Analysis of the pH Dependence of Folate Binding and Transport by Rat Kidney Brush Border Membrane Vesicles (43215)

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Abstract. **Folate reabsorption by the mammalian kidney occurs following a tight binding reaction with the renal brush border membrane. Previous studies have shown that transport of folic acid (PteGlu) by rat kidney brush border membrane vesicles occurs maximally at pH 5.6 via a saturable system that is associated with a binding component. The present studies have shown that the pH dependency of transport was due to the development of the transmembrane pH gradient (7.3 in/5.6 out), not to the acidic pH per se. The pH gradient-mediated transport was stimulated by an inwardly directed ionic gradient, either of NaCl or choline chloride. These gradients also stimulated the membrane binding of PteGlu suggesting that NaCl and choline chloride may have increased PteGlu transport by altering binding to the brush border membrane. Renal brush border membrane vesicular transport of PteGlu was not affected by induction of a relatively positive intravesicular space. Transport was inhibited by 4,4′-diisothiocyano-2,2′-disulfonic acid stilbene, an anion exchange inhibitor. The results suggest that rat kidney brush border membrane transport of PteGlu is initiated by association with a specific membrane protein, followed by transfer of folate across the membrane. The overall activity is influenced by a transmembrane pH gradient.** [P.S.E.B.M. 1991, Vol 196]

Urinary folate excretion is primarily regulated at physiologic levels by the degree of reabsorption in the proximal tubule, although secretion occurs at high plasma concentrations (1, 2). Studies of folate reabsorption have suggested an initial tight binding reaction (3), such as that which occurs with the folate binding protein (folate receptor) in the renal brush border membrane (4, 5). Clearance of folate derivatives is inversely related to their binding affinity with this protein (6), suggesting that the folate receptor can regulate proximal tubular transport, and hence renal reabsorption, of folate. In cultured mammalian cells, folate appears to be transported by a unique receptor-mediated process (7), in which folate is bound to receptors in clusters on the cell surface and then translocated by an acid pH-mediated process into the cytosol. Receptor regeneration appears to occur on the surface, not in endosomes.

Our previous studies (8) have shown that folic acid (PteGlu) is transported by isolated rat kidney brush border membrane vesicles (BBMV) in a manner consistent with a saturable system and that a binding component may be associated with the transport. Acidic extravesicular pH (5.6) markedly stimulated folate transport by renal BBMV. The role of acidic extravesicular pH in PteGlu transport in renal BBMV is not known. However, studies with rat, rabbit, and human intestinal BBMV have shown that PteGlu transport is pH dependent with stimulation by an inwardly directed H^+ gradient (9–11). In isolated rat hepatocytes, Horne (12) has shown that folate transport is also controlled by a transmembrane pH gradient, with increased transport as the medium becomes acidic (pH 5.5) compared with the cytosol.

The present studies were designed to analyze the effects of intravesicular and/or extravesicular pH on folate binding and transport by renal BBMV and the dependence of these processes on ionic (NaCl) gradients, as earlier studies have reported ionic effects on folate binding activity of renal BBMV (13).

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Materials and Methods

[3′,5′,7,9-^{3}H]PteGlu (26.0 or 40.0 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA) and used as obtained. This was 97–98% pure as determined by the manufacturer and confirmed by our high-pressure liquid chromatography analysis of the stock solution (14). Protein was determined with the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) and were of analytical grade. Metricel membrane filters (0.45 μm, 25 mm) were obtained from Gelman Sciences, Inc. (Ann Arbor, MI). Liquid scintillation complete counting cocktail 3a70B was purchased from Research Products International Corp. (Mount Prospect, IL).

