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Transport of pteroylglutamic acid into brush border membrane vesicles from rat small intestine is a partially carrier-mediated process

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Abbreviations

BBM: brush border membrane
BBMV: brush border membrane vesicles
DIDS: 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid
HEPES: N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid
HPLC: high-performance liquid chromatography
MES: 2-N-morpholinoethanesulfonic acid
PteGlu: pteroylglutamic acid
TRIS: Tris-(hydroxymethyl)-aminomethane

Summary: Intestinal transport of PteGlu was studied using BBMV from rat small intestine. Transport was neither coupled to a specific cation gradient nor was it influenced by variations of the membrane potential. In the presence of a transmembrane pH gradient ($pH_{out} < pH_{in}$) initial transport was significantly higher compared to studies without pH gradient. Under these conditions transport could be inhibited by pretreating the vesicles with DIDS, an inhibitor of anion exchange systems. Uptake of PteGlu could not be enhanced by preloading the BBMV with HPO_4^{2-} and Cl^- and was not sensitive to DIDS under these conditions. Uptake studies using different concentrations of PteGlu revealed dual transport kinetics in the presence of a pH gradient and linear uptake in its absence. It could be concluded that uptake is mediated by a $PteGlu^-/OH^-$-antiporter at low substrate concentrations and occurs by non-ionic diffusion at higher concentrations or in the absence of a pH gradient. In an additional series of experiments it could be shown that about one-third of the substrate is bound to the membrane and is not transported. The biological significance of this binding remains unclear.

Zusammenfassung: Der transmembranäre Transport von PteGlu wurde mittels BBMV aus Rattendünndarm untersucht. Der Transport war weder an einen spezifischen Kationengradienten gekoppelt noch durch Veränderungen des Membranpotentials zu beeinflussen. In Gegenwart eines transmembranären pH-Gradienten ($pH_{out} < pH_{in}$) waren die initialen Transportraten signifikant höher als in Versuchen ohne pH-Gradient. Unter diesen Bedingungen war der Transport zu inhibieren, wenn die BBMV mit DIDS, einem Hemmstoff von Anionenaustauschsystemen, vorbehandelt wurden. Die Aufnahme von PteGlu war nicht erhöht, wenn die BBMV mit HPO_4^{2-} und Cl^- vorbeladen wurden. Unter diesen Bedingungen hatte auch DIDS keinen hemmenden Effekt. Studien zur konzentrationsabhängigen

Aufnahme ergaben eine duale Transportcharakteristik in Anwesenheit eines pH-Gradienten und eine lineare Aufnahme in Abwesenheit eines pH-Gradienten. Hieraus ist zu schließen, daß die Aufnahme von PteGlu bei niedrigen Substratkonzentrationen mittels eines $PteGlu^-/OH^-$-Antiporters vermittelt wird. Bei höheren Konzentrationen oder in Abwesenheit eines pH-Gradienten erfolgt die Aufnahme hingegen durch nichtionische Diffusion. In einer zusätzlichen Versuchsserie konnte gezeigt werden, daß ein Drittel des Substrates nicht transportiert, sondern an die BBM gebunden wird. Die biologische Bedeutung dieser Bindung bleibt unklar.

Key words: pteroylglutamic acid; brush border membrane vesicles; folate-hydroxyl-antiporter; diffusion

Schlüsselwörter: Pteroylglutaminsäure, Bürstensaum-Membranvesikel, Folat-Hydroxyl-Antiporter, Diffusion

Introduction

Although intestinal uptake of folates has been studied intensively within the last few years, it still remains controversial by which mechanism the vitamin is absorbed. Judging from the data thus far, authors have either concluded that a specific carrier mediates the transport (8, 11, 19, 36, 45), that intestinal uptake occurs by non-ionic diffusion (3, 4, 13, 33, 44), or that both processes are involved (31, 46, 47, 50). Nevertheless, in most cases it was demonstrated that intestinal transport of PteGlu depends on the pH of the incubation solution.

