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Folate Transport by Prawn Hepatopancreas Brush-Border Membrane Vesicles

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The transport system of folic acid (Pte-Glu) by brush-border membrane vesicles (BBMV) isolated from prawn (*Penaeus japonicus*) hepatopancreas, was studied by measuring the uptake of Pte-Glu. This uptake was found to have two components, intravesicular transport and membrane binding. Membrane binding was not affected by the presence of a transmembrane pH-gradient at a short incubation period. However, a transmembrane pH-gradient increased membrane binding at 60 min. The transport of Pte-Glu appeared to be carrier-mediated, was stimulated by an inwardly proton gradient (pH 5.5 outside, 7.4 inside) and was unaffected by a sodium-gradient. The relationship between pH gradient-driven Pte-Glu uptake and medium Pte-Glu concentration followed saturating Michaelis–Menten kinetics. Eadie–Hofstee representation of the pH gradient-driven Pte-Glu uptake indicated a single transport system with a Km of 0.37 μM and Vmax of 1.06 pmol/mg protein/15 s. These findings indicate that BBMV isolated from prawn hepatopancreas possesses a Pte-Glu transport system similar to that described in mammalian intestine.

KEY WORDS: Folate transport; prawn; hepatopancreas; brush-border membrane vesicles; kinetics.

INTRODUCTION

Folic acid is an important vitamin that is required for normal cellular growth and function. This vitamin is found as a natural source in the diet and usually is constituted by a complex mixture of pteroylglutamates with different chain lengths of the poly-γ-glutamyl moiety, and different functional groups on the pteridine ring. More than 90% of dietary folates are found as 5-methylpteroylpolyglutamates, while 10-formylpteroylglutamates are the remainders (Stokstad *et al.*, 1977). Folate absorption requires a hydrolysis step where the pteroylpolyglutamates are transformed to pteroylmonoglutamates, which is just the form to be taken up by the intestinal mucosa (Halsted, 1979). Studies of folate transport in intestinal tissues from mammals indicate that the intestinal absorption of folate seems to be saturable and pH-dependent, and takes place by a specific transport system. Thus, previous studies

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with apical membrane vesicles from rat intestine have shown that the transport of methotrexate (amethopterin) and pteroylmonoglutamic acid is saturable and pH-dependent with a maximal rate of uptake at pH 5 (Selhub and Rosenberg, 1981). In addition, the transport of pteroylmonoglutamic acid into apical membrane vesicles from rabbit intestine is also stimulated by a pH gradient and markedly reduced by anion exchange inhibitors, suggesting the presence of hydroxyl-folate exchangers (Schron *et al.*, 1985). However, there is a lack of information about the existence of a folate specific transport system in crustaceans. In the current work we depict the transport of Pte-Glu by hepatopancreatic brush-border membrane vesicles (hBBMV) from the prawn, *Penaeus japonicus*.

MATERIALS AND METHODS

Chemicals

(^{3}H) Pte-Glu was purchased from Amersham and used as obtained. This was 97–98% pure, as described by the manufacturer and confirmed by high-performance liquid chromatography analysis of our stock solution. All other chemicals were from Sigma (St. Louis, MO) and were of analytical grade.

Animals

Adult prawns were purchased from commercial dealers (Cultivos Piscicolas Marinos S.A., San Fernando, Cádiz; and Langostinos de Huelva S.A., Cartaya, Huelva). They were transported alive to the laboratory, the wet weight and total length were recorded and midgut glands were then dissected out.

