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A carrier-mediated transport for folate in basolateral membrane vesicles of rat small intestine

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The mechanism of exit of folate from the enterocyte, i.e. transport across the basolateral membrane, is not known. In this study we examined, using basolateral membrane vesicles, the transport of folic acid across the basolateral membrane of rat intestine. Uptake of folic acid by these vesicles represents transport of the substrate into the intravesicular compartment and not binding to the membrane surface. The rate of folic acid transport was linear for the first 1 min of incubation but decreased thereafter, reaching equilibrium after 5 min of incubation. The transport of folic acid was: (1) saturable as a function of concentration with an apparent K_m of 0.6 ± 0.17 μM and $V_{max.}$ of 1.01 ± 0.11 pmol/30 s per mg of protein; (2) inhibited in a competitive manner by the structural analogues 5-methyltetrahydrofolate and methotrexate ($K_i = 2$ and 1.4 μM, respectively); (4) electroneutral; (5) Na^+-independent; (6) sensitive to the effect of the anion exchange inhibitor 4,4'-di-isothiocyanatostilbene-2,2'-disulphonic acid (DIDS). These data indicate the existence of a carrier-mediated transport system for folic acid in rat intestinal basolateral membrane and demonstrate that the transport process is electroneutral, Na^+-independent and sensitive to the effect of anion exchange inhibition.

INTRODUCTION

Folate is an important vitamin that is required for normal cellular growth and function. Folate deficiency, probably the most common vitamin deficiency throughout the world [1,2], often occurs as a complication of gastrointestinal diseases and therapy with certain drugs [1,2]. Previous studies *in vitro* and *in vivo* from our laboratory and elsewhere on the intestinal transport of physiological concentrations of folate (< 10 μM) in intact tissue preparations (everted sacs, everted rings and perfused segments) have shown that the transport process is active, carrier-mediated and pH- and Na^+-dependent [3–8]. Various folate compounds including folic acid (PteGlu), 5-methyltetrahydrofolate and the folate analogue methotrexate share the same transport system but have different affinities [4–6,8]. Transmural transport of folate, however, represents the movement of the vitamin across two different membranes: the brush border membrane and the basolateral membrane. Studies of the individual transport events are therefore important to better understand the absorption process of this vitamin under normal physiological conditions and to delineate the defect in folate absorption that occurs in certain diseases and in therapy with certain drugs. Recent studies by us and others [9–12] on transport of PteGlu across the brush border membrane using brush border membrane vesicles of human, rat and rabbit intestines have characterized the entry process of the substrate into the enterocyte. These studies have shown the involvement of a carrier-mediated system in the transport of PteGlu across the intestinal brush border membrane and showed that this system is pH-, but not Na^+-, dependent. No study, however, is available describing the transport process of folate across the basolateral membrane, i.e. the exit process of folate from the enterocyte. In this study we examined PteGlu transport across the basolateral membrane by using the basolateral membrane vesicle (BLMV) technique.

MATERIALS AND METHODS

Materials

[3',5',7,9-^{3}H]PteGlu potassium salt (20 Ci/mmol), $^{45}CaCl_2$ (15 mCi/mg) and ACS scintillation cocktail were purchased from Amersham International; D-[1-^{3}H(N)]glucose (20 Ci/mmol) was from New England Nuclear; unlabelled PteGlu, 5-methyltetrahydrofolate, methotrexate and DIDS were from Sigma; cellulose nitrate filters (0.65 μm pore size) were from Sartorius. All other chemicals and reagents were of analytical quality. [^{3}H]PteGlu was purified before use on cellulose t.l.c. plates (Eastman Kodak) with 0.1 M-phosphate buffer (pH 7) containing 5% mercaptoethanol as a solvent system. The radiochemical purity of [^{3}H]PteGlu used in this study was 96%. A similar chromatography procedure was used to determine the authenticity of the transported PteGlu.

Methods

BLMV were prepared by the self-generating Percoll gradient method of Scalera *et al.* [13] with some modifications. This method to prepare BLMV was chosen because it is relatively simple and rapid, and has been shown by many workers to produce purified BLMV

Abbreviations used: PteGlu, folic acid; DIDS, 4,4'-di-isothiocyanatostilbene-2,2'-disulphonic acid; BLMV, basolateral membrane vesicles.

