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Folate binding and transport by rat kidney brush-border membrane vesicles

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[^{3}H]Pteroylglutamic acid (PteGlu) uptake was studied using brush-border membrane vesicles isolated from rat kidney. Results on the uptake of [^{3}H]PteGlu by brush-border membrane vesicles incubated in media of increasing osmolarities demonstrated that uptake was contributed by two components, intravesicular transport and membrane binding. Both the components of the uptake exhibited similar pH dependence, with maxima at pH 5.6, and were found to be saturable mechanisms with K_m values of $6.7 \cdot 10^{-7}$ and $11.2 \cdot 10^{-7}$ M, respectively. These studies show that PteGlu is transported by isolated rat kidney brush-border membrane vesicles in a manner consistent with a saturable system and that a binding component may be functionally associated with this.

Introduction

The cell membrane of several tissues possesses a high-affinity folate-binding protein which may be involved in the transmembrane transport of folates [1,2]. The kidney is particularly enriched in a folate-binding protein that is associated with the brush-border membranes of tubular epithelial cells [1,3,4]. The folate-binding protein of the renal tubular cell membrane may play an important role in preventing urinary loss of folate by participation in folate reabsorption from the tubular fluid [1,3,5]. Control of urinary folate excretion is important since changes in it may help to regulate plasma folate levels. Acute ethanol treatment of rats produces a dose-dependent increase in urinary folate excretion [6], in amounts that would account for a subsequent decrease in plasma folate levels [7]. Other studies in pregnancy and in renal failure have also implicated changes in urinary folate levels with changes in plasma folate concentrations [8,9].

One model for renal folate reabsorption suggests that the cellular uptake of folate is mediated by a membrane carrier that either facilitates the diffusion of folate across the membrane or actively transports the molecule into the cell [10]. The renal brush-border transport of folate and its relationship to membrane-binding activity have not been determined previously. The present study was designed to examine the uptake of [^{3}H]PteGlu by isolated rat renal brush-border membrane vesicles and to determine the transport activity within the vesicles.

Abbreviations: PteGlu, pteroylglutamic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 4-morpholineethanesulfonic acid.

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Materials and Methods

Isolation of brush-border membrane vesicles from rat kidney cortex. Kidney cortices from unfasted male Sprague-Dawley rats weighing 300–400 g (obtained from Harlan, Houston, TX and maintained on Purina rat nonpurified diet) were removed and homogenized in 20 vol. (v/w) sucrose buffer (0.25 M sucrose/0.1 mM phenylmethylsulfonyl fluoride/10 mM Tris-Cl (pH 7.6) with a Potter-Elvehjem homogenizer for 10 strokes. The homogenate was fractionated for brush border isolation by a modification of the methods described by Sacktor et al. [11] and Molitoris and Simon [12]. The homogenate was subjected to centrifugation at $24000 \times g$ for 20 min and a fluffy layer was obtained over the solid pellet. This layer was resuspended in 4 ml of sucrose buffer; to this, $MgCl_2$ was added to 15 mM, and the mixture allowed to stand for 20 min at 0 °C with occasional stirring. The solution was centrifuged at $3000 \times g$ for 10 min, the pellet obtained was discarded and the supernatant was respun at $26000 \times g$ for 20 min. The resulting brush-border pellet was resuspended in 100 mM mannitol/and 10 mM potassium/Hepes buffer (pH 7.3) with eight strokes in a Potter-Elvehjem homogenizer. The last step was repeated once again and the final pellet of brush border was resuspended in mannitol buffer to contain protein at 4–5 mg/ml. Uptake of D-[1^{14}C]glucose, L-[1^{14}C]glutamate and L-[1^{14}C]glutamine was studied as described by Aronson and Sacktor [13] and Sacktor et al. [11].