The conflicting results are due to a great extent to varied methods using intact intestinal tissue which prevented the authors from differentiating between the transmembrane transport itself and the subsequent intracellular processes, i.e., metabolism, compartmentation, and binding. All these points might influence the transport step. Even the few studies using isolated BBMV revealed different data, especially concerning the driving forces of folate transport (5, 38–40, 42). The present study was aimed at clarifying which mechanisms are involved in PteGlu transport through the isolated BBM of rat small intestine.

Materials and methods

Preparation of BBMV

BBMV were prepared by a Ca^{2+}-precipitation technique (21, 30) from the small intestine of male Wistar rats (290 ± 20 g) fed a stock diet (Altromin 1324, Altromin, Lage, FRG) and fasted for 24 h before being killed by cervical dislocation. For every preparation 12 animals were used and 40 cm of gut was removed from each of them, beginning at a 10-cm distance distal to the pylorus. The gut was rinsed with Krebs bicarbonate solution and everted. The mucosa was scraped off and homogenized in a buffer solution consisting of 12 mM TRIS and 300 mM Mannitol (pH 7.1). After adding $CaCl_2$ to a final concentration of 10 mM the homogenate was centrifuged for 15 min at 7 500 g. The resulting supernatant was centrifuged again (30 min, 20 000 g) and the pellet obtained was homogenized in 70 ml buffer (20 mM HEPES, 300 mM mannitol, pH 7.4 adjusted with 1 M TRIS). After adding 10 mM $CaCl_2$ the homogenate was centrifuged for 15 min at 7 500 g and the resulting supernatant once again centrifuged at 20 000 g for 30 min. The pellet was homogenized in buffer solutions whose composition depended on the experiments (composition given in the

Table 1. Enrichment of the intestinal brush border membrane as judged by the specific activity of the marker enzymes alkaline phosphatase and maltase (n = 10).

	Crude homogenate U/mg protein	BBMV U/mg protein	Enrichment
Alkaline phosphatase	0.44 ± 0.042	8.13 ± 1.54	18.5-fold
Maltase	0.09 ± 0.001	1.28 ± 0.16	14.2-fold

legends to the figures). The BBMV-suspension was centrifuged for a last time in a table centrifuge (30 min, 15 000 g). The pellet was suspended in about 400 µl of the same buffer as used before. The BBMV-suspension was kept on ice and was used for uptake studies on the same day. Purification of the BBM was controlled by the marker enzymes alkaline phosphatase (E.C. 3.1.3.1., determined according to [49]) and maltase (E.C. 3.2.1.20, determined according to [16]) enriched by the preparation procedure. As can be seen from Table 1, specific activity of both enzymes increased, indicating that the preparation was successful.

Transport studies

All experiments were performed at room temperature by means of a rapid filtration technique (2) using 3',5',7,9-^{3}H-PteGlu (Amersham, Braunschweig, FRG; specific activity 51–62 Ci/mmol) as a substrate. It was checked by HPLC (17) and found to be 98 % pure. For every incubation about 3 µCi of the labeled substrate and differing amounts of the unlabeled compound (Sigma, Deisenhofen, FRG) were diluted in 100 µl of the substrate buffer whose composition was varied according to the experiment (given in the legends to the figures). Except for the studies concerning uptake as a function of substrate concentration all experiments were performed in the concentration range between 0.5 µM and 1 µM. Incubation was started by adding 20 µl BBMV-suspension and vortexing. At distinct intervals 20 µl of the incubate was removed from the tube, put in 5 ml of an ice-cold stopping solution whose pH was identical to that of the substrate medium (100 mM mannitol, 25 mM $MgSO_4$, 100 mM choline chloride and 20 mM HEPES pH 7.4 or MES pH 5.8 resp., pH was adjusted with 1 M TRIS). The solution was filtered under vacuum through a cellulose nitrate filter (0.45 µm, Schleicher & Schüll, Dassel, FRG) which was rinsed again withg 5 ml of the stopping solution. Filters were solubilized in methanol and the radioactivity was determined by liquid scintillation counting. Nonspecific binding of the substrate to the filters was determined by incubating a reaction mixture that contained the same media as the sample except for the BBMV suspension.