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suitable for transport studies [14–18]. Rats were killed and their jejunum was removed, washed with saline solution, then filled with warm (37 °C) buffer I (1.5 mM-KCl, 96 mM-NaCl, 8 mM-KH_2PO_4, 5.6 mM-Na_2HPO_4, 27 mM-trisodium citrate and 2 mM-dithiothreitol, pH 7.4). The segments were clamped and incubated in buffer I (without dithiothreitol) for 15 min in a shaking water bath (80 oscillations/min) at 37 °C. The clamps were then removed and the contents were emptied. The segments were filled with ice-cold buffer II (250 mM-mannitol and 24 mM-Hepes/Tris, pH 7.4) and gently palpated by hand for 5 min to release the epithelial cells. The contents were then drained into an iced beaker and the cells were collected by centrifugation for 5 min at 200 *g*. The cells were resuspended in 250 ml of buffer II to which 0.15 mM-phenylmethanesulphonyl fluoride had been added, collected by centrifugation at 200 *g* for 5 min and this process was repeated one more time. The cell pellet was homogenized in 160 ml of buffer II containing 0.15 mM-phenylmethanesulphonyl fluoride in a Waring blender-type homogenizer for 3 min. The homogenate was made up to 300 ml with buffer II and centrifuged at 22000 *g* for 25 min. The supernatant was discarded and the resulting whitish fluffy layer (which appears on top of the solid yellow pellet that is adherent to the centrifuge tube) was removed, resuspended in 90 ml of buffer II and homogenized in a glass/Teflon homogenizer (20 strokes). The resultant homogenate, which now represents 'crude plasma membranes', was then mixed with Percoll (Pharmacia Fine Chemicals) at a concentration of 15% (w/w) and then centrifuged at 48000 *g* for 75 min. A distinct band of basolateral membranes was seen at the upper one-third of the Percoll gradient, which upon fractionating the Percoll gradient proved to contain the highest activity of the basolateral marker enzyme, K^+-stimulated phosphatase, and the lowest activity of the brush border marker enzyme, maltase. This band was used to prepare BLMV and was removed with a syringe and recentrifuged at 48000 *g* for 60 min. BLMV were seen as a distinct whitish layer on the top of the glassy Percoll pellet (which is adherent to the centrifuge tube). The vesicles were removed, resuspended in 20 ml of the appropriate transport buffer (280 mM-mannitol/20 mM-Hepes/Tris, pH 7.4; changes in this buffer are mentioned in Figure legends) and centrifuged at 48000 *g* for 20 min. This washing step was then repeated twice. Following the washing procedure, the BLMV were suspended in the transport buffer and preincubated for 1 h at room temperature to load the inside of the vesicles. Transport studies were performed by a rapid filtration technique [19]. All incubations were done at 37 °C. Reaction was initiated by adding a 20 μl portion of membrane vesicle suspension to 80 μl of incubation buffer (final concentrations; 80 mM-mannitol, 100 mM-NaCl, 20 mM-Hepes/Tris, pH 7.4; changes in this buffer are mentioned in Figure legends) containing varying amounts of radiolabelled and unlabelled substrate plus other constituents. After incubation for the desired period of time, the reaction was terminated by the addition of 1 ml of ice-cold stop solution (200 mM-mannitol, 100 mM-NaCl, 5 mM-KH_2PO_4, 20 mM-Hepes/Tris, pH 7.4). The cold, diluted reaction mixture was immediately pipetted onto a prewetted filter and kept under suction. The filter was rinsed with 5 ml of ice-cold stop solution and then dissolved in 6 ml of ACS scintillation cocktail. Radioactivity was counted in a scintillation counter (model LS 3801; Beckman Instruments). Nonspecific binding of the substrate to the filter (background) was determined by filtering a reaction mixture that contains an identical solution but no vesicles and was subtracted from the transport data. Although absolute PteGlu and glucose uptake varied from one preparation to another, comparable results were obtained with different preparations. Therefore, the results of representative experiments are presented. Transport results are expressed as mean ± S.E.M. (pmol/mg of protein) of at least four observations. Protein concentration was measured by the method of Lowry *et al.* [20] with bovine serum albumin as a standard.

