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Carrier affinity as a mechanism for the pH-dependence of folate transport in the small intestine

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A mildly acidic pH in the lumen of the small intestine markedly enhances the transport of folate. This study investigated the relationship between pH and the affinity between folic acid and the apical membrane transporter using brush border membrane vesicles from rat jejunum and differentiated monolayer cultures of the colon carcinoma cell line, CaCo-2. Uptake studies with BBMV were conducted at folic acid concentrations of 0.1 to 50 μmol/l, conditions which were suitable for analyzing uptake data based on the Michaelis-Menten equation modified to include a nonsaturable component. These analyses yielded apparent K_m values of 0.6 and 12.3 μM at pH 5.5 and pH 7.4, respectively ($P < 0.05$). Values for V_{max} were lower at pH 5.5 than at pH 7.4 (0.8 vs. 1.6 pmol/mg protein per 10 s, $P < 0.05$). The studies with CaCo-2 cells employed folic acid concentrations of 0.1 to 5 μmol/l. Under these conditions the apparent K_m for folic uptake was lowest at pH 6.0, where the K_m was 0.7 μmol/l. The apparent K_m increased sharply as a neutral pH was approached; reaching a value of 13.9 μmol/l at pH 7.1. These data suggest that the prominent pH effect on intestinal folate transport is, in part, explained by an increased affinity of the folate substrate for its membrane transporter.

Introduction

The effect of pH on folate transport was first described by Smith et al. [1] in everted jejunal sacs and has since been reproduced in a number of intestinal models, including jejunal rings [2], intestinal sheets [3] and with in vivo perfusion [4]. The physiologic significance of this phenomenon is apparent since the pH of the luminal side of the brush border is between 5.8 and 6.0 [5,6] while the intracellular pH of the enterocyte has been measured to be approximately 6.8 [7]. Human studies have substantiated the clinical significance of the pH effect on folate transport [2,8,9].

Selhub and Rosenberg [10] have demonstrated that a marked pH effect on folate transport is demonstrable in rat jejunal brush border membrane vesicles, suggesting that the mechanism which underlies the pH effect might be explained by events that occur at the level of the brush border membrane. Other investigators have since demonstrated that the pH effect also exists in BBMVs from rabbit and human jejunum [11,12]. The pH optimum in BBMVs is approximately 5.0, which is slightly more acidic than that observed in intact intestinal preparations [10].

Vincent et al. [13] have recently shown that CaCo-2 cells, a colon carcinoma cell line which spontaneously differentiates in culture and displays both biochemical and morphologic characteristics of small intestinal enterocytes [14], has a folate transport system similar to that found in the intestine. When folic acid transport in these cells was determined within the pH range of 5.8 to 7.5, transport exhibited an inverse linear relationship to pH of the medium. At pH 6.3 uptake exhibited both saturable and the nonsaturable components with the nonsaturable component predominating at folate concentrations higher than 20 μM.

This study is concerned with investigating the relationship between pH and affinity of folic acid for the transporting system in jejunal brush border membrane vesicles and monolayer cultures of CaCo-2 cells. Studies by Russell et al. [2] have shown that, in everted jejunal rings, the apparent affinity for folic acid transport is higher at pH of 6.13 than at pH 7.8 ($K_m = 4.16$ μM vs. 6.05 μM, respectively). Subsequent studies by Zimmerman et al. [15], using an intestinal sheet preparation,

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have substantiated that this effect exists in preparations which utilize an intact intestinal wall. Since the folate transport system appears to be an integral part of the apical membrane, we reasoned that the increase in affinity between folate and its carrier that is observed at lower pH should be a phenomenon that is intrinsic to the brush border. The following study was designed to test this hypothesis.

Materials and Methods

Chemicals. [^{3}H]Folic acid (potassium salt, 20–50 Ci/mmol) was purchased from Amersham Corp. (Arlington Heights, IL). D-[^{3}H]Glucose (15.5 Ci/mmol) and L-[^{3}H]glucose (20 Ci/mmol) were obtained from New England Nuclear Products (Boston, MA). Non-labeled folic acid was obtained from Sigma Chemicals (St. Louis, MO). All chemicals were reagent grade or better.

