Folate Transport in Isolated Brush Border Membrane Vesicles from Rat Intestine*

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The transport of folates by the intact intestine consists of a saturable component with a luminal pH optimum of 6.0 and a nonsaturable component which predominates at high folate concentrations and/or high luminal pH. We sought to determine whether these properties reflect the behavior of the brush border membrane, free of intracellular enzyme activities, organelles, and intercellular junctions. We have studied, therefore the transport characteristics of folate, pteroylglutamic acid (PteGlu) and methotrexate in isolated brush border membrane vesicles from rat intestine. Both PteGlu and methotrexate were found to be taken up by these vesicles by a pH-dependent process with a maximum uptake at a medium pH near 5.0. Studies at pH 5.5 demonstrated both saturable and nonsaturable components for the uptake of methotrexate and PteGlu. For methotrexate, $K_m$ corrected for the nonsaturable component, was 1.5 µM and $V_{max}$ was 3.08 pmol/mg of protein/0.5 min. For PteGlu, the $K_m$ was 0.42 µM and $V_{max}$ was 0.67 pmol/mg of protein/0.5 min. Methotrexate uptake was competitively inhibited by PteGlu ($K_i = 0.6$ µM) and by 5-methyltetrahydropteroylglutamic acid (5-methyl-H$_2$PteGlu; $K_i = 1.35$ µM). Brush border membrane vesicles exhibit folate transstimulation: those preloaded with PteGlu, 5-methyl-H$_2$PteGlu or unlabeled methotrexate took up tritium labeled methotrexate at more rapid rates than did control vesicles. The data presented are consistent with a pH-dependent, structure-specific carrier or channel for folate transport across the luminal membrane of the intestinal cell shared by the three folate derivatives tested.

The absorption of folate by the intestine is a multistep process which includes the crossing of the luminal membrane, a temporary retention and/or metabolism within the enterocyte, and crossing of the basolateral membrane. Recent studies with pteroylglutamic acid and methotrexate have shown that this transport is dependent on luminal pH with a sharp peak at around 6.0 (1–5). At this pH, folate absorption is saturable and is competitively inhibited by other folate derivatives (1, 3–5). Such behavior could reflect, in part, events within the enterocyte or those at the basolateral pole of the intestinal cell in addition to events at the luminal border.

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The present study was undertaken to focus on the role of the luminal membrane in the observed characteristics of folate absorption by the intestine. For this purpose we have isolated brush border membrane vesicles from rat intestine by a modified procedure of Schmitz et al. (6) and studied their transport characteristics with respect to PteGlu and methotrexate.

MATERIALS AND METHODS

[G-3H]Methotrexate (20 Ci/mmol), [G-3H]PteGlu (26–40 Ci/mmol), [2-14C]PteGlu (58 µCi/µmol), and [3H]5-methyl-H$_2$PteGlu (65.5 µCi/µmol) were purchased from the Amersham/Searle Corp. (Arlington Heights, IL). These compounds were 97–98% pure as determined by paper chromatography. HEPES, MES, and raffinose were obtained from Sigma. Unlabeled methotrexate was from the Lederle Laboratory Division (Pearl River, NY).

Preparation of Brush Border Membrane Vesicles from Rat Intestine—Unfasted male Sprague Dawley rats (200–300 g) were killed by decapitation. The small intestine was removed through an abdominal incision and flushed with cold 0.9% NaCl solution. Thereafter, the mucosa was scraped and brush border membrane vesicles were prepared promptly by a procedure modified from Schmitz et al. (6). In this procedure the mucosal scrapings were homogenized for 30 s at 0 °C in 15 volumes (w/v) of 0.05 M potassium/HEPES, pH 7.4 (4 ml/g of original scrapings). This homogenate was centrifuged (40,000 X g) and the resulting pellet fraction was resuspended in 2 ml of the mannitol potassium/HEPES solution. This latter preparation contained nearly pure brush border membrane vesicles as detected by electron microscopy. On the basis of the brush border enzyme maltase (7) this preparation contained a 27-fold purification relative to the original homogenate. This preparation was then centrifuged (40,000 X g, 15 min) and the pellet fraction was resuspended in 2 ml of the mannitol potassium/HEPES solution. Protein was determined by the Lowry et al. procedure (9).