Isolation of BBMV from Rat Kidney Cortex. BBMV from rat kidney cortex were isolated by similar methods as described earlier (8). Basically, kidney cortices from unfasted male Sprague-Dawley rats were removed and homogenized in sucrose buffer (0.25 *M* sucrose, 0.1 m*M* phenylmethylsulfonylfluoride, 10 m*M* Tris-Cl, pH 7.6). The homogenate was subjected to centrifugation at 24,000*g* for 20 min and a fluffy layer was obtained over the solid pellet. This layer was resuspended in 4 ml of sucrose buffer and treated with 15 m*M* $MgCl_2$ for 20 min at 0°C. The solution was centrifuged at 3000*g* for 10 min and the supernatant was respun at 26,000*g* for 20 min. The resulting brush border pellet was resuspended in mannitol buffer (100 m*M* mannitol, 50 m*M* potassium/Hepes or 2-[*N*-morpholino]ethane sulfonic acid (MES) buffer of pH 7.3 or 5.6), washed once, and the final pellet of brush border was resuspended in mannitol buffer to contain protein at 4–5 mg/ml. The purity and physiologic functionality of the brush border fraction were checked as detailed earlier (8).

Measurement of PteGlu Uptake by BBMV. The uptake of PteGlu of BBMV was studied by the methods described previously (8). Vesicles were resuspended at the desired pH (5.6 or 7.3) as described above and preincubated at 25°C for 30 min to load the inside of the vesicles (11). The uptake of [^{3}H]PteGlu was determined in an incubation medium (85 μl) containing about 0.1 mg of protein of membrane vesicles, 0.16 μM [^{3}H]PteGlu, and 50 m*M* MES or Hepes of the desired pH. Included with this medium was either 100 m*M* mannitol (basal medium), 50 m*M* NaCl, or 50 m*M* choline chloride, as required. After the desired period of incubation at room temperature (25°C), an aliquot of 25 μl was removed and added into 1 ml of ice-cold solution (same composition as the incubation buffer except no [^{3}H]PteGlu). Folate uptake was then determined by rapid vacuum filtration and liquid scintillation counting. Control incubation values, performed by incubation of the vesicles with [^{3}H]PteGlu in excess stop solution at 4°C, were subtracted from each sample incubation value to obtain the total folate uptake value. Based on our previous studies (8), membrane binding and intravesicular transport of PteGlu were distinguished by a standard procedure of osmotic perturbation (15, 16). [^{3}H]PteGlu uptake by BBMV was assayed with and without increased osmolarity of media (the incubation mixtures containing 0 m*M* or 300 m*M* mannitol in addition to the constituents described above). The stop solutions also contained 0 m*M* or 300 m*M* mannitol in addition to the constituents described above. The binding component was determined by extrapolation of the uptake data to infinite osmolarity. Transport represented the difference between total uptake and binding.

Statistical Analysis. Binding and transport values at 30 sec (initial rate) and at 30 min (apparent equilibrium) were analyzed for statistical significance (overall $P < 0.05$) using a General linear models' procedure (17), followed by Duncan's multiple range test to test for difference among the means of the various groups. For comparisons of values from two treatments only, group data were analyzed for differences using Student's *t* test, with $P < 0.05$ as the level of significance.

Results

In previous studies (8), the binding as well as transport of [^{3}H]PteGlu by rat kidney BBMV were pH dependent, with peak values at an in/out pH gradient of 7.3/5.6. To investigate the role of the acidic extravesicular pH in these processes, the present studies examined the effect of various intravesicular/extravesicular pH gradients. The binding and transport values under all conditions (Figs. 1–3) were linear for the initial 1 min of incubation and generally had reached a plateau value by 15–30 min. As such, the value at 30 sec was used in all comparisons to estimate the initial rate value and that at 30 min for the equilibrium value.

PteGlu uptake by renal BBMV was first studied at pH 5.6 in the absence of a pH gradient (i.e., intravesicular and extravesicular pH were 5.6) and in the presence and absence of inward gradients of NaCl or choline chloride (50 m*M*). Results in Figure 1 show that PteGlu binding to BBMV was much greater than PteGlu transport at this pH. Transport of PteGlu at this pH was not significantly affected by NaCl or choline chloride. In contrast, PteGlu binding was significantly stimulated by the presence of either NaCl or choline chloride, with no significant difference in binding values due to the presence of sodium versus choline.