In every preparation integrity of the BBMV was tested by their ability to transport glucose (23), accompanied by an overshoot-phenomenon in a Na^+-gradient (Fig. 1).

Calculation of the results

Uptake rates were calculated with respect to the radioactivity of an unfiltered aliquot of the incubate. The data were corrected for the unspecific binding of the substrate to the filter and were referred to the protein content (6) of the BBMV suspension. All values given are the means ± SEM and represent the average of at least eight incubations from two different preparations. Statistical differences were judged by Student's *t*-test. Data were compared on the basis of the relative uptake, i.e., percentage of equilibrium, because pretests showed that the quantity of protein retained on the filter depended on the incubation pH, so that determination of absolute uptakes would not have been comparable between different pH values. This effect was also seen by others (12, 38).

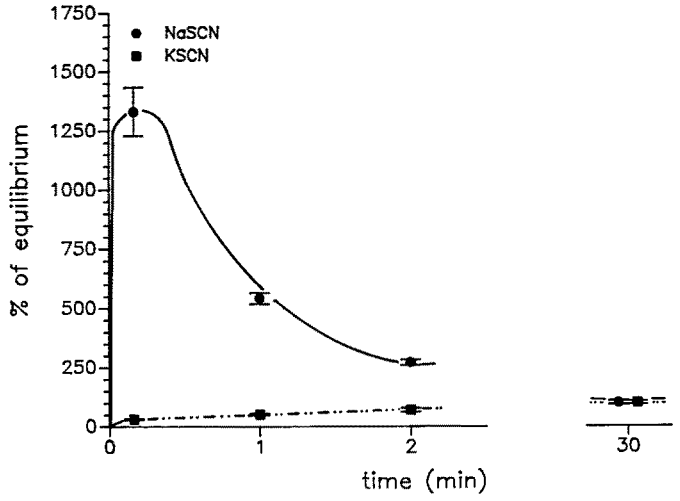

Fig. 1. Uptake of glucose into BBMV in the presence of a Na^+- or a K^+-gradient. Characteristic "overshoot" indicates Na^+/glucose-cotransport and proves functional integrity of the BBMV. Intravesicular medium: 300 mM mannitol and 20 mM HEPES (pH 7.4); substrate buffer: 100 mM mannitol, 100 mM NaSCN (or KSCN) and 20 mM HEPES (pH 7.4); glucose concentration: 12 µM.

Results and discussion

Effect of an inwardly directed cation-gradient

Transport of PteGlu was similar in the presence of a Na^+- or a K^+-gradient (Fig. 2). This is an agreement with the results obtained from

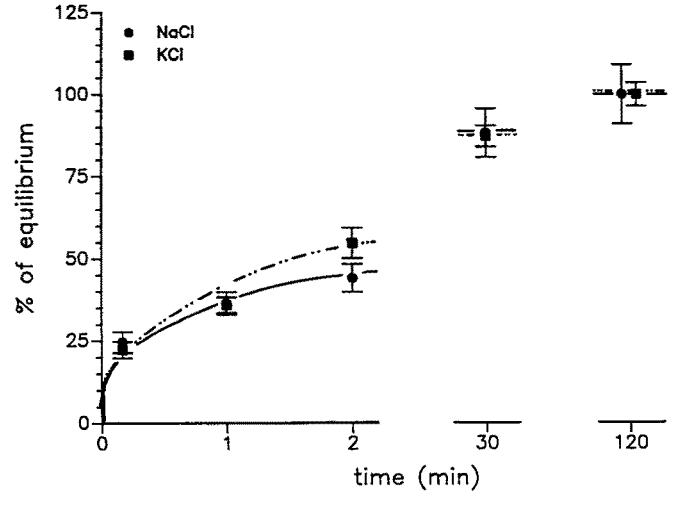

Fig. 2. Uptake of PteGlu in the presence of a Na^+- or a K^+-gradient. Incubation conditions as in Fig. 1.