Uptake Studies—Experiments were run in triplicate at room temperature in Eppendorf conical test tubes. Each incubation contained the vesicles (0.1–0.2 mg of protein) in 0.04 ml final volume with 1 µM [3H]methotrexate or [3H]PteGlu, 50 mM NaCl, 1 mM MgSO4, and a solution containing 50 mM concentrations of MES and HEPES adjusted with KOH to the desired pH. Incubation was started by the addition of the vesicles to a solution containing the other ingredients and stopped by dilution with 1 ml of cold solution containing 150 mM

The abbreviations used are: PteGlu, pteroylglutamic acid; 5-methyl-H$_2$PteGlu, 5-methyltetrahydropteroylglutamic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 2-(N-morpholino)ethanesulfonic acid.
NaCl and 20 mM potassium/HEPES at pH 7.4 followed by rapid filtration through a cellulose nitrate filter (0.45 μm, Schleicher and Schuell, Dassel, Germany). This procedure was repeated to ensure quantitative transfer of the incubation mixture onto the filter. Then the filter with the retained vesicles was washed with 3 ml of the buffered NaCl solution and dried in an 85 °C oven for 30 min. Control incubations were performed by preincubation of the vesicle preparation with 0.25 μmol of unlabeled methotrexate or PteGlu in 1 ml of the buffered NaCl solution. Radioactive methotrexate or PteGlu were then added and the mixture was filtered followed by the same washes as described above.

Radioactivity on the filters was determined as described earlier (10). In some preparations the radioactivity on the filter was removed with acetic acid (11) and analyzed by paper chromatography. No activity other than the test substance was detected.

Raffinose, when used, was added to the vesicles 30 min before the start of the incubation.

**RESULTS**

Effect of pH on the Uptake of Methotrexate and PteGlu by Brush Border Membranes—The studies described in Fig. 1 show that the brush border membrane vesicles from rat intestine take up methotrexate and PteGlu in a pH-dependent fashion. Uptake of methotrexate increases with increasing pH to a maximum at around 5.0, and decreases sharply thereafter (Fig. 1a).

The pH dependence of PteGlu uptake (Fig. 1b) is similar to that of methotrexate. Uptake is maximal at pH 4.9-5.0 and decreases with increasing pH. A difference is the small shoulder around pH 6.5 which was not observed with methotrexate. Subsequent studies were conducted at pH 5.5.

**Fig. 1.** pH dependency curves of methotrexate (MTX) and PteGlu uptake by brush border membrane vesicles. Membrane vesicles (0.2 mg of protein) were incubated in 50 mM NaCl, 1 mM MgSO4, and 100 mM MES/HEPES, adjusted with HCl or KOH to the desired pH, for 1 min with (a) 1 μM [3H]methotrexate or (b) 1 μM [3H]PteGlu before filtration through the cellulose-nitrate filters and washing of the filters as described under "Materials and Methods." Values expressed in picomoles/mg of protein are the average ± S.D. of three determinations.

In the studies presented in Fig. 2, methotrexate and PteGlu uptake by the membrane vesicles was determined at various medium osmolarity produced by the addition of the membrane-impermeant raffinose (12). The data show an inverse linear relationship between uptake and osmolarity. Uptake extrapolates to zero at infinite osmolarity, consistent with intravesicular transport rather than binding of methotrexate and PteGlu.

**Methotrexate and PteGlu Uptake in Relation to Incubation Time and Substrate Concentration**—The time course of methotrexate uptake by the membrane vesicle preparation at 0.2, 1, and 5 mM is shown in Fig. 3a. Uptake is initially rapid and then decreases to a lower velocity beyond 1 min of incubation.

At all time points, higher methotrexate concentration results in greater uptake; however, this increase in uptake with increasing concentration is nonlinear. Higher concentrations are associated with lower relative uptake.