PteGlu uptake by renal BBMV was then studied at pH 7.3 in the absence of a pH gradient (i.e., intravesicular and extravesicular pH were 7.3) and in the presence and absence of inward gradients of NaCl or choline chloride (50 m*M*). Results in Figure 2 show that the initial rates of transport and binding were similar, but

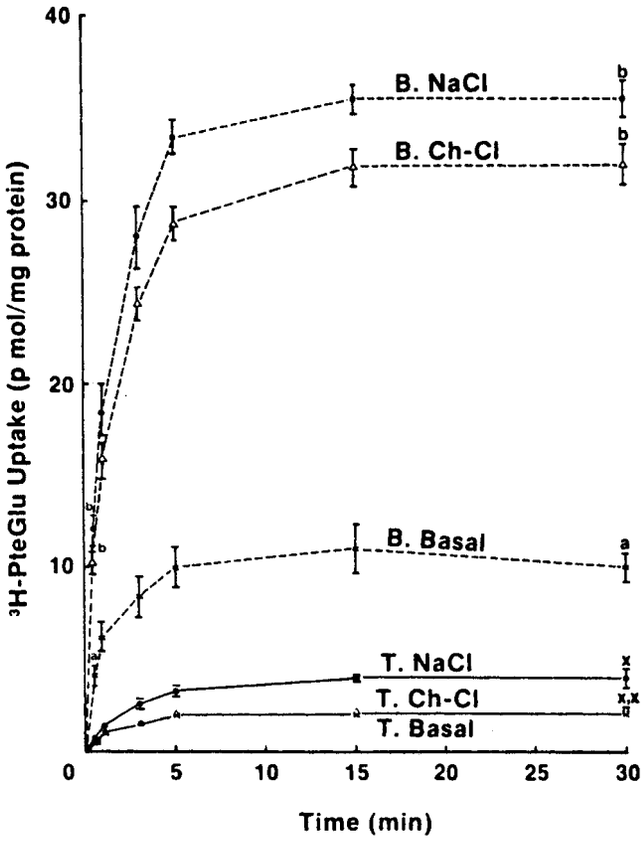

Figure 1. Effect of inwardly direct gradients of NaCl and choline chloride on PteGlu uptake by renal brush border membrane vesicles at pH 5.6 in the absence of a pH gradient. Membrane vesicles were resuspended in mannitol buffer (100 m*M* mannitol, 50 m*M* MES, pH 5.6) and aliquots (about 0.1 mg of protein) were incubated in medium (0.16 μM [^{3}H]PteGlu, 50 m*M* MES, pH 5.6) containing either 100 m*M* mannitol (basal (×), 50 m*M* NaCl (●), or 50 m*M* choline chloride (△) for different time intervals before filtration to analyze the uptake as described in Materials and Methods. Binding (dashed lines) and transport (solid lines) values were determined as described in Materials and Methods. Values represent the mean ± SE (n = 3). Means with a superscript in common (binding = abc; transport = xyz) were not significantly different from each other with an overall protection level of 0.95 (Duncan's multiple range test).