Table 1. Specific activities of marker enzymes in BLMV and mucosal homogenate

Data are expressed as means ± S.E.M. for the numbers of experiments in parentheses.

Enzyme	Activity (μmol/min per mg of protein) in: Homogenate	BLMV
K^+-stimulated phosphatase	3.45 ± 0.25 (4)	37.92 ± 4.6 (5)
Lactase	7.22 ± 0.47 (5)	4.45 ± 0.8 (5)
Sucrase	24.95 ± 1.64 (5)	15.27 ± 3.5 (5)
Maltase	6.51 ± 1.1 (5)	3.37 ± 0.9 (5)
Cytochrome *c* oxidase	8.46 ± 1.6 (4)	4.60 ± 0.7 (3)
Cytochrome *c* reductase	2.60 ± 0.5 (3)	0.88 ± 0.14 (4)

Purity of the BLMV preparation

The purity of the BLMV preparations was determined by morphological, enzymic and functional criteria. Morphological criteria were assessed by transmission electron microscopy. Enzymic criteria were determined by measuring marker enzyme activities in initial mucosal homogenates and final BLMV preparations. Functional criteria were assessed by examining the transport of D-glucose in the presence of an Na^+ and a K^+ gradient and by examining the transport of Ca^{2+} in the presence and absence of ATP. The marker enzymes measured were: lactase, sucrase, maltase, K^+-stimulated phosphatase, cytochrome *c* oxidase and cytochrome *c* reductase. Enzyme measurements were performed following previously published methods [21–23].

RESULTS

Purity of BLMV preparation

Electron microscopic examination of random BLMV preparations showed sealed membrane vesicles with minimal contamination by other cell debris. Table 1 depicts the content of various marker enzymes in crude homogenates and in BLMV. There was an 11-fold enrichment in the specific activity of the BLM marker enzyme K^+-stimulated phosphatase as compared with the initial mucosal homogenate. On the other hand, the specific activities of lactase, sucrase, and maltase, brush border marker enzymes; cytochrome *c* oxidase, a mitochondrial marker enzyme; and cytochrome *c* reductase, an endoplasmic reticulum marker enzyme,

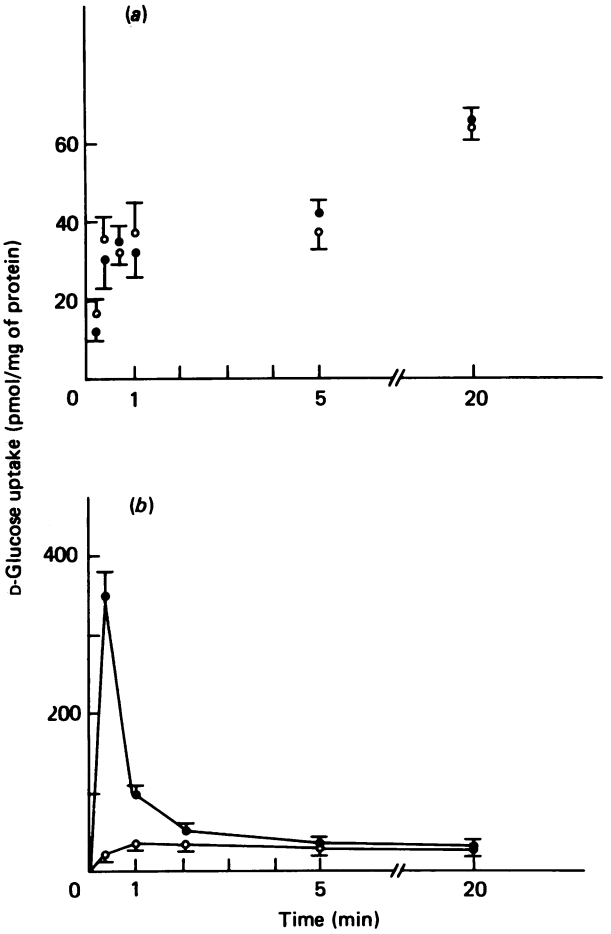

Fig. 1. Uptake of D-glucose in the presence of an Na^+ and a K^+ gradient (out > in) as a function of time in (*a*) BLMV and (*b*) brush border membrane vesicles