Brush-Border Membrane Vesicles Preparation

Hepatopancreatic brush-border membrane vesicles (hBBMV) were prepared from fresh tissue removed from individual prawns. Each membrane batch was produced from 20 organs by the method of Ahearn *et al.* (1985) for lobster hepatopancreas, with minor modifications. Hepatopancreata were minced on ice and added to a buffer containing 300 mM sucrose, 5 mM EGTA, 0.1 mM PMSF, 12 mM Tris/HCl (pH 7.4). This slurry was made hypotonic by the addition of distilled water and then homogenized using a Polytron provided with a polytron-aggregate type PTA 10S. After centrifugation for 30 min at 27,000g, the resulting pellet 1 was resuspended using a Potter–Elvehjem homogenizer in a buffer containing 300 mM sucrose and 12 mM Tris/HCl (pH 7.4), and centrifugation for 30 min at 27,000g, to remove soluble enzymes released by cellular disruption. Pellet 2 was resuspended in a buffer containing 300 mM sucrose and 12 mM Tris/HCl (pH 7.4), $MgCl_2$ added to a final concentration of 10 mM, and the resulting solution was allowed to settle on ice for 15 min. After a first centrifugation for 15 min at 3000g, the pellet 3 was discarded and the supernatant was again centrifuged for 30 min at 27,000g. Pellet 4 was resuspended in buffer containing 60 mM sucrose, 5 mM EGTA and 12 mM Tris-HCl, (pH 7.4), and the $MgCLl_2$ precipitation was repeated on this mixture, followed by

centrifugation for 15 min at 3000g. The pellet 5 was discarded and the supernatant was again centrifuged for 30 min at 27,000g. Pellet 6 was resuspended in buffer containing 30 mM sucrose and 12 mM HEPES/Tris, (pH 7.4) and finally centrifuged at 27,000g for 30 min. The resulting pellet, pellet 7, contained purified hBBMV.

Enzyme Assays

Purity of hBBMV was assessed by comparing the activities of membrane-bound enzymes in pellet 7 with the activities of the same enzymes in the resuspension of pellet 2 before addition of $MgCl_2$. Soluble digestive enzymes were recovered in supernatants 1 and 2; therefore, enzyme activities remaining in pellet 2, after two washings, represent the original total membrane-bound contribution of these proteins. Previous histochemical studies have indicated that alkaline phosphatase is localized at the brush-border membrane of hepatopancreatic epithelial cells (Ahearn *et al.*, 1985; Gibson and Barker, 1979), and it was chosen, together with maltase and sucrase, as an enzyme marker for the apical pole. Contamination of hBBMV by basolateral membranes was assessed by analyzing the enrichment of Na^+–K^+–ATPase in pellet 7. The activity of alkaline phosphatase was estimated by the method of Forstner *et al.* (1968). The activity of disaccharidase was determined by the method of Dahlquist (1984), using a 1:50, for maltase, and 1:10, for sucrase, dilution of the hBBMV preparation. The activity of Na^+–K^+ ATPase was measured by the method of Kinne *et al.* (1971). Protein was measured by a Coomassie Brilliant Blue protein assay using bovine gamma-globulin as standard (Bradford, 1976).

Pte-Glu Uptake by hBBMV

(3′,5′,7,9-^{3}H) Pte-Glu potassium salt (20 Ci/mmol) uptake was measured at 25°C by using the Millipore filtration technique (Hopfer *et al.*, 1973). hBBMV were suspended in 300 mM mannitol, 0.1 mM $MgSO_4$ and 20 mM HEPES/Tris (pH 7.4). The assay was initiated by adding 10 μl of the suspension (approximately 100 μg protein membrane vesicles) to 100 μl uptake buffer. The uptake buffer contained 0.2 μM Pte-Glu, traces of (^{3}H) Pte-Glu, 300 mM mannitol, 0.1 mM $MgSO_4$ and either 20 mM HEPES/Tris (pH 7.4) or 20 mM MES/Tris (pH 5.5). The hBBMV were separated from the incubation medium by filtering on a 0.22 μm pore size Millipore filter. Filters were then washed with 10 ml of ice-cold stop solution containing 300 mM mannitol, 0.1 mM $MgSO_4$ and 20 mM MES/Tris (pH 5.5), added to a Beckman Ready scintillation cocktail and counted for radioactivity in a Beckman scintillation counter. Nonspecific isotope binding to the filters was determined by adding stop solution to the vesicles before addition of uptake buffer, and subtracted from the total radioactivity of samples.

For the study of the effect of the osmolarity of the incubation medium on Pte-Glu uptake, mannitol was added to the incubation medium to give the indicated osmolarity.

For the kinetic analysis of Pte-Glu uptake, the Pte-Glu concentration was varied from 0.1 to 1 μM by the addition of unlabelled Pte-Glu. ph-dependent Pte-Glu uptake was determined by the difference of the Pte-Glu uptake in the presence and absence of a proton gradient.