PteGlu uptake by brush-border membrane vesicles. The method to study the uptake of PteGlu by brush border membrane vesicles was based on that described by Selhub and Rosenberg [14] for intestinal brush-border membrane vesicles. The uptake of [^{3}H]PteGlu was determined by adding about 0.1 mg protein of membrane vesicles in 0.085 ml final volume with 0.16 μM [^{3}H]PteGlu/50 mM NaCl/1 mM $MgSO_4$ and a solution containing 50 mM Mes or Hepes of the desired pH. Incubations were done at room temperature and started by the addition of the vesicles to a solution containing the other ingredients. After the desired period of incubation, an aliquot of 0.025 ml was removed and added into 1 ml of ice-cold stop solution (50 mM NaCl, 1 mM $MgSO_4$, 50 mM Mes or Hepes of a pH same as the incubation buffer), followed by rapid vacuum filtration through membrane filters. Then, the filter with retained vesicles was washed with 3 ml of stop solution and dried at room temperature. The radioactivity on the filters was determined by liquid scintillation counting using 10 ml of complete counting cocktail. The control incubations were performed by incubation of the vesicles with [^{3}H]PteGlu in 1 ml of ice-cold stop solution prior to the filtration described above. Control incubation values were subtracted from each sample incubation value to obtain the folate uptake value. To distinguish PteGlu binding and transport, a standard procedure of osmotic perturbation [15,16] was used, in that [^{3}H]PteGlu uptake was assayed in hyperosmolar media with the incubation mixture containing 300 mM mannitol in addition to the constituents described above. The stop solution also contained 300 mM mannitol in addition to the constituents described above.

Enzyme assays. γ-Glutamyltransferase was assayed by the method described by Glossman and Neville [17], alkaline phosphatase by the method of Lansing et al. [18], ($Na^+ + K^+$)-ATPase as described by Quigley and Gotterer [19].

Materials. [^{3}H]PteGlu (34.0 or 54.5 Ci/mmol) was purchased from Amersham (Arlington Heights, IL) and used as obtained. This was 97–98% pure, as described by the manufacturer and confirmed by our HPLC analysis of the stock solution [20]. γ-Glutamyl-*p*-nitroanilide was from Calbiochem. Protein was determined with the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA). All other chemicals were from Sigma 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 from Research Products International Corporation (Mount Prospect, IL).

Results

In order to investigate the renal transport of folate, brush-border membrane vesicles from rat kidney cortex were prepared as detailed in Materials and Methods. The recovery and specific activity of various marker enzymes were measured in

the brush-border fraction to assess the quality of the preparations. γ-Glutamyltransferase and alkaline phosphatase, the markers of the brush border, were enriched 13.1 ± 1.2- and 10.4 ± 0.8-fold, respectively (mean ± S.E., $n = 9$) whereas enrichment of $(Na^+ + K^+)$-ATPase, a marker of basolateral membranes, was negligible in this fraction. Recoveries of alkaline phosphatase and γ-glutamyltransferase were 13.9 ± 1.9 and 16.5 ± 1.0, respectively, whereas that of $(Na^+ + K^+)$-ATPase was only 2%. The enrichment and recovery of marker enzyme activity for mitochondria (cytochrome oxidase), microsomes (glucose-6-phosphatase) and cytosol (lactate dehydrogenase) were also estimated in the brush-border fraction and were found to be minimal. Contamination due to nuclei was assessed by assaying for DNA and was also found to be minimal.

The brush-border membrane vesicles were also evaluated functionally by testing their uptake of well-studied organic substrates. Uptake of D-[1-^{14}C]glucose by brush-border membrane vesicles was studied in incubation media containing 100 mM NaCl or choline chloride (inwardly directed gradients of NaCl or choline chloride) and the results are present in Fig. 1. Glucose uptake by brush-border membrane vesicles was stimulated by an inwardly directed electrochemical gradient of Na^+ and exhibited the characteristic 'overshoot' phenomenon [13]. Uptake of L-[^{14}C]glutamate and that of L-[^{14}C]glutamine by brush-border membrane vesicles was also studied in the presence of an inwardly directed gradient of NaCl or choline chloride. Uptake (pmol/mg protein) of L-[^{14}C]glutamate with NaCl, at incubation times of 0.5 and 5 min, was 163 ± 80 and 186 ± 51, respectively, and uptake with choline chloride (replacing NaCl) was 63 ± 6.2 and 85 ± 7.5, respectively (observations based on three independent experiments). Uptake (pmol/mg protein) of L-[^{14}C]glutamine with NaCl, at incubation times of 0.5 and 5 min, was 318 ± 37 and 349 ± 50 and