Jejunal basolateral or brush border vesicles were prepared separately by established procedures and were preloaded with 280 mM-mannitol and 20 mM-Hepes/Tris, pH 7.4. Incubation was performed at 37 °C in the presence of 0.1 mM-D-glucose in an incubation buffer of 100 mM-NaCl or KCl, 80 mM-mannitol and 20 mM-Hepes/Tris, pH 7.4. Each data point represents the mean ± S.E.M. of at least four experiments. ●, $Na^+_o > Na^+_i$; ○, $K^+_o > K^+_i$.

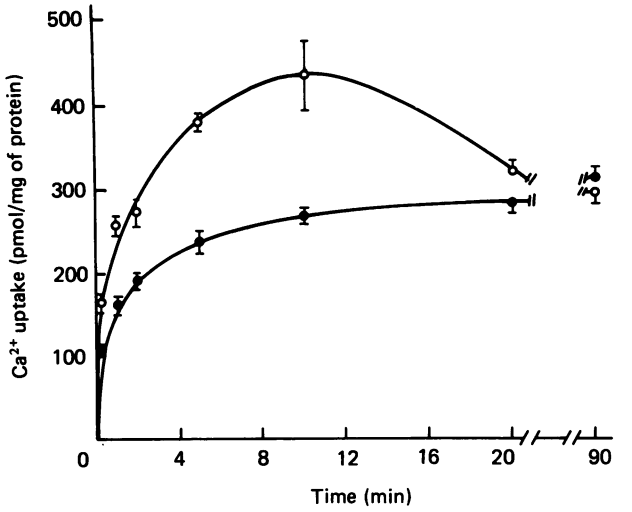

Fig. 2. Uptake of Ca^{2+} by BLMV in the presence and absence of ATP as a function of time

Jejunal BLMV were preloaded with 100 mM-KCl, 80 mM-mannitol, 5 mM-$MgCl_2$ and 20 mM-Hepes/Tris, pH 7.4. Incubation was performed at 37 °C in the presence of 50 μM-Ca^{2+} and in the presence (○) and absence (●) of 5 mM-ATP in an incubation buffer of 100 mM-KCl, 80 mM-mannitol, 2 mM-ouabain, 1 μg of oligomycin/ml, 5 mM-$MgCl_2$ and 20 mM-Hepes/Tris, pH 7.4. Each data point represents the mean ± S.E.M. of at least four experiments.

were decreased. The enrichment in BLM marker enzyme in the final vesicular preparation as compared with the initial mucosal homogenate is similar to that observed by others [14–17,24]. The basolateral membranes in our preparations were purified over 18-fold with respect to the brush border membranes in the final homogenate.

Functional criteria for determining the purity of the BLMV and their suitability for transport studies were determined by examining the transport, as a function of time, of D-glucose (0.1 mM) in the presence of an Na^+ and a K^+ gradient (inside 0 mM, outside 100 mM) and the transport of Ca^{2+} (50 μM) in the presence and absence of ATP (5 mM). Fig. 1(*a*) shows the results of D-glucose transport. In the presence of an Na^+ and a K^+ gradient the transport of D-glucose was rapid during the initial 20–40 s of incubation; following that transport occurred at a slower rate. No significant difference in the transport of D-glucose in the presence of an Na^+ and a K^+ gradient and no 'overshoot' phenomenon was observed. These observations are in contrast to D-glucose transport in brush border membrane vesicles (prepared by a Mg^{2+} precipitation method as described previously [9]) which in the presence of an Na^+ gradient showed its usual 'overshoot' phenomenon with a peak value around 20 s of incubation (Fig. 1*b*). At this point D-glucose was accumulated in the intravesicular space to approx. 11 times the equilibrium value. In the presence of a K^+ gradient D-glucose transport into brush border membrane vesicles was slower and equilibrium was reached after approx. 1 min. These observations taken collectively further emphasize that our BLMV preparations are relatively free of contamination by brush border membrane vesicles. These findings on D-glucose transport in basolateral and brush border membrane vesicles are similar to those reported by others [24–26].