The effect of Na^+-gradient on hBBMV uptake of Pte-Glu, was determined in the presence and absence (KSCN) of an inwardly directed Na^+-gradient (NaSCN).

Statistical Analysis

Individual experiments were carried out in triplicate, and using membranes prepared from different hepatopancreata. Results are presented as the mean ± SE. Comparison between different experimental groups was evaluated by the two-tailed Student's *t*-test. The level of significance was $P < 0.05$.

RESULTS AND DISCUSSION

Hepatopancreatic Brush-border Membrane Vesicle (hBBMV) Purification

Prawn hBBMV preparations were obtained by using $MgCl_2$ to aggregate nuclei, microsomes, mitochondria and basolateral membranes. Table 1 presents the characterization of these hBBMV by monitoring the activity of marker enzymes (alkaline phosphatase, maltase and sucrase for the brush-border, and Na^+–K^+–ATPase for contamination by basolateral membranes) in pellets 2 and 7, as well as the enrichment factor derived from the ratio of pellet activities. Sucrase, maltase and alkaline phosphatase in pellets 7 were enriched 6.6, 4.9 and 5.6-fold respectively, when compared with their activities in pellet 2, while enrichment of Na^+-K^+-ATPase was negative. In addition, the greatest enzyme recoveries occurred for the brush-border markers, whereas the recovery of Na^+–K^+–ATPase was low in the final pellet 7. These results are indicative of a relatively pure apical membrane preparation, which contains minimal contamination from basolateral membrane sources. These data are consistent with those reported for lobster hepatopancreas (Ahearn *et al.*, 1985).

Table 1. Enzyme Characterization of Prawn Hepatopancreatic Brush-Border Membrane Vesicles

Enzyme	Pellet 2	Pellet 7	Enrich (fold)	Recov (%)
Alkaline phosphatase	47.4 ± 6.3	264.9 ± 33.5	5.6 ± 0.7	32.3 ± 6.5
Maltase	21.7 ± 5.4	105.1 ± 12.3	4.9 ± 0.5	28.2 ± 5.3
Sucrase	43.4 ± 5.2	285.0 ± 31.6	6.6 ± 0.2	38.3 ± 6.9
Na^+–K^+–ATPase	216.7 ± 40.6	163.3 ± 27.8	0.7 ± 0.3	4.4 ± 1.8

Data represents means ± SE of six different preparations. Enzyme activities are in nmol inorganic phosphate released.

mg protein^{-1} min^{-1} for alkaline phosphatase and Na^+–K^+–ATPase, and in nmol glucose formed mg protein^{-1} min^{-1} for maltase and sucrase. Enrichment (Enrich) factors are means of individual pellet 7 activities/individual pellet 2 activities. Enzyme recoveries (Recov) represent comparisons between content of pellet 2 and 7.

Transmembrane pH Gradient and Pte-Glu Uptake into hBBMV

To determine the effect of pH of the extravesicular medium on Pte-Glu uptake into hBBMV, the vesicles were incubated with 0.2 μM Pte-Glu for different times in extravesicular medium made to either pH 7.4 or pH 5.5, as described in Materials and Methods. Pte-Glu uptake was greater in the presence than in the absence of a proton gradient at all time points (Fig. 1). Although no overshoot was observed, the ratio of Pte-Glu uptake in the presence of the pH gradient to that in the absence of the pH gradient was approximately twofold at all time points.

This observation can be explained by the existence of a Pte-Glu:H^+ cotransporter as well as by a direct effect of the pH gradient on Pte-Glu transporter (Blakeborough and Salter, 1988; Said *et al.*, 1987; Schron, 1991). In fact, an acidic microenvironment around the hepatopancreatic luminal space would contribute to an increase of the proton influx and in turn to facilitate the transport of different nutrients (Gibson and Barker, 1979), including Pte-Glu transport.