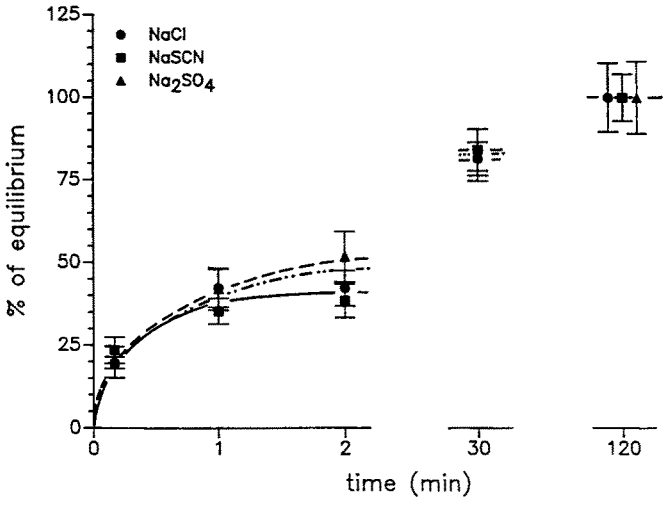

Fig. 3. Effect of membrane potential on the uptake of PteGlu. Potential was varied by the anion substitution technique. Intravesicular medium: 300 mM mannitol and 20 mM HEPES (pH 7.4); substrate buffer: 100 mM mannitol, 100 mM NaSCN (or 100 mM NaCl or 50 mM Na_2SO_4).

studies with BBMV from rabbit (39) and human (38) intestine, and argues against a $folate^-/Na^+$-cotransport, as has been assumed in experiments using gut tissue (36, 46) or isolated epithelial cells (11), and also against a

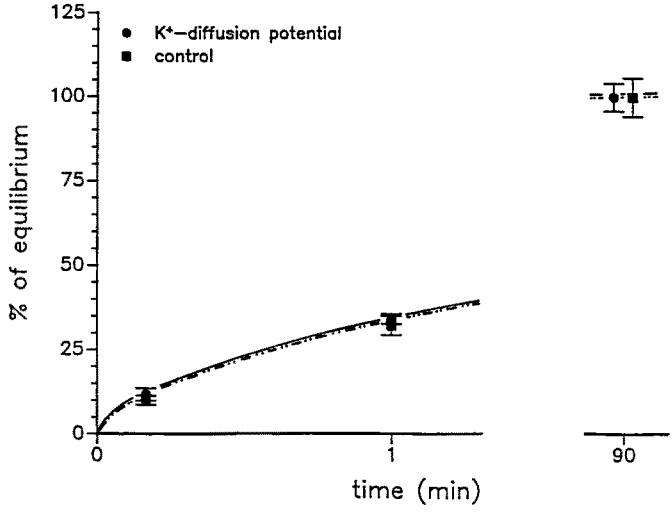

Fig. 4. Influence of an inside positive potential on PteGlu-transport. BBMV were pretreated for 60 min in the intravesicular buffer (10 µM valinomycin, 300 mM mannitol and 20 mM HEPES, pH 7.4). Valinomycin was dissolved in ethanol (final concentration: 1 % in the intravesicular buffer). In control experiments intravesicular buffer also contained 1 % of ethanol. Substrate buffer: 50 mM NaCl, 100 mM KCl and 20 mM HEPES (pH 7.4).

folate conductance as proposed by one group (50). Therefore, it must be assumed that the decreased folate uptake, observed in experiments with intact tissue when removing Na^+ from the buffer, is a secondary phenomenon. This might be due either to a diminished uptake of metabolizable substrates, like glucose, with a subsequent break-down of the energy delivery, or to changes of the intestinal surface pH because of the lack of metabolizable sugars (10). It might also be caused by a reduced activity of Na^+/H^+-antiport (32) which could lead to changes in intracellular pH as well as in intestinal surface pH. The latter is known to be an important factor for intestinal folate uptake and increases when the Na^+ content of the buffer is reduced (4, 27).