Fig. 2 shows the results on the transport of Ca^{2+} in the presence and absence of ATP as a function of time. In the presence of ATP, initial Ca^{2+} uptake was rapid with a transient accumulation 'overshoot' of Ca^{2+} in the vesicles being observed. Equilibrium was reached after 90 min of incubation. In the absence of ATP, initial Ca^{2+} uptake was slower with no accumulation (i.e., no 'overshoot') being observed and equilibrium being reached after 90 min of incubation. These observations are similar to those reported by others on the transport of Ca^{2+} in this vesicular preparation [17] and further indicate the suitability of our BLMV for transport studies.

Effect of osmolarity

Membrane vesicles, including the intestinal BLMV, are osmotically active structures, i.e. they respond to changes in medium osmolarity. Using this characteristic

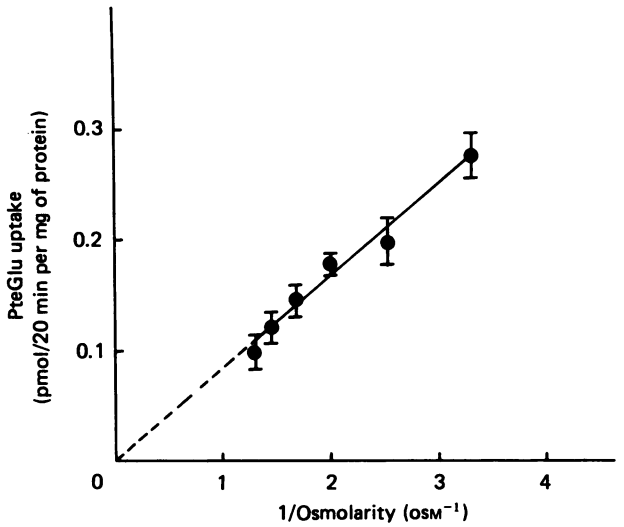

Fig. 3. Effect of incubation medium osmolarity on PteGlu uptake by BLMV

Jejunal BLMV were preloaded with 280 mM-mannitol and 20 mM-Hepes/Tris, pH 7.4. Incubation was performed for 20 min at 37 °C in an incubation buffer of 100 mM-NaCl, 80 mM-mannitol and 20 mM-Hepes/Tris, pH 7.4, with sufficient amounts of mannitol to give the indicated osmolarity. PteGlu (0.38 μM) was added to the incubation medium at the beginning of the incubation. Each data point represents the mean ± S.E.M. of at least four experiments; $y = mx+b$, where m = slope (0.859), $b = y$ intercept (−0.0046) and r represents the correlation coefficient (0.99).

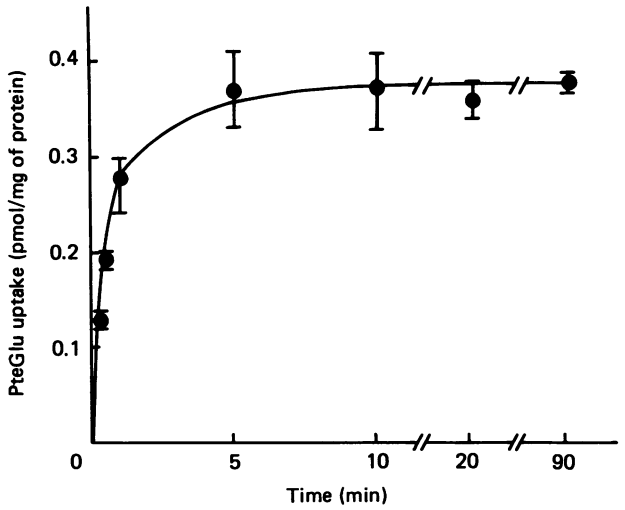

Fig. 4. Transport of PteGlu as a function of time in BLMV

Jejunal BLMV were preloaded with 280 mM-mannitol and 20 mM-Hepes/Tris, pH 7.4. Incubation was performed at 37 °C in an incubation medium of 100 mM-NaCl, 80 mM-mannitol and 20 mM-Hepes/Tris, pH 7.4. PteGlu (0.25 μM) was added to the incubation medium at the beginning of the experiment. Each point represents the mean ± S.E.M. of at least four experiments.

