and cellular accumulation of folate

In the presence of FBP, the transport (Ap > Bl) and cellular accumulation of folic acid and 5-CH_3-H_4folate was decreased (Table 1). This inhibitory effect of FBP appeared to be concentration-dependent and maximal at a FBP concentration of 1 µM. At higher FBP concentrations (1.5 and 2 µM), no significant ($P > 0.05$) further decrease was found in the transport and cellular accumulation of folic acid and 5-CH_3-H_4folate. The effect of FBP on the transport of folate was more pronounced for 5-CH_3-H_4folate than for folic acid, resulting in a higher Ap > Bl transport of folic acid under all test conditions. No difference in inhibitory effect of FBP was found between folic acid and 5-CH_3-H_4folate with respect to cellular accumulation. At FBP concentrations above 0.75 µM, the sum of transported and accumulated folic acid was significantly ($P < 0.05$) higher than that of 5-CH_3-H_4folate, while at lower FBP concentrations no difference was found.

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 were 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 (Fig. 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 (Fig. 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.

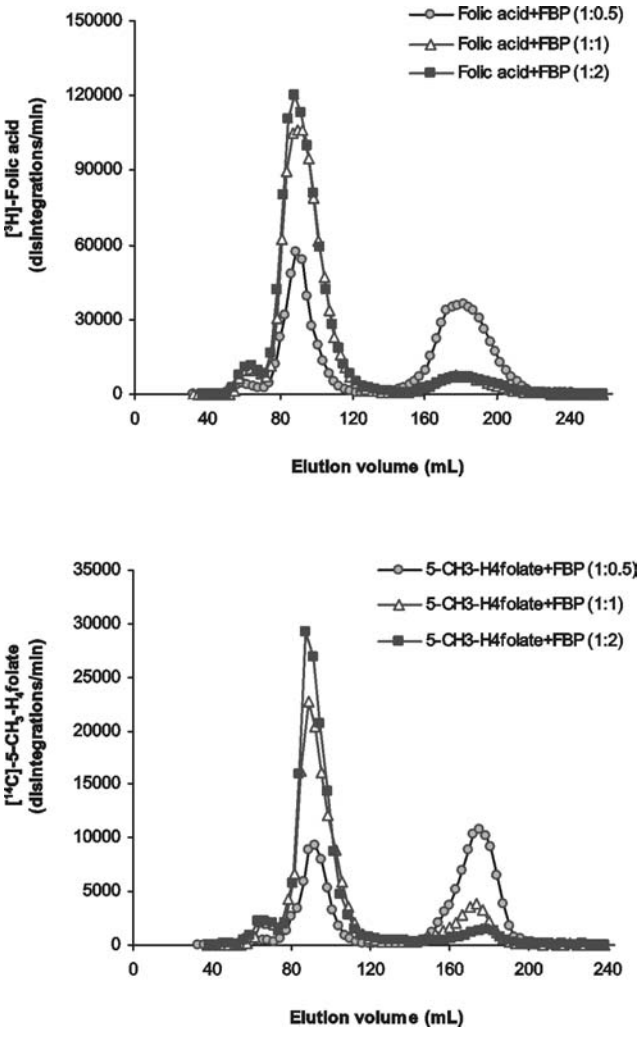

Fig. 3 Sephadex gel filtration chromatography of [^{3}H]-folic acid or [^{14}C]–5-CH_3-H_4folate in 1 µM folic acid or 5-CH_3-H_4folate test solutions with 0.5, 1 or 2 µM FBP. The folate content in the fractions is plotted against the elution volume

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 (Fig. 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 2 h 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 the 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 into the systemic distribution of folate and the overall effect of FBP on these processes.

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