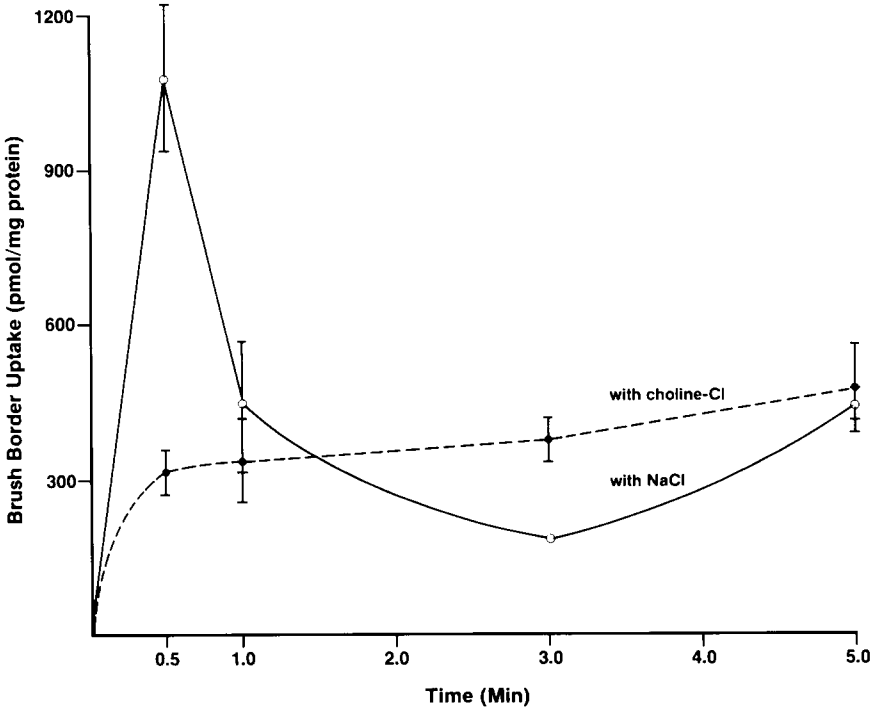

Fig. 1. D-Glucose uptake by brush-border membrane vesicles. The brush-border membrane vesicles were incubated at room temperature in incubation medium containing 100 mM mannitol, 5 mM Hepes-Tris (pH 7.3) and 750 μM D-[^{14}C]glucose with 100 mM NaCl (———) or choline chloride (— — —) for different time intervals and then analyzed for amount of radioactivity taken up by rapid vacuum filtration technique [13]. Values expressed as the mean ± S.E. ($n = 3$).

uptake with choline chloride (replacing NaCl) was 93 ± 7 and 153 ± 19, respectively, (observations based on nine independent experiments). Thus uptakes of L-glutamate and L-glutamine by brush-border membrane vesicles were also stimulated by inward Na^+ gradients. These results suggest the physiological functionality of the membrane vesicles.

Brush-border membrane vesicles are osmotically active vesicles, i.e., they respond to changes in medium osmolarity [15]. This characteristic of brush-border membrane vesicles has been widely used to differentiate between binding of the substrate to the membrane and transport into the intravesicular space [16]. The general procedure for this purpose [15,16] has been to measure the vesicular uptake under conditions of increasing external osmolarity and to extrapolate the data to infinite osmolarity. Preliminary studies on the effect of pH of the extravesicular medium on [^{3}H]PteGlu uptake by brush-border membrane vesicles (intravesicular pH, 7.3) suggested maximal uptake at pH 5.6. Subsequent studies on uptake were therefore carried out at this pH in the extravesicular medium. To determine whether the uptake of [^{3}H]PteGlu by brush-border membrane vesicles represented transport into an intravesicular space or binding to the membranes, the vesicles were incubated with 0.16 μM [^{3}H]PteGlu for 0.5 minutes in media of increasing osmolarities, achieved by addition of mannitol to decrease the intravesicular space [21]. As shown in Fig. 2, a plot of [^{3}H]PteGlu uptake versus 1/osM was linear and the uptake of [^{3}H]PteGlu decreased as osmolarity increased, indicating transport of [^{3}H]PteGlu into an intravesicular space. However, extrapolation of the regression line to infinite osmolarity, and a presumed intravesicular volume of zero, suggested a binding component of 2.60 pmol/mg protein. This binding component would