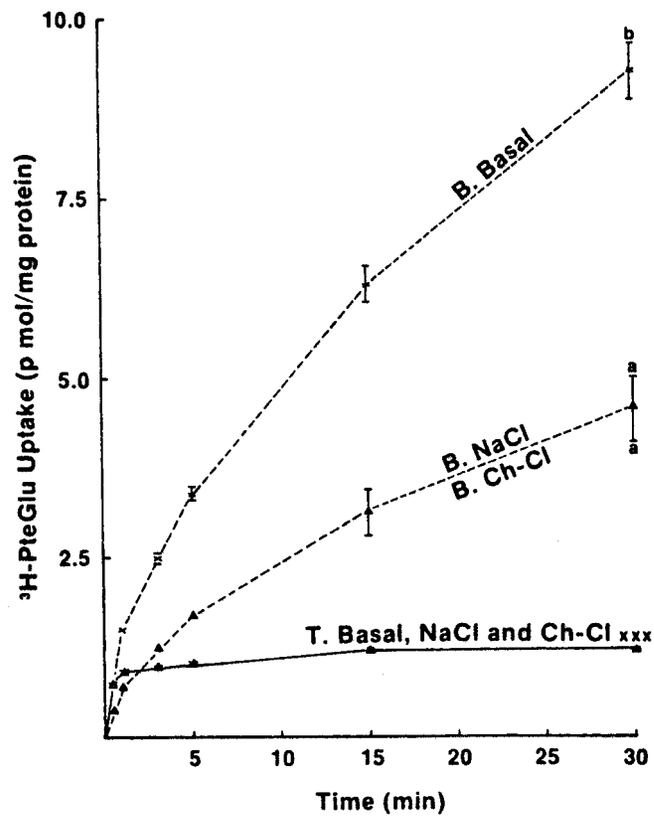

Figure 2. Effects of inwardly directed gradients of NaCl and choline chloride on PteGlu uptake by renal brush border membrane vesicles at pH 7.3 in the absence of a pH gradient. Membrane vesicles were resuspended in mannitol buffer (100 m*M* mannitol, 50 m*M* Hepes, pH 7.3) and aliquots (about 0.1 mg of protein) were incubated in medium (0.16 μM [^{3}H]PteGlu, 50 m*M* Hepes, pH 7.3) containing either 100 m*M* mannitol (basal (×), 50 m*M* NaCl (●), or 50 m*M* choline chloride (△) for different time intervals before filtration to analyze the uptake as described in Materials and Methods. Binding (dashed lines) and transport (solid lines) values were determined as described in Materials and Methods. Values represent the mean ± SE (n = 3). Means with a superscript in common (binding = abc; transport = xyz) were not significantly different from each other with an overall protection level of 0.95 (Duncan's multiple range test). Comparisons at 30 sec were the same as those at 30 min and were not included on the figure for clarity.

at 30 min the binding was greater than transport at this pH. PteGlu transport was not affected by the presence of a NaCl or a choline chloride inward gradient. PteGlu binding to renal BBMV at pH 7.3 was remarkably and equally decreased by the presence of NaCl or choline chloride.

Finally, the effect of the pH gradient, intravesicular pH 7.3 and extravesicular pH 5.6, on PteGlu uptake by renal BBMV was studied in the presence and absence of inward gradients of NaCl or choline chloride (50 m*M*). Results in Figure 3 show that a major portion of PteGlu uptake was due to transport in the presence of the pH gradient. In the presence of the pH gradient, the transport of PteGlu was significantly stimulated by either a NaCl or a choline chloride gradient. The effect of NaCl on the pH-dependent transport was somewhat greater than the effect of choline Cl. PteGlu binding to renal BBMV in the presence of this pH gradient was also increased by the presence of an inward gradient of NaCl or choline chloride, but this increase was smaller than that observed for PteGlu transport.

Since an inwardly directed H^+ gradient may generate a transmembrane electrical potential (relatively positive inside) and possibly stimulate the transport of negatively charged folate (electrical coupling), experiments were performed to assess the effects of an inwardly directed positive gradient on folate uptake by renal BBMV. An inwardly directed positive gradient was established in the absence of a pH gradient (pH 5.6 in and out) by exposing the vesicles to an inwardly directed 50 m*M* K^+ gradient in the presence of the K^+ ionophore, valinomycin (10 μg/mg protein) (10, 11). Uptake under these conditions was compared with