Studies with brush border membrane vesicles (BBMVs). Vesicles were prepared in a buffered mannitol solution (300 mmol/l mannitol + 10 mmol/l Hepes (pH 7.4)) from nonfasting male Sprague-Dawley rats (180–280 g body weight) by the divalent cation precipitation method [16] as modified by Knickelbein et al. [17]. The final vesicle preparation had a protein concentration of approx. 10 mg/ml as determined by the method of Lowry et al. [18].

The vesicle preparation was enriched 13.2 ± 3.0 fold with respect to alkaline phosphatase activity [19] and 14.2 ± 4.8 fold with respect to sucrase activity [20] compared to the crude homogenate. The intravesicular volume at 300 mM mannitol, as determined by the L-glucose space [21], was 0.54 μl/mg protein. The vesicle preparation exhibited an initial sodium-dependent overshoot of D-glucose uptake which was 10.4 ± 0.2 fold higher than at equilibrium. The phenomenon of membrane binding, as opposed to intravesicular transport, comprised a very small proportion of the total folic acid uptake in these vesicles: studies with osmotic manipulations of the vesicles, as described by Schron et al. [11], confirmed that less than 20% of the observed uptake was due to membrane binding. Uptake studies were conducted as previously described [10]. The vesicle suspension in 0.3 M mannitol, 10 mM Hepes-Tris, pH 7.4 (20 μl, 0.1–0.25 mg protein) was added to a mixture (20 μl) containing [^{3}H]folic acid, 250 mM mannitol and 50 mM of either Mes-Tris or Hepes-Tris at pH 5.5 and 7.4, respectively [10]. The mixture was incubated at room temperature for the time indicated. The incubation was terminated by addition of a 4°C stop solution (100 mmol/l mannitol, 100 mmol/l NaCl and 10 mmol/l Hepes (pH 7.4)) and then immediately filtered through a 0.45 μm nitrocellulose filter under vacuum. The filter was then washed with 4 ml of the stop solution, dried in an oven at 100°C for 10 min, placed in 10 ml of toluene-based scintillant fluid and counted on a scintillation spectrophotometer (Tricarb 2000CA, Packard Instr. Co., Sterling, VA) as previously described [10]. Blank incubations were performed by adding vesicles and stop solution at the same time and then immediately proceeding to vacuum filtration. All experiments were performed in either triplicate or quadruplicate.

Studies with CaCo-2 cells. CaCo-2 cells were grown at 37°C in a 5% CO_2/95% air atmosphere in 75 cm plastic flasks containing DMEM supplemented with 20% fetal calf serum and a mixture of penicillin (10000 U/ml) and streptomycin (10000 μg/ml) [14]. The cells were routinely split according to the following protocol: each flask of cells was rinsed with calcium and magnesium-free Hanks' balanced salt solution (Gibco) and split 1 : 5 with 0.25% Trypsin and 0.2 g/l EDTA. Cells were seeded into plastic wells (35 mm in diameter) and allowed to grow until they reached confluency (about 6 days). Thereafter the media was changed daily until the 16th day, at which time the monolayer cultures were well differentiated [13,14] and were used for uptake studies.

For uptake studies the growth medium was removed and the monolayer cell culture was washed twice with a pH 6.3 phosphate-buffered saline solution [13]. The wells were then filled with a 1 ml solution containing [^{3}H]folic acid (0.1–5 μM), [^{14}C]inulin (which served as a nonabsorbable marker), 0.15 M NaCl, 0.025 M sodium phosphate buffer of the indicated pH and 11 mM D-glucose. The cells were incubated at 37°C for the time indicated. The incubation medium was removed and the cells were washed five times with 2 ml pH 7.4 phosphate-buffered saline containing 0.2 mM EGTA. The cells were then solubilized with 2 ml 1% Triton X-100. Aliquots of 0.5 ml were mixed with Ready-Solve (Beckman Instrument Inc., Fullerton, CA) and counted in the abovementioned scintillation counter which was programmed for simultaneous counting of ^{3}H and ^{14}C. Experiments were conducted in triplicate or quadruplicate and data were expressed as pmol of folate uptake per mg protein. Protein was determined as described by Lowry et al. [18].