Effect of membrane potential

In the studies presented in Figs. 3 and 4, an electrical gradient was established either by the anion substitution technique (29) or by an inside positive potential caused by a valinomycin induced K^+-diffusion (15). In both cases no variations in PteGlu transport were observed and it can thus be concluded that intestinal transport of PteGlu in rat intestine, as well as in rabbit (39) and in human intestine (38) is not due to an electrogenic mechanism. This means that transport either occurs by non-ionic diffusion or is mediated by an electroneutral transport system.

Influence of pH

Figure 5 shows that initial transport rates were significantly higher ($p \leq 0.001$) in the presence of a transmembrane pH gradient ($pH_{out} = 5.8/pH_{in} = 8.0$) compared to studies without a pH gradient ($pH_{out} = pH_{in} = 5.8$ or

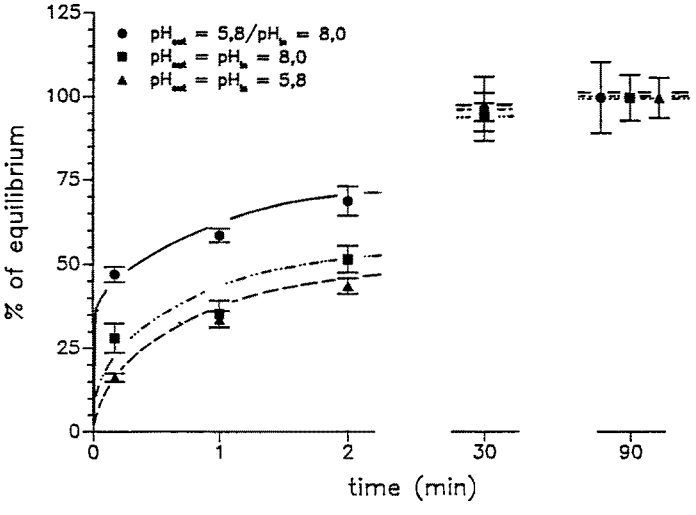

Fig. 5. Effect of pH on the transport of PteGlu. BBMV were either incubated in a buffer whose pH was identical to the intravesicular one (300 mM mannitol/20 mM HEPES, pH 8.0 or 300 mM mannitol/20 mM MES, pH 5.8) or BBMV were incubated under pH gradient conditions ($pH_{out} = 5.8/pH_{in} = 8.0$).

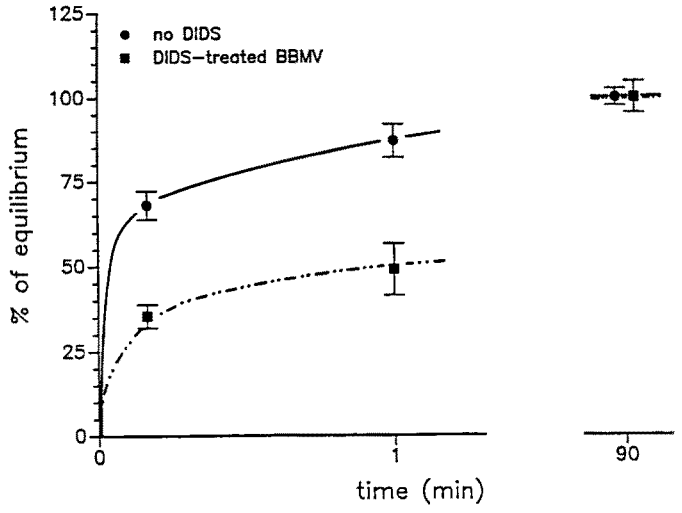

Fig. 6. Uptake of PteGlu under pH gradient (see Fig. 4) conditions after DIDS-treatment of the BBMV. DIDS treatment was carried out as described in the text.