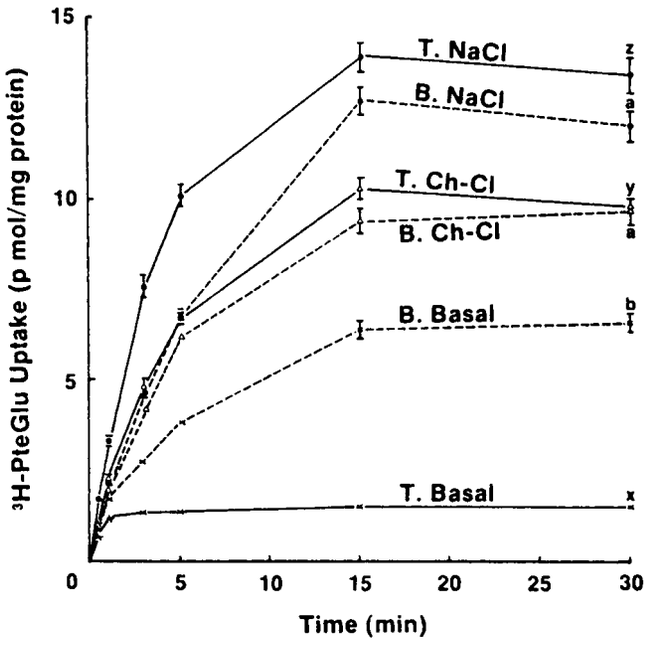

Figure 3. Effect of inwardly directed gradients of NaCl and choline chloride on PteGlu uptake by renal brush border membranes vesicles in the presence of a pH gradient (7.3 intravesicular/5.6 extravesicular). Membrane vesicles were resuspended in mannitol buffer (100 m*M* mannitol, 50 m*M* Hepes, pH 7.3) and aliquots (about 0.1 mg of protein) were incubated in medium (0.16 μM [^{3}H]PteGlu, 50 m*M* MES, pH 5.6) containing either 100 m*M* mannitol (basal) (×), 50 m*M* NaCl (●), or 50 m*M* choline chloride (△) for different time intervals before filtration to analyze the uptake as described in Materials and Methods. Binding (dashed lines) and transport (solid lines) values were determined as described in Materials and Methods. Values represent the mean ± SE (n = 3). Means with a superscript in common (binding = abc; transport = xyz) were not significantly different from each other with an overall protection level of 0.95 (Duncan's multiple range test). Comparisons at 30 sec were the same as those at 30 min and were not included on the figure for clarity.

Table I. Effect of an Electrogenic Potential on PteGlu Binding and Transport by Rat Renal BBMV[a]

Time (min)	Binding (pmol PteGlu/mg protein)		Transport (pmol PteGlu/mg protein)	
	K^+ gradient	No gradient	K^+ gradient	No gradient
0.5	11.6 ± 2.2	9.4 ± 1.5	1.02 ± 0.11	1.08 ± 0.10
30.0	32.0 ± 4.3	27.5 ± 1.2	5.20 ± 0.80	4.98 ± 0.22

[a] Incubations were designed to establish a transmembrane electrical potential (relatively positive inside) in the absence of a pH gradient. K^+ gradient signifies that BBMV were suspended in mannitol buffer (pH 5.6, no K^+) containing valinomycin (10 μg/mg protein) and then folate uptake was measured in incubation medium (pH 5.6) containing 50 m*M* KCl. No gradient signifies that BBMV were suspended in mannitol buffer containing 50 m*M* KCl (100 m*M* mannitol was removed) in addition to valinomycin. Values represent the mean ± SE (n = 3). There were no statistically significant differences between values with and without gradient (Student's t test, $P < 0.05$).

uptake in the absence of a K^+ gradient ([K^+]) in and out at 50 m*M*, plus valinomycin), i.e., under voltage-clamped conditions. Results in Table I show that neither PteGlu binding nor transport was affected by the presence of an inwardly directed positive gradient.