Statistical analysis. Data in the figures and text are expressed as mean ± S.E. 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 was less than 0.05. Kinetic modelling was performed by a weighted, non-linear least-squares method on an RS1 statistical program (BBN Software Products Corp.).

Results

Kinetics of folic acid uptake in brush border membrane vesicles

Experiments were performed to examine the uptake

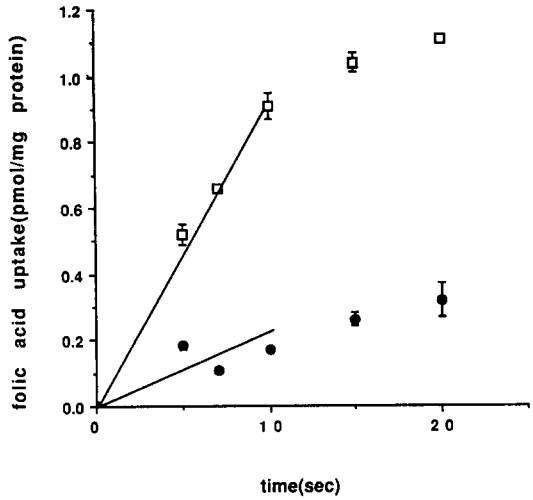

Fig. 1. The time-dependent uptake of folic acid in brush-border membrane vesicles (BBMV) at pH 5.5 and 7.4. Data were obtained by incubating 20 μl of BBMV (Approx. 0.2 mg protein) in 20 μl of incubation solution containing 250 mmol/l mannitol, 1.0 μmol/l [^{3}H]folic acid, and either 50 mmol/l Tris-Hepes, pH 7.4 (●) or 50 mmol/l Tris-Mes, pH 5.5 (□). Means and standard error bars represent the data from at least three incubations. Error bars are absent in those instances where the size of the data symbol exceeds the standard error.

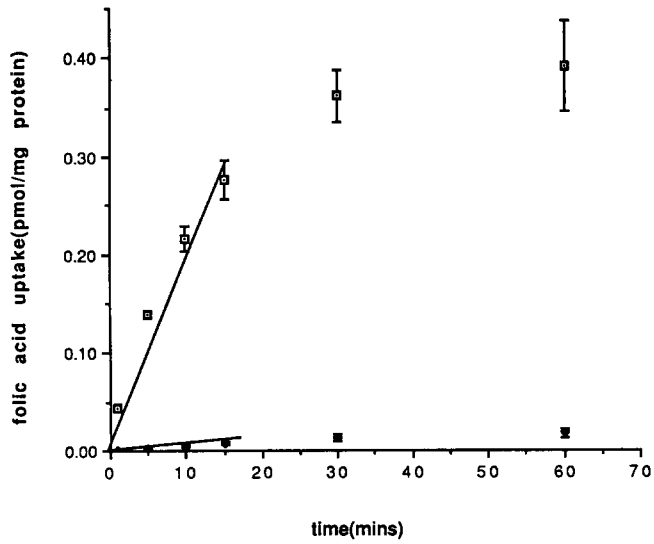

Fig. 3. Time dependence of folic acid uptake by CaCo-2 cells at pH 5.5 (□) and 7.4 (●). The monolayer cultures were in incubated at 37 °C with 0.01 μmol/l [^{3}H]folic acid in phosphate buffered saline for the time indicated. Data in the figure represents the means and standard errors of at least three incubations. Error bars are absent in those instances where the size of the data symbol exceeds the standard error.

of folate at very short time points (Fig. 1). These experiments revealed than uptake of folic acid was linear over the first 10 s when the extravesicular pH was either 5.5 ($r = 0.98$, $P < 0.01$) or 7.4 ($r = 0.79$, $P < 0.01$).