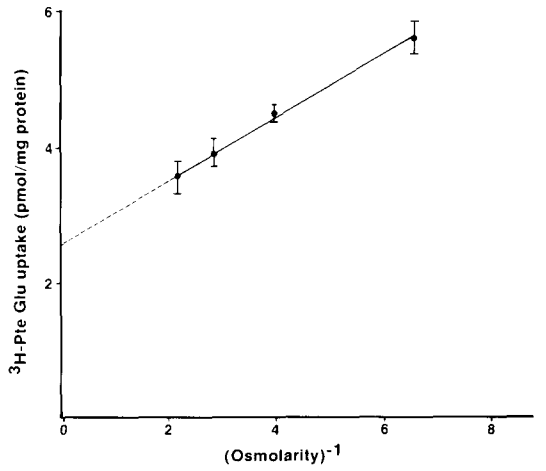

Fig. 2. Effect of increasing osmolarity on the uptake of PteGlu. Aliquots of brush-border membrane vesicles suspension (about 0.1 mg protein) were incubated in mixtures (0.16 μM [^{3}H]PteGlu/50 mM NaCl/1 mM $MgSO_4$/50 mM Mes (pH 5.6) containing increasing concentrations of mannitol) for 0.5 min before filtration to analyze the uptake as described in Materials and Methods. Uptake values mean $\pm$ S.E. ($n = 3$), were plotted against the reciprocal of total osmolarity.

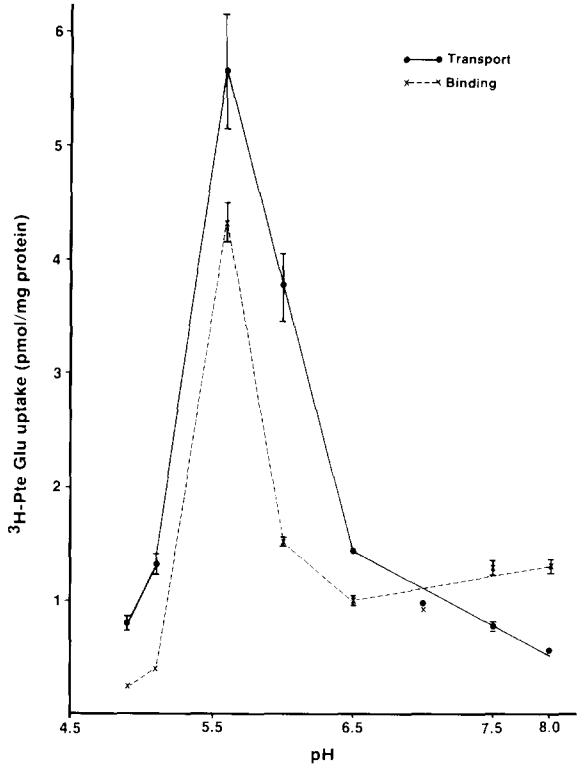

Fig. 3. pH dependency curves of PteGlu uptake by brush-border membrane vesicles. Membrane vesicles (about 0.1 mg protein) were incubated in mixtures (containing 0.16 μM [^{3}H]PteGlu, 50 mM NaCl, 1 mM $MgSO_4$, 50 mM Mes or Hepes of the desired pH, either with or without 300 mM mannitol) for 1 min and analyzed for amount of radioactivity taken up. Separate control incubations were run for each pH value. Binding (— — —) and transport (———) values were calculated as described in text. Values expressed as the mean $\pm$ S.E. ($n = 3$).