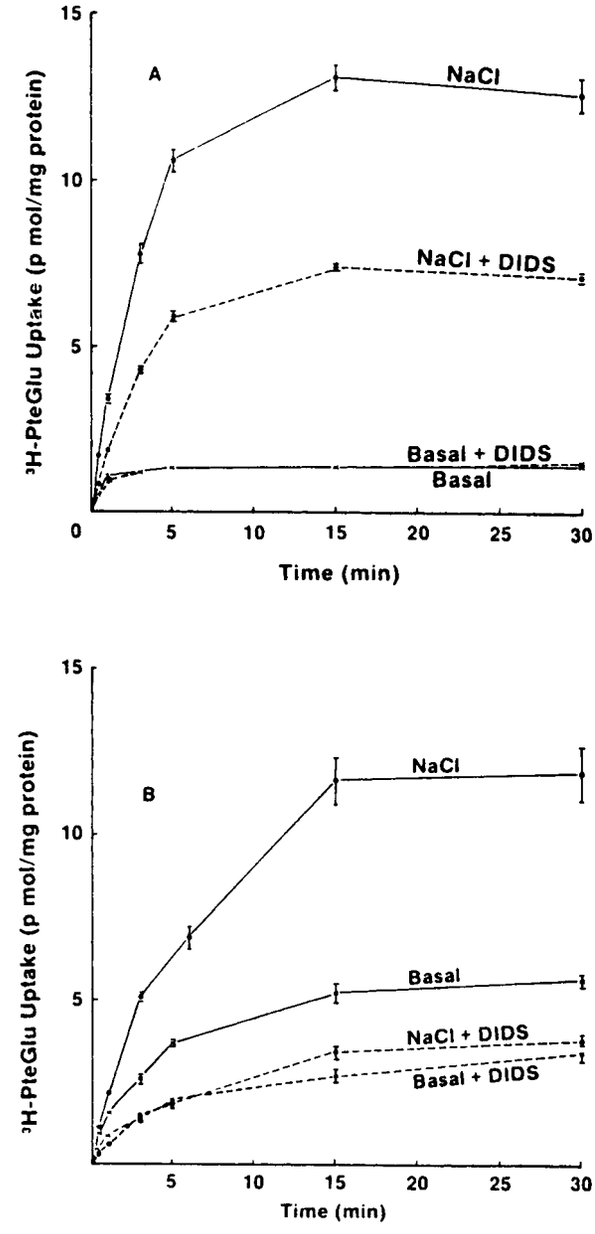

Figure 4. Effect of DIDS on PteGlu uptake by renal brush border membrane vesicles in the presence of a pH gradient (7.3 intravesicular/5.6 extravesicular). Membrane vesicles were resuspended in mannitol buffer (100 m*M* mannitol, 50 m*M* Hepes, pH 7.3) and aliquots (about 0.1 mg of protein) were incubated in medium (0.16 μM [^{3}H] PteGlu, 50 m*M* MES, pH 5.6) containing either 100 m*M* mannitol (basal) (×) or 50 m*M* NaCl (●) with (dashed lines) and without (solid lines) DIDS at 1 m*M* for different time intervals. Transport (A) and binding (B) values were determined as described in Materials and Methods. Values represent the mean ± SE (n = 3). All means with DIDS were significantly different from respective means without DIDS (Student's t test, $P < 0.05$), except for transport values under basal conditions where DIDS had no effect.

Earlier studies on the transport of PteGlu into rat, rabbit, and human intestinal BBMV (10, 11) have shown that the process is inhibited by anion exchange inhibitors. The effects of 4,4′-diisothiocyano-2,2′-disulfonic acid stilbene (DIDS) on PteGlu uptake by renal BBMV were studied in the presence of a pH

gradient (in/out = 7.3/5.6), with and without an inward gradient of NaCl (50 m*M*). The results in Figure 4 (A, transport; B, binding) show that pH gradient-mediated PteGlu transport was markedly (44%) inhibited by DIDS, but only in the presence of an inward gradient of NaCl. In contrast to its effects on transport, DIDS altered binding by renal BBMV in basal medium as well as in the presence of NaCl.

Discussion

This study has shown that the acid pH stimulation of folate transport by rat kidney BBMV (8) resulted from the presence of the transmembrane pH gradient (7.3 in/5.6 out). Acidic pH per se did not increase transport. Transport was a major portion of the total PteGlu uptake by renal BBMV in the presence of the pH gradient and, in its absence, PteGlu binding was the major contributor. The presence of certain ions (Na^+ and/or Cl^-) in the extravesicular medium was also needed for maximal transport of folate by renal BBMV. This effect of ions on PteGlu transport into BBMV occurred only in the presence of the pH gradient and was associated with the concomitant stimulation of PteGlu binding to the BBM. The effects of NaCl and choline chloride, whether involving Na^+ and choline as cations or Cl^-, remain to be clarified. At pH 5.6 (in = out), NaCl and choline chloride markedly stimulated binding, but did not increase transport. Thus, stimulation of PteGlu binding by these ions might provide a basis for transport, but increased binding itself was not sufficient for increased transport of PteGlu. We can speculate that PteGlu transport across the renal BBM first involves association with a specific membrane protein (4, 5), which is regulated by the presence of various ions (13). A second activity involved in folate transport would be the pH gradient-mediated transfer of folate across the membrane. These studies were carried out at a folate concentration of 160 n*M*, which would be slightly above the physiologic range for rat plasma (about 60–130 n*M*) (18). In this range, folate is primarily reabsorbed by the renal proximal tubule (1) so that the renal BBMV transport of folate would represent a model of the reabsorptive process.