However, Pte-Glu transport has also been described as a Na^+-dependent process for intact intestinal tissue (Said *et al.*, 1986). To study whether Pte-Glu uptake into hBBMV from prawn was Na^+-dependent, Pte-Glu uptake was measured in the presence or absence (KSCN) of an inwardly directed Na^+-gradient (NaSCN). As shown in Fig. 2, no differences were found in Pte-Glu uptake in the presence and absence of Na^+. These results were in accordance with previous studies using BBMV obtained from mammalian intestine (Halsted, 1979; Bhandari *et al.*, 1988).

Binding Versus Transport of Pte-Glu

hBBMV are well known as osmotically active vesicles, i.e., they respond to changes in medium osmolarity. By using this characteristic of the hBBMV, one can

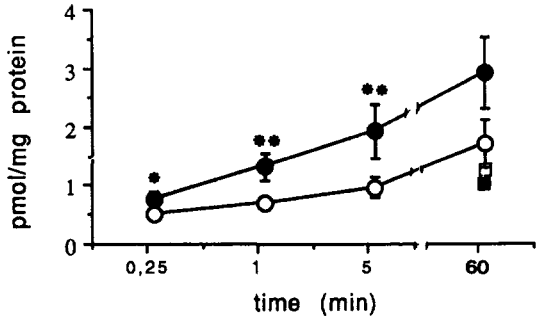

Fig. 1. Effect of pH-gradient on time course of Pte-Glu uptake by prawn hBBMV. Uptake studies were performed as described in Materials and Methods. (○) and (●) indicate the uptake in the absence and in the presence of a pH-gradient. (□, ■) represent uptake corrected for binding. Values are means ± SE of twelve different preparations. $*P < 0.01$ and $**P < 0.001$ compared to values obtained in the absence of a pH-gradient.

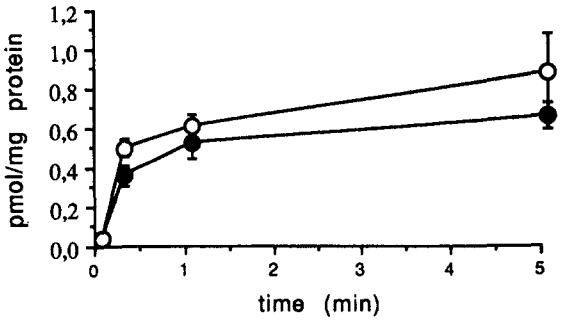

Fig. 2. Effect of sodium-gradient on time course of Pte-Glu uptake by prawn hBBMV. (○) and (●) indicate the uptake in the presence of K^+ gradient and Na^+ gradient respectively. Values are means ± SE of twelve different preparations.

distinguish between binding of the substrate to the membrane surface and its transport into the intravesicular space. This can be done by examining the substrate uptake as a function of incubation medium osmolarity. Pte-Glu uptake at 15 s was decreased with an increasing osmolarity of the medium (Fig. 3A), indicating that Pte-Glu is taken up into an osmotically sensitive vesicular space. The relationship between uptake and the reciprocal of osmolarity was fitted to a straight line. The intercept on the ordinate (zero intravesicular volume) is a measure of binding. It can be seen that extrapolation of the regression line to infinite osmolarity, intercepts the ordinate axis at 0.136 pmol/mg protein/15 s (in the presence of a pH gradient) and at 0.142 pmol/mg protein/15 s (in the absence of a pH gradient), revealing that the binding component, after 15 s incubation period, was not affected by the presence (18.8%) or absence (17.4%) of a pH gradient.

However, binding after a 60 min incubation period (Fig. 3B), represented 28% of the Pte-Glu uptake in the absence of a pH gradient (intercepts the ordinate axis at 0.453 pmol/mg protein/60 min), and 19% in the presence of a pH gradient (intercepts the ordinate axis at 0.539 pmol/mg protein/60 min). Therefore, the binding component at 60 min was affected by the presence and absence of a pH gradient. The Pte-pGlu uptake corrected for binding under standard conditions 2.9 osm^{-1}), showed an overshoot in the presence of a pH gradient (see Fig. 1), with an equilibrium time at 60.min. A pH-dependent binding of folate has previously been demonstrated using pig brush-border membrane vesicles (Reisenauer *et al.*, 1986.