Fig. 2 shows the concentration-dependent uptake of folic acid over 10 s by the membrane vesicles at pH 5.5

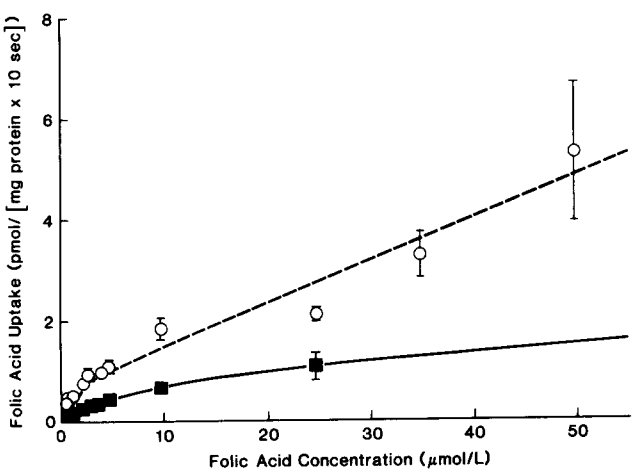

Fig. 2. The concentration dependence of folic acid uptake in brush-border membrane vesicles (BBMV) at pH 5.5 and 7.4. Data were obtained by incubating 20 μl of BBMV (approx. 0.2 mg protein) for 10 s with [^{3}H]folic acid at the concentrations indicated in 250 mmol/l mannitol and either 50 mmol/l Tris-Mes, pH 5.5 buffer (— — —) or 50 mmol/l Tris-Hepes buffer pH 7.4 (———). Means and standard error bars represent the data from at least three incubations. Error bars are absent in those instances where the size of the data symbol exceeds the standard error. The lines in the figure are the least-squares fits of the data using the modified Michaelis-Menten equation as previously described [10].

and 7.4. These data are consistent with transport that proceeds by both saturable and nonsaturable processes: a model which has previously been shown to appropriately describe the uptake of folic acid in BBMV [10]. The curvilinear functions seen in Fig. 2 are the graphic representations of this kinetic modelling. The numerical values obtained for K_m at pH 5.5 and 7.4 were 0.62 ± 0.37 and 12.31 ± 2.23 μmol/l, respectively ($P < 0.05$). The values obtained for V_{max} at these two pH values were 0.80 ± 0.18 and 1.62 ± 0.21 pmol/mg protein per 10 s, respectively ($P < 0.05$). K_m increases by approximately 20-fold at pH 7.4 compared to the value obtained at an extravesicular pH of pH 5.5. The magnitude of change in V_{max} is considerably less: at 7.4 V_{max} is approximately double what it is at 5.5.

Studies with CaCo-2 cells

Fig. 3 shows the time-dependent uptake of folic acid by CaCo-2 cell monolayer culture. Uptake was linear for up to 15 min at both pH 5.5 ($r = 0.98$, $P < 0.01$) and pH 7.4 ($r = 0.94$, $P < 0.01$). The marked effect of pH on folate transport in CaCo-2 cells in this experiments is similar to previous observations [13].

The data presented in Fig. 4 were obtained from studies which determined folic acid uptake by CaCo-2 cells after an incubation time of 5 min and at substrate concentrations that did not exceed 5 μM. These conditions were selected specifically to minimize the contribution of the nonsaturable component of transport to a negligible level so that the observed uptake was essentially all due to saturable transport. According to