8.0, resp.). The differences between the two series obtained without a pH gradient were not statistically significant. These data indicate that the increase in folate transport by lowering pH and the decrease of the uptake by elevating pH, which has been observed in clinical studies as well as in experiments using intestinal tissue (1, 37, 43, 45, 46), is caused by the transmembrane pH difference and not by the H^+-concentration itself. In contrast to BBMV from rabbit (39) and human gut (38), we did not observe an overshoot in the presence of a pH gradient. This might be at least partially due to a lower incubation temperature and a lower fluidity of the membrane under the conditions applied by us.

It was assumed that transport of PteGlu which exits predominantly in the monoanionic form at physiological pH (34) was facilitated by an exchange with OH^-. To confirm this assumption BBMV were pretreated with DIDS prior to incubation. DIDS is known to inhibit anion exchange systems (7) such as Cl^-/HCO_3^--transport und was also shown to inhibit folate transport into rabbit (39) and human BBMV (38). BBMV were incubated for 60 min in a substrate buffer containing 100 μM DIDS, centrifuged at 15000 g for 30 min and the pellet was resuspended in a buffer without DIDS. Pretests revealed that it was necessary to wash out the surplus of DIDS, because high concentrations of this stilbene derivative in the substrate buffer led to a high unspecific binding of PteGlu to the membrane. As can be seen from Fig. 6, transport was significantly inhibited ($p \leq 0.01$) when BBMV were DIDS-treated, and the resulting uptake kinetics were similar to that observed in the absence of a pH gradient. In contrast, Na^+-dependent glucose transport was not affected by DIDS.

These data emphasize that uptake of PteGlu into BBMV is facilitated under pH gradient conditions by an exchange with OH^-. For further characterization of the transport step BBMV were preloaded with 10 mM

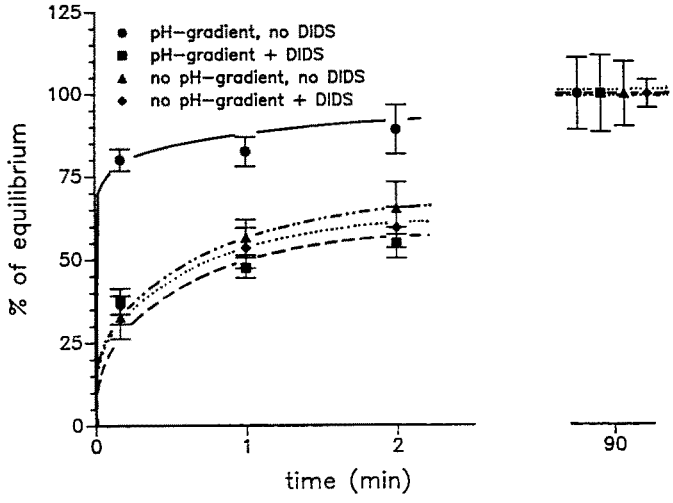

Fig. 7. Transport of PteGlu in the presence of an outwardly directed HPO_4^{2-}/Cl^--gradient. Intravesicular medium: 80 mM mannitol, 100 mM KCl, 10 mM KH_2PO_4 and 20 mM HEPES, pH 7.4. Substrate buffer: 300 mM mannitol/20 mM HEPES, pH 7.4 or 300 mM mannitol/20 mM MES, pH 5.8. DIDS treatment was carried out as described in the text.

HPO_4^{2-} and 10 mM Cl^-. Under these conditions (Fig. 7) transport rates were identical to those obtained in the absence of these anions and transport was not DIDS sensitive. On the other hand, when a simultaneous pH gradient ($pH_{out} < pH_{in}$) was also established, transport was significantly higher and could be inhibited by pretreating the BBMV with DIDS.