Time dependency studies of PteGlu uptake at three different medium concentrations yielded curves similar to those of methotrexate (Fig. 3b). Uptake is also nonlinear with respect to concentration.

The relationship between uptake and substrate concentration under conditions which approximate initial velocity, i.e., after 0.5 min of incubation, is shown in Fig. 4. The uptake of either methotrexate or PteGlu exhibits a saturable component at low concentrations and a nonsaturable component which becomes noticeable at high concentrations. An appropriate expression of this uptake system uses the Michaelis-Menten equation altered to correct for the nonsaturable component (13). This expression, which also fits the concentration-dependent uptake of PteGlu by everted rings from rat intestine (14) is given below:

\[ v = \frac{V_{max} S_o}{K_v + S_o} \]

**Fig. 2.** Effect of increasing osmolarity using raffinose on the uptake of methotrexate (MTX) and PteGlu. Brush border membrane vesicles were preincubated at room temperature for 15 min with increasing concentrations of raffinose. Aliquots (0.2 mg of protein) were added to a salt mixture (pH 5.5) containing 1 mM [3H]methotrexate or 1 μM [3H]PteGlu after a 1-min incubation for the amount of radioactivity taken up. Uptake values ± S.D. were plotted against the reciprocal of total osmolarity.
were incubated for 0.5 min at pH 5.5 with increasing concentrations of substrate, where component, of follows. For methotrexate, linear regression system. The constants obtained were as K1 = K, \( \text{min/pmol} \); the correlation coefficient was 0.991. For PteGlu, \( \text{mg of protein/0.5 min} \), and \( \text{coefficient was 0.993.} \)

Preloading the vesicles with unlabeled PteGlu, 5-methyl-H4PteGlu, or methotrexate prior to addition of the test \([3H]\)methotrexate causes stimulation of the initial rate of uptake of the label (Fig. 6). When vesicles are preloaded with PteGlu, \([3H]\)methotrexate uptake at 0.5 and 1 min is increased by 36 and 26%, respectively, compared to controls. After preloading with 5-methyl-H4PteGlu, uptake at 0.5 and 1 min is increased by 80 and 54%, respectively. Preloading with unlabeled methotrexate caused a 35% greater uptake in 0.5 min of incubation. Eventually, after 7.5 min of incubation to permit requilibration, the effect of preloading was lost, i.e. uptake of radioactivity with preloaded and unloaded membrane vesicles was the same.

**FIG. 4.** Concentration dependency curves of methotrexate (MTX) and PteGlu uptake after 0.5 min of incubation. Results were obtained from membrane vesicles (0.18 mg of protein) which were incubated for 0.5 min at pH 5.5 with increasing concentrations of methotrexate or PteGlu.

\[
u = V_{\text{max}} + K_1 K_m + K_1 S_0 - \frac{K_m}{S_0}
\]  

(2)

where \( \nu \) denotes uptake, \( S_0 \) is the initial concentration of substrate, \( V_{\text{max}} \) is the maximum uptake for the saturable component, \( K_m \) is the dissociation constant for the saturable component, and \( K_1 \) is the nonsaturable constant. The values of \( \nu, S_0, \) and \( \nu/S_0 \) from Fig. 4 were analyzed by a trivariate linear regression system. The constants obtained were as follows. For methotrexate, \( K_m = 1.54 \mu M, V_{\text{max}} = 3.08 \text{ pmol/mg of protein/0.5 min}, \) and \( K_1 = 0.415 \text{ pmol/mg of protein/0.5 min} \); the correlation coefficient was 0.991. For PteGlu, \( K_m = 0.42 \mu M, V_{\text{max}} = 0.67 \text{ pmol/mg of protein/0.5 min}, \) and \( K_1 = 0.154 \text{ pmol/mg of protein/0.5 min} \); the correlation coefficient was 0.993.