Kinetic Study of the Pte-Glu Transport

Pte-Glu uptake into hBBMV was measured, in the presence and absence, respectively, of an inwardly directed transmembrane pH gradient, at different external Pte-Glu concentrations (from 0.1 to 10 μM). In the presence of a pH gradient Pte-Glu uptake plotted against its concentration agrees initially with a saturable process that becomes linear when the substrate concentration increases, not yielding a saturation curve (Fig. 4A). In the absence of a pH gradient, Pte-Glu uptake

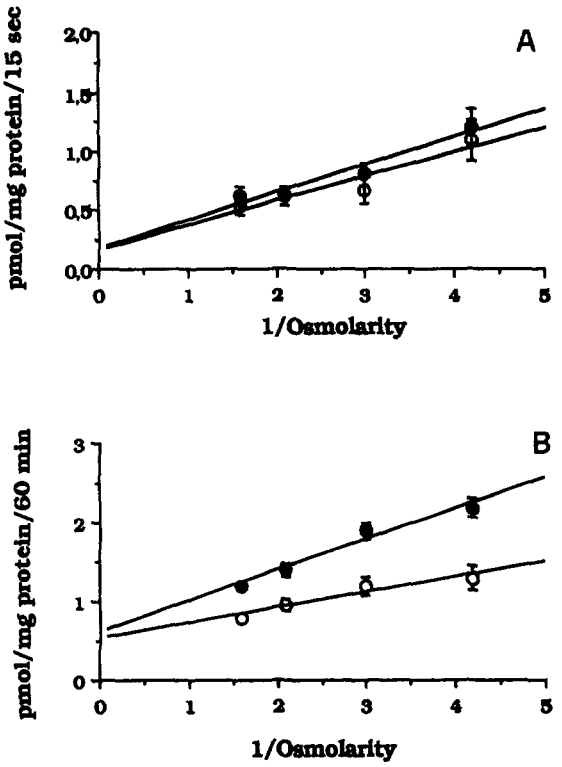

Fig. 3. Effect of increasing osmolarity on the uptake of Pte-Glu by prawn hBBMV. Aliquots of hBBMV suspensions were incubated for 15 s (A) or 60 min (B). (○) and (●) indicate the uptake in the absence and in the presence of a pH-gradient. Values are means ± SE for five different preparations.

showed a linear relationship with its extravesicular concentrations. The difference between total Pte-Glu uptake and that observed in the absence of a pH-gradient follow first-order kinetics (Fig. 4A, difference curve). The kinetic analysis with an Eadie–Hofstee plot (Fig. 4B) yielded a linear relationship, consistent with the existence of a single Pte-Glu transporter. The apparent transport constants, Km, and maximal rates of transport, Vmax, were 0.37 μM and 1.06 pmol/mg protein/15 s, respectively. The apparent Km is similar to that reported for the Pte-Glu transporter of rat (Selhub and Rosenberg, 1981) and rabbit (Schron, 1991) intestine, and lower than that reported for rat kidney (Bhandari *et al.*, 1988).

In summary, the present results suggest that, as in other epithelial cells, in the brush-border of prawn hepatopancreatic cells there exists a Pte-Glu transporter that is dependent of an inwardly directed pH-gradient, similar to that described in mammalian intestine.

More studies are needed for molecular characterization of this transporter, in prawn hepatopancreas.