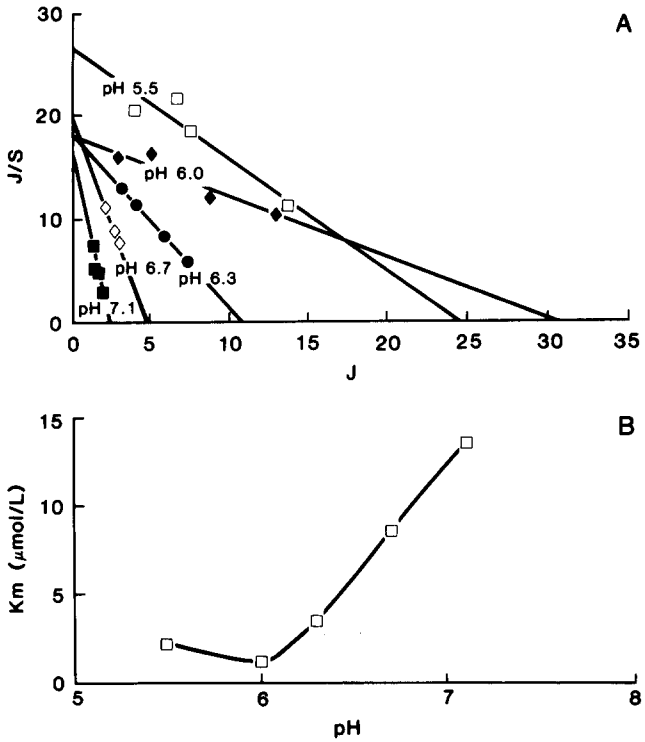

Fig. 4. The concentration of folic acid uptake by CaCo-2 cells at various pH. (A) Eadie-Hofstee plot; (B) plot of the relationship between pH and K_m for folic acid determined from the Eadie-Hofstee plot. Each data point represents the average of triplicate experiments.

Vincent et al. [13] a nonsaturable component becomes predominant only at folic acid concentrations greater than 10 μM. This was substantiated by kinetic modelling which was performed in the same manner as described above and which showed no significant nonsaturable component. The maximum rate of folate uptake occurred at pH 6.0 which is very similar to the acidic pH optimum observed by Vincent et al. [13]. The Eadie-Hofstee plot (Fig. 4A) and the calculated affinity constants obtained from this plot (Fig. 4B) demonstrate that K_m is lowest at pH 6.0, where its value is 0.7 μmol/l, and that the K_m increases sharply with an increase in pH, reaching a value of 13.9 μmol/l at pH 7.1. V_{max} values did not change substantially with pH and what changes were present were not in a consistent pattern.

Discussion

One of the most characteristic features of carrier-mediated folate absorption in the intestine is the marked enhancement that occurs in the presence of a mildly acidic environment. Two mechanisms have been proposed to explain this pH effect on intestinal folate absorption: (a) the transmembrane pH gradient serving as a chemical driving force [11,12] and (b) changes in the affinity between folate and its membrane carrier [2]. The first explanation is supported by recent data demonstrating an enhancement of transport in the presence of a transmembrane gradient but not in the presence of equally acidic environments on both sides of the membrane [11,12]. The data presented in this paper provide strong evidence that the second mechanism plays a significant role as in the pH effect on intestinal folate transport. Analyzing the data from both the BBMV and CaCo-2 cells reveals that a very substantial consequence of changing the incubation pH is the change that occurs in the apparent affinity of folate for its 'carrier'. In BBMV this change is manifested by a 20-fold increase in K_m when the pH is increased by 2 units from pH 5.5 to pH 7.4. The data with CaCo-2 cells corroborate the data obtained with the BBMV. The CaCo-2 data also identify that pH range where the affinity between folic acid and its 'carrier' attains its maximum value. In fact the relationship between affinity (which is the inverse of K_m) and pH closely correlates with the known relationship between intestinal folate transport and luminal pH [1–4,8–10]. In both cases the maximum activity is around pH 6.0: further increases in pH are associated with marked decreases in affinity and transport. The fact that the change in K_m occurs in a graduated fashion, as is shown in Fig. 3B, suggests that the mechanism that underlies the increase in affinity proceeds in a cumulative fashion rather than by one which proceeds in a one-step process. This is consistent with the relationship that is observed between luminal pH and folate transport.

Incubation pH also affects the apparent V_{max} values with the BBMV. However, these changes in V_{max} are relatively small and, since the apparent V_{max} increases at higher pH, pH-induced alterations in V_{max} cannot explain the enhancement of transport at an acidic pH.

Our data demonstrate that an increased affinity between folic acid and its membrane carrier contributes to the enhancing effect of an acidic environment on folate transport in the intestine. Further studies will be necessary to determine the relative contribution to transport made by this mechanism compared to that mechanism which relies on the driving force provided by the transmembrane pH gradient.

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