From these results it can be concluded that intestinal transport of PteGlu is highly specific and only acts with OH^-, but not with Cl^- or HPO_4^{2-} as a counterion. This is in agreement with previously published data from rabbit jejunum (40). Therefore, intestinal transport of PteGlu is mediated by a specific folate/hydroxyl-antiporter which is distinguishable from other anion exchange systems such as band-3-protein in erythrocyte membrane (14), which is known to transport different anions (18), but which could not be detected in the intestinal BBM (48).

Uptake of PteGlu as a function of substrate concentration

Unidirectional uptake in relation to the substrate concentration was studied under short-circuit conditions at an incubation time of 10 s in the presence ($pH_{out} = 5.8/pH_{in} = 7.4$) and in the absence of a pH gradient ($pH_{out} = pH_{in} = 7.4$). Short-circuit conditions were obtained by pretreating the BBMV with valinomycin and adding 50 mM KCl to the substrate buffer, as well as to the intravesicular medium.

As can be seen from Fig. 8 transport was a linear function of substrate concentration under non-gradient conditions and relative uptake was similar at all concentrations. Least square regression analysis revealed a transport according to the equation $V = P \times S$, with V representing the

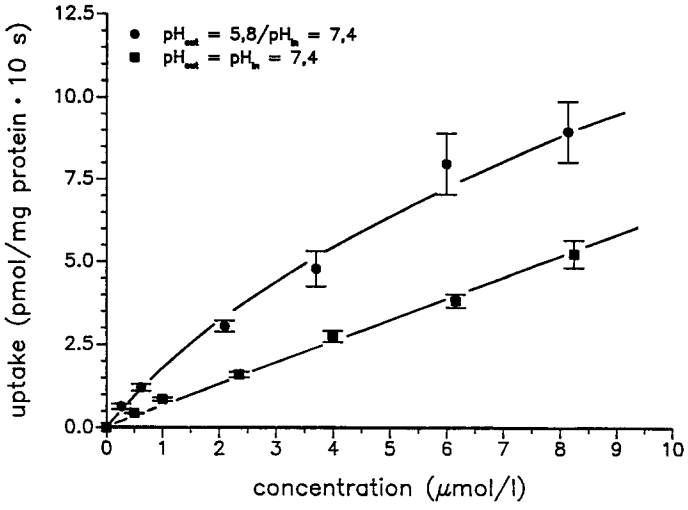

Fig. 8. Uptake of PteGlu as a function of substrate concentration. BBMV were pretreated for 60 min in the intravesicular buffer (100 mM mannitol, 100 mM KCl, 10 μM Valinomycin, 20 mM HEPES, pH 7.4.). Valinomycin was dissolved in ethanol (final concentration: 1 % in the intravesicular buffer): Incubation solution: 50 mM NaCl, 100 mM KCl, 20 mM HEPES, pH 7.4 or 50 mM NaCl, 100 mM KCl, 20 mM MES, pH 5.8.

uptake, S the substrate concentration, and P the permeation coefficient. P was calculated to be 0.64 pmol × mg protein^{-1} × 10 s^{-1} × μM^{-1} with a correlation coefficient of 0.889 (n = 123).

In the presence of a pH gradient, transport exhibited dual characteristics, i.e., a saturable component at low and a nonsaturable part which becomes noticeable at higher substrate concentrations. Relative uptake decreased at higher substrate concentrations. Thus, the uptake under these conditions may be described by the Michaelis-Menten equation with an additional term for the non-saturable component:

$$V = \frac{V_{max} \times S}{k_T + S} + P \times S,$$

where V denotes uptake, S the initial substrate concentration, k_T the transport constant for the saturable part, and P the permeation coefficient for the linear component.