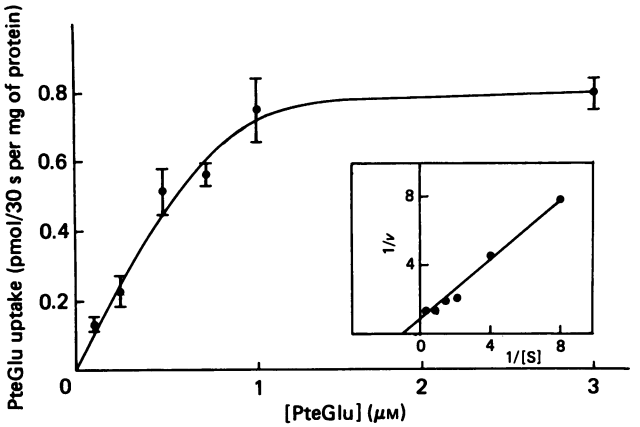

Fig. 5. Transport of PteGlu as a function of concentration in BLMV

Jejunal BLMV were preloaded with 280 mM-mannitol and 20 mM-Hepes/Tris, pH 7.4. Incubation was performed for 30 s at 37 °C in an incubation medium of 100 mM-NaCl, 80 mM-mannitol, 20 mM-Hepes/Tris, pH 7.4, and different concentrations of PteGlu. Each point represents the mean ± S.E.M. of at least four experiments. The insert is a plot of $1/v$ against $1/[S]$; $y = mx+b$, where m = slope (0.88), $b = y$ intercept (0.68) and r represents the correlation coefficient (0.99).

of membrane vesicles, one can differentiate between binding of the substrate to membrane surface and transport into the intravesicular space. This can be done by examining uptake of the substrate as a function of changing medium osmolarity. Fig. 3 shows the relationship between transport of 0.38 μM-PteGlu into jejunal BLMV after 20 min of incubation (equilibrium) and 1/osmolarity of the incubation medium (osmolarity was varied by changing the mannitol concentration). The relationship appeared to be linear with a correlation coefficient (r) of 0.99. Extrapolating the line to infinite osmolarity showed a zero y-intercept, indicating that uptake of the substrate by the BLMV vesicles was the result of transport of the substrate into the intravesicular space and not of binding to the membrane surface.

In another experiment, t.l.c. was used to examine the metabolic form of the radioactivity transported into BLMV following incubation for 20 min with 0.25 μM-[^{3}H]PteGlu. The results showed a single peak which contained > 93% of the transported radioactivity and which was eluted at the same position as standard [^{3}H]PteGlu, indicating that no metabolism of the substrate occurred.

Transport as a function of time and concentration

Fig. 4 shows the transport of 0.25 μM-PteGlu in BLMV as a function of time. Transport of PteGlu was linear during the first 1 min of incubation but decreased thereafter, reaching equilibrium after 5 min of incubation.

Fig. 5 shows the results on the transport of PteGlu in BLMV as a function of concentration (0.125–3.25 μM). Transport in PteGlu was saturable as a function of concentration with an apparent K_m and $V_{max.}$ (calculated from the Lineweaver–Burk plot; insert of Fig. 5) of 0.6 ± 0.17 μM and 1.01 ± 0.11 pmol/30 s per mg of protein, respectively.