In terms of the effects of ions (NaCl and choline chloride) and pH on PteGlu binding by renal BBMV, the results are more complex. In the absence of pH or ionic gradients (in basal media), the amount of the PteGlu binding was similar at pH 7.3 and pH 5.6 at 30 min of incubation, although the rate of binding was apparently faster at pH 5.6. The increased rate of PteGlu binding at pH 5.6 may have been due to an acid-induced release of endogenously bound folates, which would open more binding sites for PteGlu (5, 13). In our study, BBMV used were not acid treated before incubation so that we could study the characteristics of the transport and binding of PteGlu in freshly prepared, untreated membrane vesicles. The effects of NaCl and choline chloride on binding at pH 7.3 (in = out) were distinctly different from those at pH 5.6 (in = out). Unlike the stimulation at pH 5.6, NaCl and choline chloride caused a remarkable decrease in PteGlu binding by renal BBMV at pH 7.3. These results are consistent with that of Selhub *et al.* (13), who suggested that this inhibition by ions was due to an anion-induced stabilization of the endogenous folate-binding protein complex. Such a stabilization would reduce the rate of exchange with exogenous labeled PteGlu.

In the method of osmotic pertubation (15, 16), the response of BBMV to exposure to external osmolality is used to distinguish binding from intravesicular transport. In the present study, it is possible that the vesicles exposed to acidic pH could have responded differently to external osmolarity. However, Pratz *et al.* (19) have shown that the rate of shrinkage of rat renal BBMV due to exposure to increasing osmolarity is not affected by an acidic pH. In the present use of osmotic pertubation, PteGlu transport was the same in the absence of a pH gradient whether vesicles were prepared at pH 5.6 or 7.3, yet increased values were noted at the latter pH when a gradient existed. PteGlu binding values were also differentially affected by ionic gradients at different pH (Figs. 1 and 2). Thus, the osmotic pertubation technique could detect changes in binding and transport under various conditions, irrespective of the pH.

This study has shown that the pH dependency of PteGlu transport into renal BBMV was related to a pH gradient-mediated mechanism. Studies of PteGlu uptake at pH 5.6 or at pH 7.3 with no gradient showed that PteGlu transport was much smaller than that observed with the pH gradient (7.3 in/5.6 out). These data suggest that the acidic extravesicular pH of 5.6 was not producing a direct stimulatory effect on the PteGlu transport mechanism and that the intravesicular pH 7.3 was not important per se in folate transport. The stimulation of PteGlu transport by the pH gradient was not due to an electrical coupling mechanism because PteGlu transport under the influence of an inside-positive electrical potential (inwardly directed K^+ gradient in the presence of the K^+ ionophore valinomycin) was not different from that under voltage-clamped conditions (absence of K^+ gradient and presence of valinomycin).

Transmembrane pH gradients apparently energize uptake of folates in intestinal BBMV from rabbits (10) and humans (11) and in isolated rat hepatocytes (12). According to Horne (12), the role of H^+ in stimulating transport like that observed in the present study may be related to a cotransport mechanism or to a coupling mechanism, by which H^+ interacts with the carrier to stimulate folate transport without a concomitant H^+

transport. Mason *et al.* (20) suggested that the enhancement of PteGlu transport into rat intestinal BBMV and into a colon carcinoma cell line (CaCo-2) in the presence of an inward H^+ gradient may result from pH-associated changes in the apparent affinity of folates for the carrier (folate-binding protein). Rothberg *et al.* (7) concluded, on the basis of their studies on a monkey kidney cell line (MA 104), that folates move across the membrane via a transport mechanism, after binding to a folate receptor, by using the energy generated from a H^+ gradient. Thus, there are several possibilities by which the H^+ gradient could stimulate folate transport into different cells. Our results definitely suggest a role for the H^+ gradient in PteGlu transport into renal BBMV, but further studies are required to define the specific mechanism of the H^+ in this transport mechanism. The marked inhibition of PteGlu transport observed with DIDS in the presence of the pH gradient might indicate a possible role of an anion exchange mechanism in folate transport. But this conclusion is clouded by the fact that PteGlu binding to renal BBMV was also inhibited by DIDS and thus the effect of DIDS on folate transport may not necessarily result from an inhibition of anion exchange. Organic anion exchange mechanisms for PteGlu transport across cellular membranes have been suggested for numerous systems, including intestinal BBMV (10, 11), L1210 cells (21), and bacterial cells (22).

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