The non-linear component is due to diffusion of PteGlu across the BBM. Thus, it can be equated to the uptake under non-gradient conditions, i.e., without the driving force for $PteGlu^-/OH^-$-exchange. By subtracting these uptake rates from the transport rates observed under pH gradient conditions the saturable part can be calculated. Saturable component was linearized according to Hanes (20) and the transport parameters were calculated by least square regression analysis. V_{max} was found to be 6.13 pmol × mg protein^{-1} × 10 s^{-1} and apparent k_m was 4.2 μM.

It is thus understandable that intestinal transport of PteGlu under physiological conditions is energized by the pH difference across the

BBM, which is established by the intestinal surface pH being relatively lower (26, 28) compared to the intracellular pH which was found to be 6.8 (24). Furthermore, it is evident that changes in intestinal intraluminal pH, with corresponding changes in surface pH, affect intestinal uptake of PteGlu by diminishing or enhancing the transmembrane pH difference and thus the driving force for transport. Taking into account that effective intraluminal concentration of folates can be assumed to be in the lower micromolar range, as has been shown for pyridoxine and riboflavin (9), this transport system mediates intestinal uptake of physiological doses of folates, whereas higher amounts of substrate predominantly enter the enterocyte by simple diffusion.

Binding of PteGlu to the BBM

Because of the existence of folate binding proteins in the intestinal BBM (22, 25, 35, 41) it must be assumed that PteGlu is not only transported into the BBMV, but also bound to the membrane. To differentiate between binding and transport, BBMV were incubated in solutions with increasing osmolarity under equilibrium conditions. Extrapolating the results to an infinite osmolarity, i.e., zero intravesicular space, the amount of substrate "transported" under these conditions represents binding to the membrane.

As can be seen from Fig. 9, at both pH values, 5.8 and 7.4, uptake decreases linearly by increasing osmolarity and about one-third of the PteGlu is not transported, but bound to the membrane. This is in agreement with other experiments in which about 50 % of the substrate was bound to the BBM of rabbit jejunum. Selhub and Rosenberg (42) reported that PteGlu was not bound to the BBM, which is probably due to their

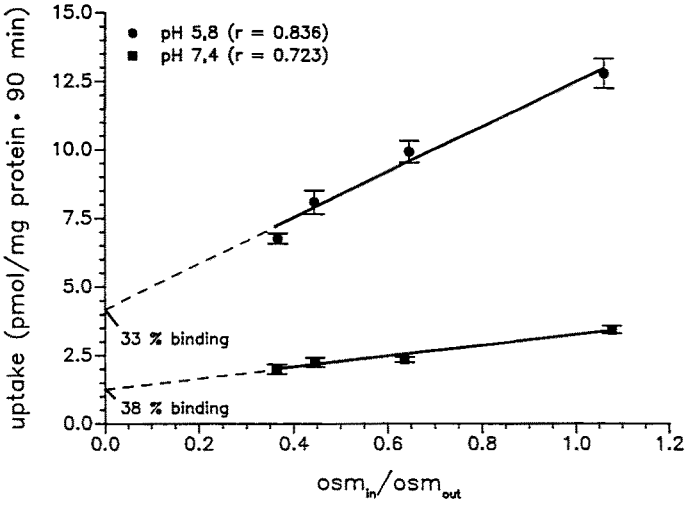

Fig. 9. Uptake of PteGlu as a function of medium osmolarity. Intravesicular medium: 300 mM mannitol and 20 mM HEPES, pH 7.4. Incubation solutions: 20 mM HEPES (pH 7.4) and increasing concentrations of mannitol.

incubation conditions, i.e., they did not examine binding under equilibrium conditions, but only during the initial uptake period. Considering the fact that several investigators detected folate binding proteins in the intestinal BBM, a certain binding of the substrate must be expected. It is not yet possible to judge the physiological role of this binding. In one case it was concluded that binding is not involved in folate uptake (22), but it might be possible that binding serves as an intermediate store to retain folates.

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