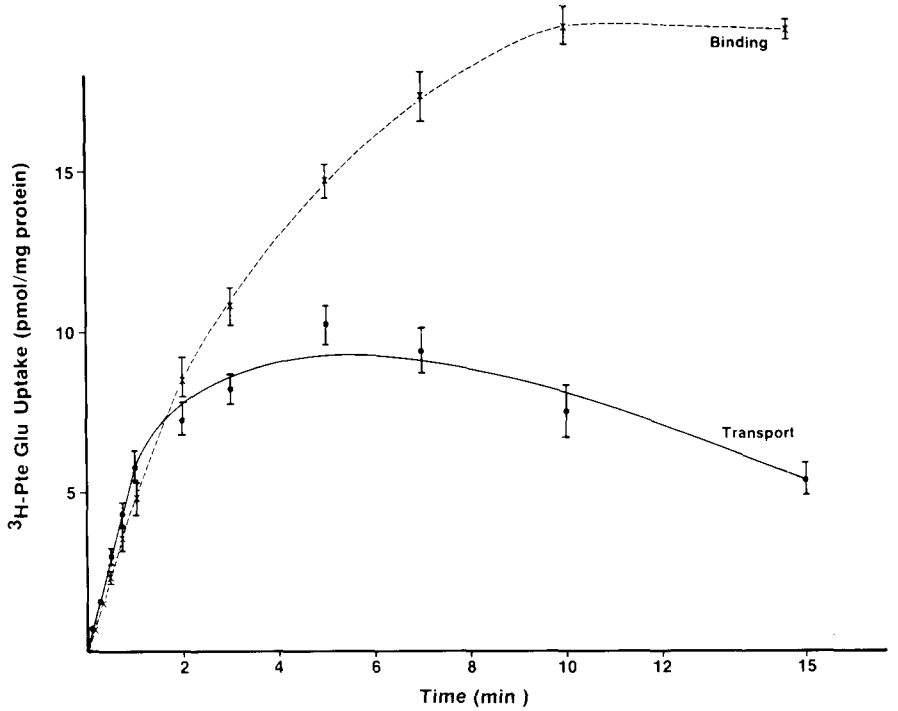

Fig. 4. Time dependency of PteGlu uptake by brush-border membrane vesicles. Membrane vesicles (about 0.1 mg protein) were incubated in a mixture (containing 0.16 μM [^{3}H]PteGlu/50 mM NaCl/1 mm $MgSO_4$/50 mM Mes (pH 5.6) with and without 300 mM mannitol) for different time intervals and analyzed for amount of radioactivity taken up. Separate control incubations were run for each time point. Binding and transport values were calculated as described in text. Values expressed as the mean $\pm$ S.E. ($n = 3$).

account for 46% of the total uptake of [^{3}H]PteGlu (at pH 5.6), with transport accounting for the remaining. On the basis of this experiment, [^{3}H]PteGlu uptake by brush-border membrane vesicles was investigated with and without increased osmolarity of media (6.6 and 2.2 osM^{-1}) and the binding component was determined by extrapolation of the data to infinite osmolarity. Transport represented the difference between total uptake and binding. This scheme was a common protocol in subsequent studies.

To determine the effect of pH of the extravesicular medium on brush-border membrane vesicles uptake of [^{3}H]PteGlu, the vesicles were incubated with 0.16 μM of [^{3}H]PteGlu for 1 min in extravesicular media made to varying pH. The studies described in Fig. 3 show that brush-border [^{3}H]PteGlu binding as well as intravesicular transport were pH-dependent, with a sharp peak at pH 5.6.

The time course of [^{3}H]PteGlu uptake by brush-border membrane vesicles at 0.16 μM PteGlu concentration is shown in Fig. 4. The binding as well as intravesicular transport were initially rapid and linear for 1 min of incubation. The binding continued to increase till 10 min of incubation and then remained unchanged, whereas the amount of intravesicular transport increased till 5 min and decreased thereafter. PteGlu binding as a percentage of total uptake remained between 45 and 49% during the initial 1 min of incubation, but after that it gradually increased to a value of 78% at 15 min of incubation.

To examine the effect of PteGlu concentration on brush-border membrane vesicles uptake, vesicles were incubated in media containing [^{3}H]PteGlu of the desired concentration for 0.5 min. The relationship between uptake and substrate concentrations under conditions which approximate initial velocity is shown in Fig. 5.