Effect of PteGlu and 5-Methyl-H4PteGlu on Methotrexate Uptake—When added simultaneously with \([3H]\)methotrexate, unlabeled PteGlu or 5-methyl-H4PteGlu inhibits the uptake of the labeled compound. The Dixon plots show a competitive mode of inhibition with apparent \( K \), values of 0.6 \( \mu M \) for PteGlu (Fig. 5a) and 1.35 \( \mu M \) for 5-methyl-H4PteGlu (Fig. 5b).

It is important to note, therefore, that all of these characteristics are retained in the isolated brush border membrane vesicles and may then be attributed to events at or within the membrane itself. Characteristics of folate transport across the intestine include: 1) a prominent \( \text{pH dependence; 2) saturable uptake at low folate concentrations with a linear relationship between transport and concentration at higher initial concentrations; and 3) competition among various folate derivatives.} \)

**FIG. 3.** Time and concentration dependencies of methotrexate (MTX) or PteGlu uptake at pH 5.5. Membrane vesicles (1.1 mg of protein) were incubated at pH 5.5 with (a) \([3H]\)-methotrexate or (b) \([3H]PteGlu at the concentrations indicated. At time intervals, aliquots of the mixture were withdrawn and analyzed for uptake.

**DISCUSSION**

The capacity to study transport of folates across the isolated brush border membranes of intestine provides a means of determining which of the characteristics of folate transport in intact intestine are attributable to events at or within the membrane itself. Characteristics of folate transport across the intestine include: 1) a prominent \( \text{pH dependence; 2) saturable uptake at low folate concentrations with a linear relationship between transport and concentration at higher initial concentrations; and 3) competition among various folate derivatives.} \)

Some or all of these characteristics could be influenced by metabolic events within the enterocyte or even at the basolateral pole of the intestinal cell. For example, two components for uptake at high versus low substrate concentrations could theoretically reflect saturation of intracellular enzymes of folate metabolism (15, 16) occurring at low concentrations. The nonsaturable component of transport which predominates at high concentrations could represent paracellular rather than transcellular movement of folates. If this explanation for the linear relationship between substrate concentration and transport were correct, then membrane vesicles which lack intercellular junctions should not exhibit such a linear relationship. Further, if the saturable component reflects association with enzymes of folate metabolism, we would see no saturable uptake by vesicles since these vesicles are devoid of intracellular cytoplasm and organelles.

It is important to note, therefore, that all of these characteristics are retained in the isolated brush border membrane vesicles and may then be attributed to events at or within the behavior of the luminal membrane. Table I summarizes the characteristics of folate transport by the vesicles and intact intestine preparations. Except for small differences, which will be dealt with below, the two systems have the same characteristics.

That uptake by the vesicles, at pH 5.5 employed in this study, represents intravesicular transport, and not just binding, is supported by the results of the experiments which used raffinose (Fig. 2). Raffinose is a nonpermeant sugar and its inhibitory effect on the uptake of small solute molecules is...
attributed to its capacity to shrink intravesicular spaces with increasing osmolarity of the bathing medium (12). A zero or near zero uptake level at infinite osmolarity as extrapolated from the present study (Fig. 2) is consistent with the total exclusion of the two folate derivatives when the vesicular space tends to zero. Binding to the membrane surface, which could be confused for uptake, is negligible under these conditions, even though binding to membrane proteins may be significant at higher pH particularly in the case of PteGlu (17). The shoulder on the PteGlu pH dependence uptake curve (Fig. 1) could represent this binding activity.

The affinity and inhibition constants reported with the vesicle preparations are lower than those reported using the intact intestine (Table I). Such differences are present only when corrections are made for the nonsaturable components
presence in the luminal membrane of a structure specific mechanism for folate transport. The stimulation of the rate of methotrexate transport observed with membrane vesicles pre-loaded with these folate derivatives is consistent with a structure-specific mechanism of exchange between intravesicular unlabeled folates and external methotrexate (21). A similar mechanism with different properties has been reported to be responsible for folate transport by plasma membrane vesicles from L1210 murine leukemia cells (27).

REFERENCES