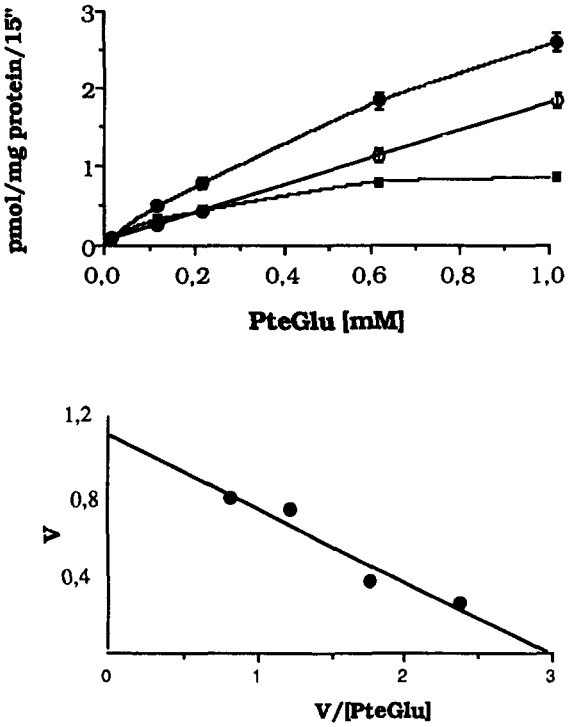

Fig. 4. Initial rate of Pte-Glu uptake (15 s) as a function of external Pte-Glu concentration. (A) Uptake in the presence (●) and in the absence (○) of a proton gradient. Difference curve represents total uptake minus that in the absence of proton gradient (■). (B) Eadie–Hofstee plot of the difference curve. Kinetic parameters (apparent Km and Vmax) of the H^+-dependent Pte-Glu transport system were calculated using linear regression analysis ($y = 1.06 - 0.37 \times$, $r = 0.94$). Each point represents mean values ± SE of triplicate assays using five membrane vesicle preparations.

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REFERENCES

Ahearn, G. A. Grover, M. L., and Dunn, R. E. (1985). Glucose transport by lobster hepatopancreatic brush-border membrane vesicles. *Am. J. Physiol.* **248:**G133–G141.

Bhandari, S. D., Joshi, S. K., and McMartin, K. C. (1988). Folate binding and transport by rat kidney brush-border membrane vesicles. *Biochim. Biophys. Acta* **937:**211–218.

Blakeborough, P. and Salter, D. N. (1988). Folate transport in enterocytes and brush-border membrane vesicles isolated from small intestine of the neonatal goat. *British J. Nutr.* **59:**485–495.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:**248–254.

Dahlquist, A. (1984). Assay of intestinal disaccharidases. *Scand. J. Clin. Lab. Invest.* **44:**169–172.

Forstner, G. G., Sebesin, S. M., and Isselbacher, K. J. (1968). Rat intestinal microvillus membranes. Purification and biochemical characterization. *Biochem. J.* **106:**381–390.

Gibson, R. and Barker, P. L. (1979). The decapod hepatopancreas. *Oceanograf. Mar. Biol.* **17:**285–346.

Halsted, C. H. (1979). The intestinal absorption of folates. *Am. J. Clin. Nutr.* **32:**846–855.

Hopfer, U., Nelson, K., Perroto, J., and Isselbacher, K. J. (1973). Glucose transport in isolated brush-border membrane from rat small intestine. *J. Biol. Chem.* **248:**25–32.

Kinne, R., Schmitz, J. E., and Kinne-Saffran, E. (1971). The localization of the Na^+–K^+–ATPase in the cells of rat kidney cortex. A study on isolates plasma membranes. *Pfluegers Arch.* **329:**191–206.

Reisenauer, A. M., Chandler, C. J., and Halsted, C. H. (1986). Folate binding and hydrolysis by pig intestinal brush-border membranes. *Am. J. Physiol.* **251:**G481–G486.

Said, H. M., Blair, J. A., Lucas, M. L., and Hilburu, M. E. (1986). The intestinal surface and microclimate *in vitro* and *in vivo* in the rat. *J. Lab. Clin. Med.* **107:**420–424.

Said, H. M., Ghishan, F. K., and Redha, R. (1987). Folate transport by human intestinal brush-border membrane vesicles. *Am. J. Physiol.* **252:**G229–G236.

Schron, C. M., Washington, Jr., and Blitzer, B. L. (1985). The transmembrane pH gradient drives uphill folate transport in rabbit jejunum. *J. Clin. Invest.* **76:**2030–2033.

Schron, C. M. (1991). pH modulation of the kinetics of rabbit jejunal brush-border folate transport. *J. Membr. Biol.* **120:**192–200.

Selhub, J. and Rosenberg, I. H. (1981). Folate transport in isolated brush-border membrane vesicles from rat intestine. *J. Biol. Chem.* **256:**4489–4493.

Stokstad, E. L. R., Shin, I. S., and Tamura, T. (1977). In: Folic Acid Biochemistry and Physiology in Relation to the Human Nutrition Requirement, Washington, DC, National Academy of Sciences, p. 56.