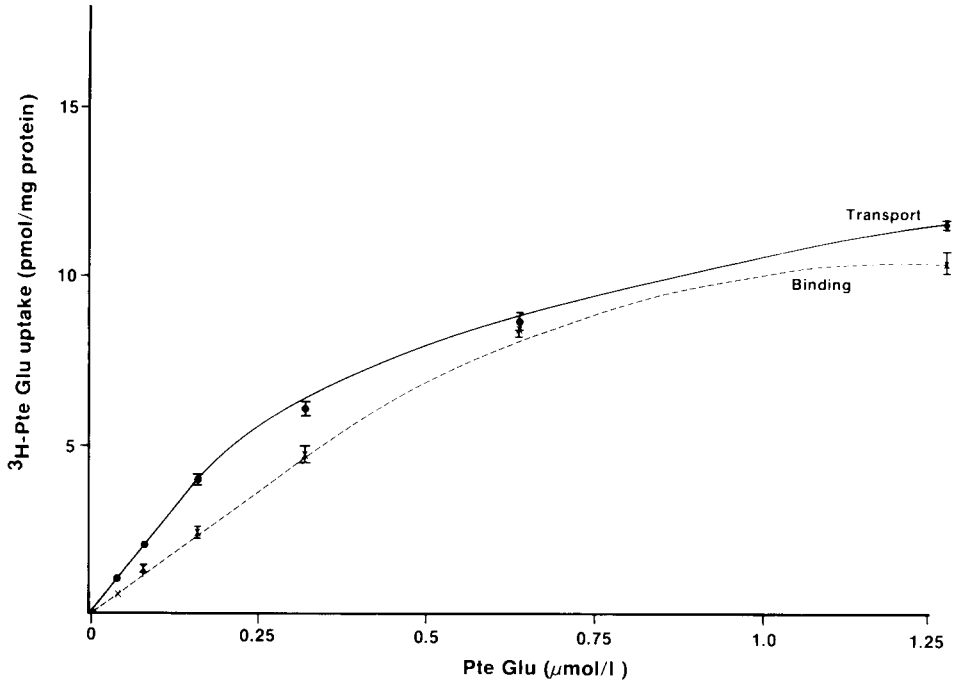

Fig. 5. Concentration dependency curve of PteGlu uptake by brush-border membrane vesicles. Results were obtained from membrane vesicles (0.1 mg protein) which were incubated for 0.5 min with increasing concentrations of PteGlu in mixtures (50 mM NaCl/1 mM $MgSO_4$/50 mM Mes (pH 5.6) with and without 300 mM mannitol) and analyzed for radioactivity taken up. Separate control incubations were run for each folate concentration. Binding (— — —) and transport (———) values were calculated as described in text. Values expressed as the mean ± S.E. ($n = 3$).

[^{3}H]PteGlu binding as well as intravesicular transport were saturable functions of the external [^{3}H]PteGlu concentration. A double-reciprocal plot of binding and transport as a function of the external PteGlu concentration gave a straight line. From these data, K_m values for binding and transport were $11.2 \cdot 10^{-7}$ and $6.7 \cdot 10^{-7}$ M, respectively, whereas V_{max} values were 20.3 and 19.2 pmol/mg protein, respectively.

Discussion

These studies demonstrate transport of PteGlu by isolated rat kidney brush-border membrane vesicles in a manner consistent with a saturable system and that a binding component may be functionally associated with the transport. The demonstration of PteGlu transport by renal brush-border membrane vesicles in the present study is important because binding to the renal brush-border membrane has been reported in earlier studies [3,4,22], but this is the first demonstration of intravesicular transport of PteGlu by renal brush-border membrane vesicles. This in vitro model may be useful for studies of transmembrane transport of folate by renal proximal tubular luminal membranes.

These studies of the uptake of PteGlu by brush-border membrane vesicles under conditions of increasing external osmolarity suggested that the uptake was contributed by two distinct components, intravesicular transport and membrane binding. The presence of a significant binding component as a major contributor to PteGlu uptake by renal brush-border membrane vesicles was expected because high-affinity folate-binding protein activity has been demonstrated in renal brush border membranes in several earlier studies [3,4,22]. Such studies first proposed a role for this protein in folate transport, although no transport activity was reported in those studies. Our studies have shown that the two components of PteGlu uptake by renal brush-border membrane vesicles, i.e., binding and transport, share similar character-

istics in initial rates, pH maxima and dependence on substrate concentration.