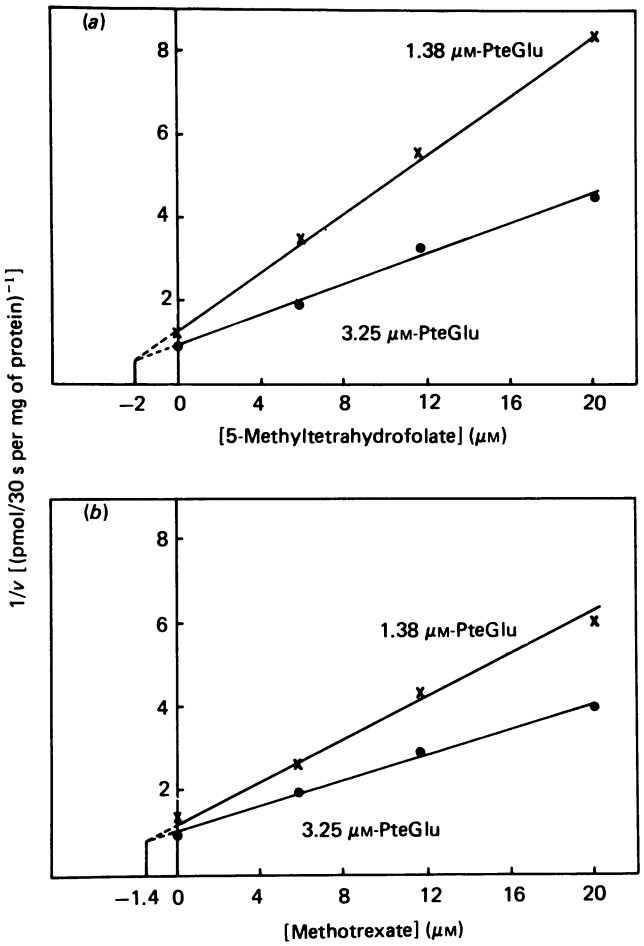

Fig. 6. Dixon plot of the effect of structural analogues on PteGlu transport in BLMV

Jejunal BLMV were preloaded with 280 mM-mannitol and 20 mM-Hepes/Tris, pH 7.4. Incubation was performed for 30 s at 37 °C in an incubation medium of 100 mM-NaCl, 80 mM-mannitol and 20 mM-Hepes/Tris, pH 7.4. PteGlu (1.38 and 3.25 μM) and different concentrations of the structural analogue under study were added to the incubation medium at the beginning of incubation. Each point represents the mean ± S.E.M. of at least four experiments. Inhibition constants for 5-methyltetrahydrofolate (*a*) and methotrexate (*b*) were 2.0 and 1.4 μM, respectively.

Effect of structural analogues

Fig. 6 shows the effect of the structural analogues 5-methyltetrahydrofolate and methotrexate on the transport of PteGlu in BLMV. Both structural analogues inhibited the transport of PteGlu in a competitive manner. The inhibition constants were calculated from the Dixon plots (Fig. 6) and were found to be 2 and 1.4 μM for 5-methyltetrahydrofolate and methotrexate, respectively.

Effect of transmembrane electrical potential, Na^+ and DIDS

To determine the electrical nature of the transport process of PteGlu into BLMV, we examined the transport of PteGlu in the presence and absence of a valinomycin-induced transmembrane electrical potential [9,18,27,28]. Transport of PteGlu (0.25 μM) was compared in the presence of a K^+ gradient (K^+_o = 100 mM, K^+_i = 0 mM) and the K^+ ionophore valinomycin (10 μg/mg of protein) with that observed in the absence of a K^+ gradient ($K^+_o = K^+_i$ = 100 mM) and the presence of valinomycin (voltage clamp condition). The rapid diffusion of K^+ in the first experiment will generate a transmembrane electrical potential with a relatively positive intravesicular compartment [18,28] thereby affecting any electrical component of PteGlu transport. No significant difference was observed in the transport of PteGlu between the two conditions (0.16 ± 0.02 and 0.16 ± 0.01 pmol/30 s per mg of protein, respectively). This observation suggests that the transport of PteGlu into BLMV is by an electroneutral process.

In another experiment we examined the transport of PteGlu in the presence of Na^+ and K^+ gradients (in = 0 mM, out = 100 mM) and in the presence of Na^+ but in the absence of a gradient ($Na^+_i = Na^+_o$ = 100 mM). The results showed similar uptake under these conditions (0.17 ± 0.02, 0.18 ± 0.02 and 0.18 ± 0.03 pmol/30 s per mg of protein, respectively), indicating that the transport of PteGlu into the BLMV is Na^+-independent.