During the initial 1 min of uptake, membrane binding of PteGlu represented about 45% of the total uptake; this percentage increased with increasing incubation time, suggesting that, with time, the relative amount of transport decreased. Assuming some gradient (ionic, electrochemical, etc) powers this transport, one explanation of this relative change in binding and transport could be a time-related dissipation of the gradient. A similar time-related increase in binding has been reported by Hsu et al. [16], who observed, by osmotic perturbation and other methods, that the percentage of cystine bound by rat renal brush-border membrane vesicles increased considerably after 1 min of incubation.

Renal brush-border PteGlu binding as well as intravesicular transport were pH-dependent, both exhibiting a sharp peak at pH 5.6 of extravesicular medium. In an earlier study [1] on rat kidney brush-border membranes, folate binding was found to increase sharply above pH 5.0, with a maxima at pH 6.4–7.7. However, Kamen and Caston [22] observed a broader maximum binding of PteGlu to porcine kidney folate binding protein at pH 5.5–7.6. In both studies, brush-border membranes were prepared using different techniques and were acid-treated prior to binding; such differences in methodology from our studies may account for the differences in observed pH maxima. The physiological relevance of the acidic pH optima for transport observed in the present study remains unknown. No studies have been done in the kidney, but in studies on rat intestine, an acid microclimate (pH around 5.5–5.8) immediately adjacent to the brush-border membrane has been documented [23]. The presence of such a layer near the membrane could support a greater amount of transport in the acidic pH range. Another explanation for the acidic maxima could be that the transport of folate across the membrane could be driven by a limited in/out pH gradient [24], such as that of 7.3/5.6 in our studies. Schron et al. [25] have observed that an outwardly directed (OH^-) gradient can stimulate PteGlu uptake by intestinal brush-border membrane vesicles. Clarification of the role of other ionic and electrochemical gradients is needed before renal folate transport can be associated with a pH gradient.

PteGlu binding as well as transport by brush-border membrane vesicles were found to be saturable mechanisms. The K_m for binding was $11.2 \cdot 10^{-7}$ M and that for vesicular transport was $6.7 \cdot 10^{-7}$ M. Since the K_m values are close to the concentration of folate in rat serum [7], and hence, in the glomerular filtrate, folate binding and transport by kidney brush-border membrane should be efficient under physiological conditions. The K_m value obtained for transport of PteGlu by brush-border membrane vesicles agrees well with those reported in other mammalian systems. The K_m reported for PteGlu transport by rat intestinal brush-border membrane vesicles [14] is $4.2 \cdot 10^{-7}$ M, that for rat hepatocytes [26] is $6.6 \cdot 10^{-7}$ M, and that for human erythrocytes [27] is $7.5 \cdot 10^{-7}$ M. However, K_m values reported in earlier studies [1,3] for renal brush-border membrane binding of PteGlu are lower than that obtained in the present study. The difference probably is related to the acid treatment given by earlier workers to brush-border membranes in order to remove endogenous bound folate and to release other binding sites of higher affinity for folic acid. Corrocher et al. [3] have shown that, prior to acid treatment of renal membranes, there exist both high- and low-affinity binding sites. Selhub et al. [28] have shown that the untreated membranes contain binding sites that are partially occupied with reduced folate derivatives, and that acid stripping enhances the binding activity of these sites. Our studies were designed to determine the brush-border membrane vesicles transport of folic acid, and, thus, acid treatment of the membrane was not done as it could have affected the transport property of the brush-border membrane vesicles. As such, our studies of binding would include the low-affinity sites present in the native membrane.

The similar characteristics of PteGlu binding and transport by brush-border membrane vesicles found in the present study may indicate a role of binding in renal epithelial cellular transport of folate across the brush-border membrane. A similar suggestion has earlier been made by Selhub and co-workers [1,4], Corrocher et al. [3] and Kamen and Capdevila [29]. Reisenauer et al. [21] also observed similar characteristics of folate binding and transport by intestinal brush-border mem-

brane vesicles and concluded that there is a role of the binding protein in the intestinal transport of PteGlu. The characteristics of the folate transport in this study suggest that a major component is carrier-mediated. However, the data do not exclude a contribution from simple diffusion.

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