Table 2 shows the effect of different concentrations of the anion exchange transport inhibitor DIDS [29–31] on the transport of 0.25 μM-PteGlu and 0.1 mM-D-glucose into BLMV. DIDS inhibited, in a concentration-dependent manner, the transport of PteGlu. DIDS did not inhibit the transport of D-glucose, indicating the specificity of the DIDS effect.

Table 2. Effect of DIDS on the transport of PteGlu and D-glucose in BLMV

Jejunal BLMV were preloaded with 280 mM-mannitol and 20 mM-Hepes/Tris, pH 7.4. Incubations were performed for either 30 or 20 s at 37 °C in an incubation medium of 100 mM-NaCl, 80 mM-mannitol and 20 mM-Hepes/Tris, pH 7.4. PteGlu (0.25 μM) or D-glucose (0.1 mM) and different concentrations of DIDS were added to the incubation buffer at the beginning of the experiment. Data are expressed as means ± S.E.M.; *P* values were determined by using Student's *t*-test.

Condition	PteGlu transport (pmol/30 s per mg of protein)	Glucose transport (pmol/20 s per mg of protein)
Control	0.25 ± 0.03	85 ± 24
DIDS (0.1 mM)	0.14 ± 0.02 ($P < 0.01$)	83 ± 21
(0.5 mM)	0.11 ± 0.01 ($P < 0.01$)	81 ± 11

DISCUSSION

The present study examined for the first time the transport of PteGlu across the basolateral membrane of rat enterocytes using vesicles prepared by a previously published procedure. Uptake of PteGlu by the BLMV represents transport of the substrate into an osmotically active intravesicular space rather than binding to the membrane surface (Fig. 1). The transport of PteGlu across the basolateral membrane may not require a specialized carrier system, since the negative intra-

vesicular electrical potential favours the movement of the anionic PteGlu out of the cell and down its concentration gradient. The results, however, showed that this is not the case. Transport of PteGlu in BLMV appeared to be saturable as a function of concentration with an apparent K_m of 0.6 ± 0.17 μM and $V_{max.}$ of 1.01 ± 0.11 pmol/30 s per mg of protein. This observation indicates that the transport of PteGlu in BLMV is via a carrier-mediated system. The apparent K_m for the transport of PteGlu observed in the present study with rat jejunal BLMV is approx. 9-fold less than the previously reported apparent K_m for transport of PteGlu in rat jejunal brush border membrane vesicles of 5.63 μM [10]. These findings indicate that the PteGlu transport system at the basolateral membrane has a relatively higher affinity for the substrate than has the system at the brush border membrane, suggesting that the enterocyte is adapted for efficient absorption of this essential nutrient. The transport of PteGlu into BLMV was inhibited in a competitive manner by the addition to the incubation medium of the reduced substituted structural analogue 5-methyltetrahydrofolate and the oxidized substituted structural analogue methotrexate. These findings further indicate the involvement of a carrier-mediated system for the transport of PteGlu into BLMV. The inhibition constants of these analogues (2 and 1.4 μM for 5-methyltetrahydrofolate and methotrexate, respectively) were close to the apparent K_m value of PteGlu transport (0.6 μM), suggesting that these compounds may utilize the same transport carrier as PteGlu in BLMV.

The transport of PteGlu in BLMV was electroneutral and Na^+-independent, as shown by the lack of an effect of a valinomycin-induced transmembrane electrical potential and of an Na^+ gradient on the substrate transport. An interesting observation was the ability of the anion exchange transport inhibitor DIDS to inhibit the transport of PteGlu. DIDS did not inhibit the transport of D-glucose, indicating that it had no nonspecific effect on transport systems in general. This observation raises the possibility that PteGlu transport across the basolateral membrane is by an anion exchange mechanism. An anion exchange mechanism for transport of folates has been shown in other mammalian cells (L 1210 mouse leukaemia cell [32,33]) and has been suggested for the transport of folate across intestinal brush border membrane vesicles [9,10,12]. Further studies, however, are required to confirm this suggestion.

In summary, the present study shows that transport of PteGlu across the intestinal basolateral membrane is carrier-mediated and shows that the transport process is electroneutral, Na^+-independent and is DIDS-sensitive.

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